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Bioremediation of Copper-Ions by Polymer Encapsulated and Immobilized *Micrococcus Luteus*

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Bioremediation of copper (Cu^{2+}) with immobilized *Micrococcus luteus* in polymer matrices has been broadly studied for a wide range of applications including wastewater treatment. Herein, the bioremediation efficiency based on modified immobilization techniques and by the addition of Cu^{2+} is investigated. Porous composite nonwovens with living *M. luteus* (living polymer composites) are prepared by encapsulation of the bacterial cells in poly(vinyl alcohol) (PVA) microparticles (*M. luteus*/PVA microparticles) produced by spray drying method. The *M. luteus*/PVA microparticles are chemically cross-linked. The hydrogel microparticles with encapsulated *M. luteus* are embedded in a nonwoven of poly (lactic acid) (PLA) electrospun short fibers provided by wet-laid method. Two different models of composite nonwovens are reported, in which the place position of the hydrogel PVA microparticles with encapsulated *M. luteus* and PLA nonwoven can affect the bioremediation process.

1. Introduction

Copper (Cu), as a trace element like many other heavy metals, beyond a certain amount, is capable of providing nutrients for the metabolism of microorganisms and regulating plant functionality. The human activities have altered the environmental copper content, resulting in copper pollution in the aquatic system. The excess amount of copper and especially highly toxic copper (II) (Cu^{2+}), is affecting human bodies and is known as a harmful ion in wastewater.[1]

Removal of Cu^{2+} from wastewater is performed with various physicochemical methods, such as absorption, cementation, nonwoven filtration, and dialysis. Absorption of Cu^{2+} with modified natural carriers like biopolymers has attracted numerous attentions in wastewater treatment. Modified biopolymers are widespread viable and environmentally friendly. Polysaccharide polymers as a well-known group of biopolymers are industrially attractive for reducing the Cu^{2+} concentration in wastewater upon absorption to the functional groups.[2] Parkash et al. showed the absorption ability of chitosan to uptake Cu^{2+} with the uptake amount of 87 mg per g of chitosan.[3]

Another prevalent method of Cu^{2+} removal is bioremediation, which is a non-costly and least harmful method. Bioremediation with microorganisms is highly potential for the decontamination of wastewater. Microorganisms such as fungi, bacteria, algae, protozoa, or viruses are breaking down the chemical components and using them as a source of carbon, whereas in bioremediation of heavy metals, biological degradation is not possible. The mechanism with microorganisms is based on the change from oxidative state or complex to another state (valance transformation), volatilization, and extracellular chemical precipitation.[4–9] Bacteria are confessed as the most abundant microorganisms in the ecosystem with a high surface to volume ratio.[10] The bioremediation efficiency is depending not only on the ambient conditions, such as pH and temperature, but also to the type of bacteria.[11] The most attractive feature of bacteria and a key to achieve excellent bioremediation is the presence of chemisorption sites in the cell wall. The presence of negative chemisorption sites in gram-positive bacterial cell wall provides higher sequestration of metal cations.[1] *Micrococcus Luteus* (*M. luteus*) is an aerobic gram-positive bacterium, which is exclusively withstanding the environmental stress.[12] In the study of Letnik et al. it was shown that only 10% of *M. luteus* cells with the initial concentration of 10^8 CFU are able to survive Cu^{2+} by the concentration of 370 ppm (6 mM).[13] In contrary, a Cu^{2+} solution of 0.05–0.1 mM has no inhibition effect on bacterial growth. The maximum bioremediation of Cu^{2+} by *M. luteus* cell after 1 h was determined as 59mg (Cu^{2+}/g of bacterial cells) in the solution concentration of 1 mM (80% uptake), which decrease to 14% for Cu^{2+}solution of 10 mM.

Despite the merits of bacterial bioremediation, continuous bioremediation has not been studied so far. The bioremediation process can be achieved by biologically active as well as inactive bacterial cells. Besides that, bacteria ability to self-producing is remarkable. These features can provide infinite possibilities for self-renewing bioremediation.[14,15]

Another essential factor maintaining the biological activity of bacteria, for higher bioremediation achievement is to protect

