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Reusable Bacteria Immobilized Electrospun Nanofibrous Web for Decolorization of Methylene Blue Dye in Wastewater Treatment

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In our study, electrospun cellulose acetate nanofibrous webs (CA-NFW) was found to be quite effective in immobilizing bacterial cells. Here, decolorization of methylene blue (MB) dye in aqueous medium was achieved by using three types of bacteria (Aeromonas eucrenophila, Clavibacter michiganensis and Pseudomonas aeruginosa) immobilized on CA-NFW. The decolorization time (0–48 h) and different MB dye concentrations (20–500 mg L−1) were studied to elucidate the maximum MB dye removal by the bacteria immobilized CA-NFW. The effective dye decolorization was achieved within 24 hours and MB dye removal was ~95%. Interestingly, MB dye decolorization performance of bacteria immobilized CA-NFW was quite close to that of free bacteria. We have also tested the reusability of bacteria immobilized NFW after four cycles and ~45% of the dye decolorization capacity was obtained at the end of the 4th cycle. These results are quite promising and therefore suggest that bacteria immobilized electrospun NFW could be quite applicable for the decolorization of dyes in wastewater due to their versatility and reusability.

Introduction

Discharge from industrial plants usually consists of many unwanted effluents, and part of it is taken by dyes. Synthetic dyes are widely used in textile, leather, paper, pulp, plastic and printing industries. At the moment, approximately 8000 chemical problems are known to be associated with the dyeing process. Annually, 700,000 tons of dye related products are fabricated in the world.14 Apart from esthetic viewpoint, most of dyes contain carcinogens such as; benzidine which requires treatment before it is drained into the environment. Furthermore, untreated water reduces solubility of the gas in water resources.5,7 Methylene blue (MB) is one of the common dyes used in the textile industry.8 Yet, the efficient and cost-effective treatment of MB from aqueous systems remains to be a challenge. A range of conventional techniques have been extensively investigated, such as activated sludge,9 carbon adsorption,10 chemical coagulation,11 reverse osmosis,12 electrochemical treatment13, hydrogen peroxide catalysis14 and photocatalysis.15,16

Although some of the techniques are proven to be effective, none of them were successful in complete removal of dye. Furthermore, each of them have their own limitation. Recent research has focused on the development of cost-effective, renewable, eco-friendly, locally available and efficient alternatives such as microorganisms which are capable of biodegrading and biosorbing of the dyes in wastewater. Notably, they are bacteria, fungi and algae, which can decolorize a wide range of dyes with high efficiency.17-20 On the other hand, when compared to algae and fungi, bacteria are easier for culturing, they grow faster and they are able to accumulate contaminants under a broad range of external conditions.21

MB is a basic, thiazine cationic dye which has widespread applications in coloring paper, temporary hair colorant, dyeing cottons, wools and coating for paper stock. It is also used in microbiology, surgery and diagnostics and as a sensitizer in photo-oxidation of organic pollutants.22-27
Electrospinning has recently emerged as a promising technique for the production of nanofiber/nanoweb due to its simplicity, versatility and cost-effectiveness. Electrospun nanofibrous webs (NFW) exhibit unique properties including very large surface area to volume ratio along with nanoscale porosity which makes them promising as filter and membrane materials for environmental applications. The integration of electrospun nanofibers with microorganism can enhance the potential of these NFW for the filtration and purification purposes. Balamurugan et al. summarized the trends in NF membranes and their suitability for air and water filtration. Especially the potential applications are in textile industry, air cleaning in hospitals and other domains. Yet, there are very few studies in the literature on the incorporation of microorganisms in electrospun nanofibers. The results from these studies suggest that, the functional nanofibers containing bacteria or algae have great potential for the environmental practices. For instance, in a study by Klein et al. Pseudomonas sp. ADP was encapsulated in the core part of core-shell nanofibers for the degradation of atrazine which is a widely used herbicide. In another study of Eroglu et al. algal cells were effectively immobilized on electrospun chitosan nanofiber mats to generate a hybrid system for nitrate removal. Moreover, one of the related study was performed by our group in which Acinetobacter calcoaceticus STB1 cells were immobilized on electrospun cellulose acetate nanofibrous webs (CA-NFW) in order to achieve enhanced ammonium removal in aqueous environments. In this study, it was shown that, STB1/CA-NFW can be reused without significant loss of their ammonium removal capacity. It is notable that bacteria is employed in treating wastewater, there are no reports in the context when such bacteria were immobilized on electrospun NFW for the decolorization of dyes. Hence, in this study we comparatively investigated three bacteria for their decolorization efficiency, namely