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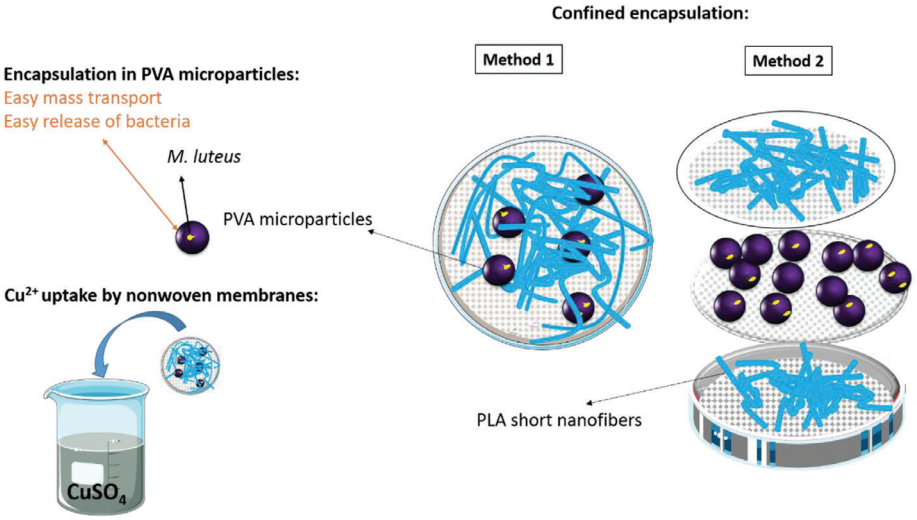

Scheme 1. Production of porous composite nonwovens containing *M. luteus*. Encapsulation of *M. luteus* in PVA microparticles followed by embedding in fiber nonwovens. Method 1: randomly mixed *M. luteus*/PVA microparticles and PLA short fibers. Method 2: *M. luteus*/PVA microparticles placed between 2 PLA nonwoven layers. Biosorption of Cu^{2+} with nonwoven membrane.

bacterial cells against unwanted environmental stresses. Immobilization of bacteria in polymer matrices offers advantages in terms of higher resistance to unfavorable environmental impacts, higher storage time, and reusability.[4,16,17] "Living composites" of bacteria/polymers promise to change the way of bioremediation methods with various advantages. Polymer particles and fibers are well known for encapsulation of active materials, such as catalysts, enzymes, microorganisms, nanoparticles, and pharmaceuticals, antibiotics, vitamins for a wide variety of applications. A wide variety of processing methods is well known for particle production, including emulsion polymerization, evaporation, and spray drying. Among all the mentioned methods, spray drying has attracted significant attention for its easy and fast manufacturing procedure. In this method, a polymer mixture in the form of a solution, emulsion, or suspension is atomized with atomization nuzzle, following by exposure to a heated gas stream. As a result, the formation of powder of solid particles by evaporation of the solvent is achievable.[18–21] Reich et al. introduced the encapsulation of bacteria in polymer microparticles by spray drying of gram-positive and negative bacteria in different polymer solutions.[22]

Another commonly used method for the preparation of polymer composites is electrospinning, in which fibers in the range of 10–100 nm in diameter is achievable.[23–26] Electrospun fibers have the unique feature highly porous structure and a wide variety of different fiber morphologies. As a result, the emerging use of fiber nonwovens in the production of nonwoven for traditional filtration, tissue engineering, biosensors, and composite materials is observed. Besides that, fibers are widely used for the immobilization of enzymes, bacteria, viruses, proteins, and other biologically active materials.[8,16,17,27–30] Salalha et al. reported the first work on encapsulation of biological materials like bacteria and viruses in electrospun PVA fibers in 2006.[30] In the work of Sarioglu et al. bacteria were trapped in fibers by natural adhesion force, which leads to the formation of biofilms.[31] Gensheimer et al. studied the double encapsulation of bacterial cells in polymer particles and fibers.[29] In more detail, *M. luteus* was firstly encapsulated in PVA particles, followed by electrospinning of a suspension of particles in another polymer/ethanol solution. Although the double encapsulation retards the release of bacteria, the essential nutrient transports through the particles were also reduced.

However, the preparation of high surface area nonwoven composites with electrospun fibers is a time-consuming process, which can be optimized by preparation of similar nonwoven by use of short fibers' dispersion by a wet-laid method.[32] The procedure offers additional advantage of mixing other additives, in short fiber dispersion just by mixing and forming the additive immobilized nonwoven.[32,33]

The objective of the current work is to emphasize on immobilization techniques of *M. luteus* cells for continuous sequestrating of Cu^{2+}. Encapsulation of *M. luteus* in PVA microparticles (*M. luteus*/PVA microparticles) was achieved by the spray-drying method. The microparticles were chemically cross-linked, to attain hydrogel structure that swells in exposure to aqueous media. This swelling behavior grants the elasticity to the microparticles upon bacterial growth. In this work, for retarding the bacterial leakage from PVA microparticles, a carrier of electrospun PLA fibers was used (Scheme 1). The effect of porous composite nonwoven preparation on mass transport for bioremediation was studied with two methodologies. Following **Scheme 1**, the immobilization of *M. luteus*/ PVA microparticles in PLA short fibers was made in two different forms (the *M. luteus*/ PVA microparticles were sandwiched or randomly mixed in PLA short fibers). Bioremediation of Cu^{2+} by alive encapsulated *M. luteus*, as well as encapsulated dead cells was studied, and identified by analytical methods.

2. Result and Discussion

Two different strategies were adopted in order to immobilize *M. luteus*/PVA microparticles in nonwovens with the aim to study

the effect of the preparation method on the bioremediation efficiency. In the first approach (the work of Genscheimer et al.), *M. luteus*/PVA microparticles can be encapsulated in electrospun polymer fibers, which means double encapsulation of *M. luteus*. As a result, fibers act as a shell for encapsulated microparticles.[29] The mass transport for this immobilization model is retarded with the fibers, which cover the microparticles surfaces. Therefore, a nonwoven is desirable, which makes no hindrance for nutrient passage through the PVA microparticles.

In the second approach, *M. luteus*/PVA microparticles are dispersed in short length fibers by help of wet-laid method (Scheme 1). In this method, nonwoven matrix has less limiting effect on mass transport.

For both strategies, and to protect *M. luteus* from environmental stress, and increase the storage time with respect to the biological activity, *M. luteus* was successfully encapsulated in PVA microparticles using spray drying method (Sample 1, Table 2, **Figure 1**a,b). The proof of the efficiency of the encapsulation in the PVA microparticles was shown in the work of Reich et al. and by a Raman-atomic force microscopy (AFM) measurement.[22] In this study, the presence of *M. luteus* inside the PVA microparticles was shown. The diameter size of the PVA microparticles with encapsulated *M. luteus* is in the range of 2.58 ± 0.89 μm (Figure S1, Supporting Information). *M. luteus*/PVA microparticles were cross-linked (Sample 3, Table 2, Figure 1c), and survival of *M. luteus* was tested with bacterial culture in agar plate test. The observation of characteristic yellow colonies revealed the fact of the biologically active *M. luteus* in the cross-linked PVA microparticles. The cross-linked microparticles were kept in water for 48 h in order to study the cross-linking efficiency (Figure 1d). The cross-linked PVA microparticles were immobilized in PLA short fibers nonwoven. Electrospun PLA fibers with average diameters of 240 ± 115 nm (Sample 4, Table 2) (Figure 1e, Figure S2, Supporting Information) underwent a fiber cutting process to get short fibers of an average length of 0.45 ± 0.29 mm (Sample 5, Table 2) (Figure 1f). PLA short fibers nonwoven were prepared by wet laid procedure.[32–34] The method utilizes a dispersion of short fibers, and subsequent filtration leading to the formation of a self-standing nonwoven by the percolation of fibers. In the first method, the *M. luteus*/PVA microparticles were dispersed together with PLA short fibers and filtered through a filtration funnel to obtain PLA nonwoven immobilizing the microparticles (Samples 6 and 7, Table 2). Whereas, in second method the *M. luteus*/PVA microparticles were sandwiched between two PLA nonwovens by a three step procedure (Sample 8 and 9, Table 2): making PLA nonwoven by wet-laid process using a dispersion of short PLA fibers, filtration of *M. luteus*/PVA microparticles through PLA wet-laid nonwoven followed by covering the particles with another layer of PLA made by wet-laying short PLA fiber dispersion. The thickness of the nonwoven formed by short-fiber and microparticles dispersion filtration in both methods is in the range of 1.68 ± 0.12 mm. The pore size measurement of the PLA nonwoven showed the size range of the pores roughly about 119.95 ± 67.55 μm.

SEM micrographs of wet-laid nonwovens with PVA microparticles containing the encapsulated *M. luteus* are shown in Figure 1g–j. The cross-section of the wet-laid nonwoven in Figure 1h,j showed the distribution of *M. luteus*/PVA microparticles throughout the PLA short fibers nonwovens.

Bacterial leaching test was performed by agar plate tests for porous composite nonwovens of two different approaches (Method 1 and 2), and also with different amounts of *M. luteus*/PVA microparticles (**Figure 2**a). The release behavior was studied for the time period of 72 h, and the formation of yellow colonies confirmed the release of *M. luteus*. The release study showed less leaching of *M. luteus* as observed by the formation of less colonies for composite nonwovens of sandwich samples (Method 2, Samples 8 and 9) in comparison to the randomly mixed nonwovens (Method 1, Samples 6 and 7). As a result of the investigation in release behavior, and in order to prevent leakage of *M. luteus*, the bioremediation ability of encapsulated *M. luteus* was required to be studied in 48h. In this step, composite samples (made with different approaches and amount of components) with the size of 1 cm × 1 cm were placed in 10 mL Cu^{2+} solutions with different concentrations (5, 10, and 15 mM) at 37 °C for 48 h.

In order to analyze the functionality of the composite nonwoven, we have investigated the remediation of Cu^{2+} by the encapsulated *M. luteus*. The remediation of Cu^{2+} was quantified by ICP-OES. To compare the passive and active bioremediation (with live and dead cells, respectively), control samples of encapsulated dead cells of *M. luteus* in polymer composites with methods 1 and 2 were used (Sample 10, Table 2). The result of bioremediation with control samples made in different models (method 1 and 2) were approximately in the same range (narrow error bar in Figure 2b). The Cu^{2+} uptake with the composite nonwovens with living *M. luteus* was 3 to 4 times more than the uptake with the dead cells (for all Cu^{2+} concentrations). A low concentration of Cu^{2+} solution (5 mM) resulted in approximately 90% uptake by the composite nonwovens of all types. The uptake for 10 mM Cu^{2+} solution was different for different composites, and a higher amount of Cu^{2+} solution (15 mM) led to a decrease in uptake. In Cu^{2+} concentration of 10 mM and 15 mM, the significantly reduced amount of uptake for Samples 6 and 7 (Table 2) is due to the release of the encapsulated *M. luteus* from the surface of the samples, which was also observed in the study of the release behavior. The increased release of *M. luteus* for Samples 6 and 7 (Table 2) was studied by Agar plate test (Figure 2a), and observation of more yellow colonies compared to Samples 8 and 9. However, for Samples 8 and 9 (Table 2), the PLA short fibers covering the top and bottom of the composites made by model 2 were reducing the exposure of *M. luteus*/PVA microparticles with nutrient medium or Cu^{2+} solution, which was playing role in preventing the release of *M. luteus*. The bioabsorbed Cu^{2+} in the nonwoven composites with encapsulated *M. luteus* were identified by SEM using an energy dispersive X-ray detector SEM using a back scattered electron (BSE) detector (Figure 2c,d).

The study on continuous bioremediation of *M. luteus* by the addition of Cu^{2+} and the self-renewing bioremediation ability was carried out in this work. Composite nonwoven samples (the same size and same procedure as explained before) were kept in 5, 10, and 15 mM Cu^{2+} solutions (10 mL) at 37 °C for 48 h, followed by the addition of the second batch of Cu^{2+} solution with the same concentrations after 48 h. After the second addition of Cu^{2+}, the amount of Cu^{2+} bioabsorbed was quantified by ICP-OES measurement. The control sample was prepared as mentioned in the previous section. The result (**Figure 3**) is revealing the ability of encapsulated *M. luteus* to grow and continue the uptake process.

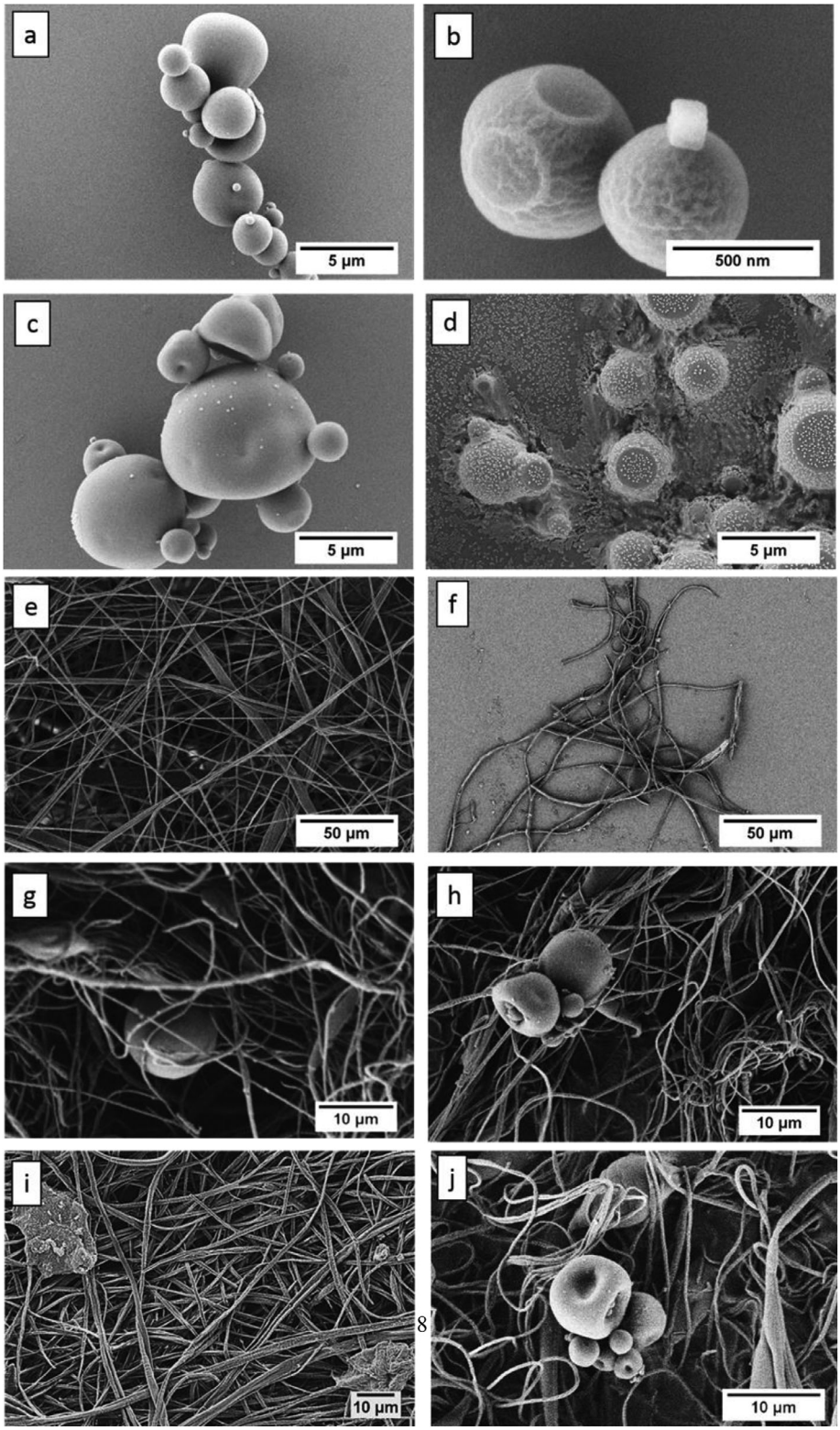

Figure 1. SEM micrograph of samples in Table 2. a) *M. luteus*/PVA microparticles after spray drying, Sample 1. b) Surface of the dry *M. luteus*/PVA microparticles (the salt crystal is due to the use of PBS for spray drying), Sample 1. c) *M. luteus*/ PVA microparticles after cross-linking with glutaraldehyde, Sample 3. d) *M. luteus*/ cross-linked PVA microparticles after 48 h immersion in water, Sample 3. e) Electrospun PLA fibers, Sample 4. f) PLA short fibers, sample 5. g) *M. luteus*/PVA microparticles in short fiber nonwoven, the surface of Sample 6 (the gel formed on top of the sample is due to the cross-linking of $CaCl_2$ and sodium alginate). h) The cross-section of the nonwoven, Sample 6. i) *M. luteus*/PVA microparticles in short fiber nonwoven, the surface of sample 8; j) the cross-section of the nonwoven, Sample 8.

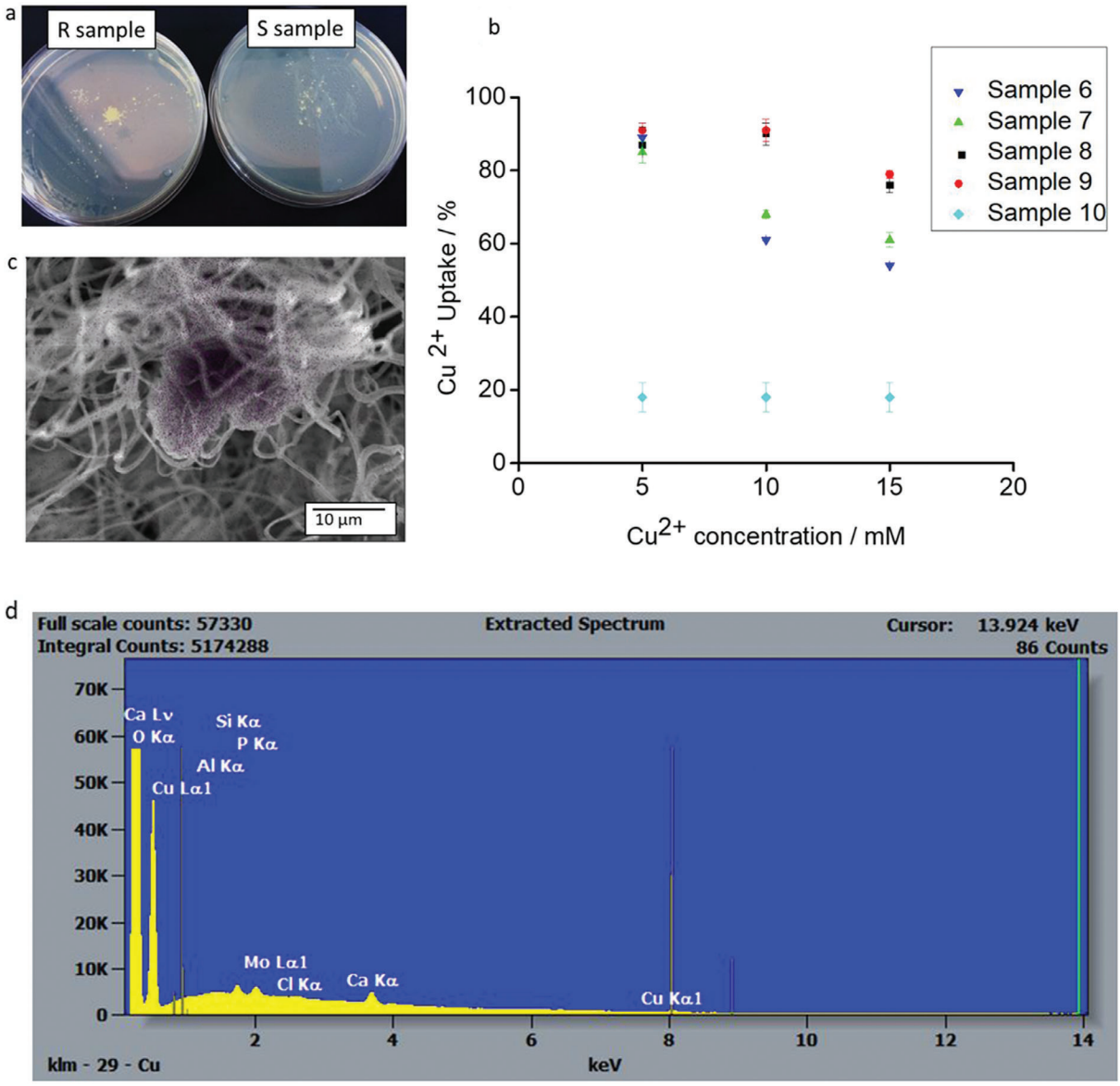

Figure 2. a) Release of *M. luteus* from porous composite nonwovens of different methods.) Characterization of Cu^{2+} uptake by composite nonwovens after 48 h at 37 °C, Comparison of the Cu^{2+} uptake of different samples (the control sample (Sample 10, Table 2) consists of the dead cell of *M. luteus*) measured by ICP-OES at different Cu^{2+} concentrations (5, 10, and 15 mM). c) SEM/BSE image of PVA/PLA nonwoven Sample 9 (Table 2). d) SEM/EDX spectra of *M. luteus*/PVA microparticles embedded in PLA short fibers, Sample 9.

Figure 2 shows that the sandwich composites (method 2, Samples 8 and 9) perform the bioremediation better than the randomly made composites (method 1, Samples 6 and 7), which is also observed in the current experiment. After 96 h and by the addition of Cu^{2+} salt to the composite samples with living *M. luteus*, bioremediation of roughly 60–70% for Samples 8 and 9 and 25–45% for Samples 6 and 7 were observed.

3. Conclusion

Two different porous composite nonwovens with encapsulated living *M. luteus* were prepared, and the bioremediation of Cu^{2+} as an existing heavy metal in wastewater by encapsulated *M. luteus* was quantified. The porous composite nonwovens were prepared by the encapsulation of *M. luteus* in PVA microparticles with a diameter of 2.58 ± 0.89 µm, followed by embedding the microparticles in PLA short fibers with average diameter of 240 ± 115 nm and length of 0.45 ± 0.29 mm. PLA nonwoven used as a carrier and made by wet-laid method. Two types of samples with alive and dead *M. luteus* cells were prepared. 1:By filtering the microparticles and short fibers together, and 2: a sandwich structure of two layers of short fibers with a middle layer of microparticles. The amount of microparticles with encapsulated *M. luteus* was doubled for the composite nonwovens of each model (model 1 and 2) to show its impact on bioremediation efficiency *M. luteus* remained active for 48 h without leaching from porous composite

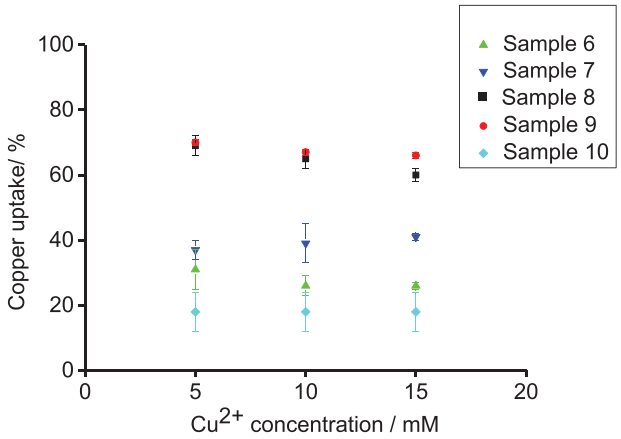

Figure 3. ICP-OES measurement of Cu^{2+} uptake after 96 h: the effect of additional Cu^{2+} to the ability of different composite samples with living *M. luteus* and dead *M. luteus* (for control samples) for second time bioremediation, continuous bioremediation was operated at 37 °C and pH = 5.

nonwovens of two different models, and samples were successfully applied for Cu^{2+} removal. The bioremediation of Cu^{2+} by encapsulated *M. luteus* was quantified by ICP-OES. The uptake for composite nonwovens of all types with initial Cu^{2+} concentration of 5 mM was approximately 90%, which reduced by increasing the initial Cu^{2+} concentration to 10–15 mM. The continuous uptake was quantified by prolonging the time of the experiment for the second 48 h and by the addition of Cu^{2+} salt to the porous composite samples with living *M. luteus*. The Cu^{2+} bioremediation of 60–70% for method 2 and 25–45% for method 1 were observed. Therefore, the composite nonwovens made by method 2 were more efficient for bioremediation applications, due to the higher protection of *M. luteus*/PVA microparticles with two layers of PLA nonwovens. The current work confirmed the ability of encapsulated *M. luteus* in polymer composite nonwovens for continuous bioremediation of Cu^{2+} in aqueous media. With the present concept, a new system is available which will provide optimum mass transport for bioremediation while the unwanted escape of the bacteria from their carrier is suppressed and thereby the environmental pollution by the microbes.

4. Experimental Section

Materials: PVA (M_w = 13 000–23 000 g·mol^{-1}, 98–99% hydrolysis, Sigma Aldrich) and PLA (IngeoTM 4043D, NatureWorks LLC) were obtained as indicated. Glutaraldehyde (70% in H_2O) (Sigma Aldrich), copper sulfate ($CuSO_4$) (Roth ≥98%), calcium chloride ($CaCl_2$) (Grüssig GmbH), alginic acid sodium salt ($NaC_6H_7O_6$) (Merck), and acetic acid (CH_3COOH, 100%, VWR) were used as received. Tetrahydrofuran (THF), chloroform ($CHCl_3$), dimethylformamide (DMF) were distilled before use.

M. luteus (DSM-No. 20030) from DSMZ Braunschweig was cultivated in a mixture of Lysogeny broth (LB) (Roth) in extra pure water provided by a Milli-Q Plus system (conductivity = 0.072 µS·cm^{-1}, pH = 7) at 37 °C for 72 h.

Analytical Methods: Scanning electron microscopy (SEM) (Zeiss LEO 1530, Jena, Germany) was used to analyze the morphology of microparticles and fibers with a secondary electron (SE2) detector, and an acceleration voltage of 3 kV and a working distance of ≈4.6 mm. The samples were stuck onto a sample holder with double-sided adhesive tape and subsequently coated with 2.0 nm of platinum by a high-resolution sputter coater (208 HR, Cressington).

Table 1. Parameters of spray dryer for particle producing.

Aspirator [%]	Inlet temperature [°C]	Inlet temperature [°C]	Pump [mL min^{-1}]	PVA solution [%]
100	110	67	2.5	2.5

Energy-dispersive X-ray spectroscopy (EDX) measurements were performed with a Zeiss Ultra Plus (Jena, Germany) with a Schottky field-emission cathode with an acceleration voltage of 10 kV. The samples were vapor coated with platinum using a Balzer Union MED 010 before measurement.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) was performed on a VARIAN Vista-Pro (40 MHz, Argon Plasma) equipped with an ASX-510 autosampler, an Echelle polychromator, and an argon humidifier. Before measurement, samples were dissolved in 0.5 mL nitric acid and diluted with 9.5 mL of ultrapure water. A CCD semiconductor detector was used for the detection.

Encapsulation of M. Luteus in PVA Microparticles and Cross-Linking Procedure: Cultivated *M. luteus* pellet was centrifuged (10 min, 4000 rpm), washed, and re-suspended in the solution of 2.5 wt% PVA in phosphate buffered saline (PBS) (with 8.0 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, and 0.24 g KH_2PO_4 per liter of millipore water, pH = 7.3). The ratio between *M. luteus* and PVA polymer for one setup of spray drying was set as 1:20 g/g. The PVA solution was sterilized by autoclaving at 121 °C for 20 min.

A mini spray dryer b290 (Büchi, Switzerland) was utilized to encapsulate *M. luteus* in PVA microparticles. The bacteria/polymer solution was fed to the device with a feed rate of 2.5 mL·min^{-1} and the atomization gas of 600 L·h^{-1}. The inlet temperature based on the boiling point of water was set to 110 °C. The experiment was carried out under an ambient atmosphere. Further parameters used for spray drying are listed in **Table 1**. The encapsulated *M. luteus* in PVA microparticles (*M. luteus*/PVA microparticles) were collected and stored at 4 °C for further analysis (Sample 1, **Table 2**, Scheme 1A).

Besides the production of PVA microparticles with biologically active *M. luteus*, dead cell encapsulation was also performed. In this case, the centrifuged pellet of *M. luteus* obtained after cultivation was washed three times with ethanol. The encapsulation of dead cells in PVA microparticles was similar to the encapsulation of live *M. luteus* (Sample 2, Table 2).

The spray-dried PVA microparticles were chemically cross-linked with glutaraldehyde (Sample 3, Table 2). An argon-flushed flask was charged with glutaraldehyde (70% in H_2O) (0.035 g) and THF (25 mL). The amount of glutaraldehyde was decided based on testing its effect on *M. luteus* by minimum bactericidal concentration (MBC)/minimum inhibitory concentration (MIC) tests. Considering the result, the amount of glutaraldehyde below 0.14 mg L^{-1} has no inhibition effect on the growth of *M. luteus*. *M. luteus*/PVA microparticles (0.29 g) (Sample 1, Table 2) was added to the glutaraldehyde-THF solution under stirring for 4 h at RT.

Electrospinning of PLA: Electrospinning was carried out using a 7 wt% PLA solution in the mixture of $CHCl_3$/DMF/CH_3COOH (v/v/v, 70/15/15). The solution feeding rate was 1 mL h^{-1}. The applied voltage was 20 kV. The fibers (Sample 4, Table 2) were collected on a collector covered with a parchment paper (cellulose-based paper) with the distance of the collector to the nozzle of 12 cm.

Preparation of PLA Short Fibers Dispersion and M. Luteus/PVA Microparticles Immobilized in Porous Composite Nonwovens by a Wet-Laid Method: PLA fibers (8 g) (Sample 4, Table 2) were dispersed in water (1 L) and cooled down to −18 °C. The cutting process was operated by adding the dispersion in a blender for 1 h at 4000 rpm. The PLA short fibers (Sample 5, Table 2) were collected for the next step.

Porous composite nonwovens of PLA with immobilized *M. luteus*/PVA microparticles (Scheme 1) were prepared by two methods.

Method 1. Both *M. luteus*/PVA microparticles (Sample 2, Table 2) and short PLA fibers (Sample 5, Table 2) were re-dispersed together in water

Table 2. Details of components used for the preparation of *M. luteus*/PVA microparticles immobilized porous composite nonwovens.

Samples	Components
1	Live *M. luteus* /PVA microparticles
2	Dead *M. luteus* /PVA microparticles
3	Cross-linked PVA microparticles with encapsulated live *M. luteus*
4	PLA nanofibers
5	PLA short nanofibers nonwoven

Samples	Composite nonwovens and method	Amount of PLA short fibers [g]	Amount of microparticles [g]
6	Live *M. luteus*/PVA microparticles /PLA short fibers made by method 1	0.5	0.5
7	Live *M. luteus*/PVA microparticles /PLA short fibers made by method 1	0.5	1
8	Live *M. luteus*/PVA microparticles /PLA short fibers made by method 2	0.5	0.5
9	Live *M. luteus*/PVA microparticles /PLA short fibers made by method 2	0.5	1
10	Dead cells of *M. luteus*/PVA microparticles/PLA short fibers made by method 1 and 2	0.5	0.5 or 1

and filtered through a mesh of poly (ethylene terephthalate) (PET) (Franz Eckert GmbH) with a pore size of 100 µm. In this method, the position of *M. luteus*/PVA microparticles and short PLA fibers were randomly distributed (Samples 6 and 7 were made by this method, Table 2).

Method 2. Firstly, PLA short fiber dispersion (Sample 5, Table 2) was filtered through a PET mesh (pore size 100 µm) to make a PLA porous nonwoven by the percolation of short fibers as shown in the previous works for different applications,[32,33] on top of which *M. luteus*/PVA microparticles (Sample 2, Table 2) were filtered. Afterward, they were covered by filtration of another batch of PLA short fibers. Consequently, *M. luteus*/PVA particles were sandwiched between two layers of PLA porous nonwovens (Samples 8 and 9, Table 2). To study the effect of encapsulated *M. luteus*, the amount of microparticles was varied, while the amount of short PLA fibers was kept the same for each method (Table 2). To prevent the unwanted release of the *M. luteus*/PVA microparticles from porous nonwovens, a solution of 1 and 2 wt% Alginic acid sodium salt and $CaCl_2$, respectively, were added in the last step and after the porous nonwoven composites of microparticles and short fibers were made (5 mL of each solution). The cross-linking of alginic acid by $CaCl_2$ is well-known in the literature.[35] Table 2 represents the preparation methods and the amount of the components used in sample preparation.

All the steps of cross-linking or preparation of porous nonwovens were carried out for Sample 2, Table 2 for comparison purposes.

Cu^{2+} Uptake by Encapsulated M. Luteus: To quantify the Cu^{2+} depletion by encapsulated *M. luteus* in porous composite nonwovens with ICP-OES, 10 mL solutions of 5, 10, and 15 mM Cu^{2+} in extra pure water (milli-Q system) were prepared. Porous composite nonwoven samples made as explained previously with the size of 1 × 1 cm and weight of 0.1g were kept in the solutions for 48 h (temperature 37 °C, pH = 5, and shaking speed 100rpm). After the first interval (48 h), the polymeric matrices (PLA and PVA matrix) were destroyed by keeping the samples in aqua regia for 24 h. This is part of the sample preparation for required for the destruction of measurement by ICP-OES.

To measure the continuous sequestration of Cu^{2+} by bacteria/polymer porous composite nonwovens, the first experiment was repeated with the same size and weight of the samples (37 °C and pH = 5). After 48 h, the samples were taken out of the solutions and were washed with water. The washed samples were placed again in newly made 10 mL Cu^{2+} solutions of 5, 10, and 15 mM. The second interval of 48 h was considered, followed by removing the polymeric matrices.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Keywords

bioremediation, encapsulation, polymer microparticles, wet-laid nonwovens

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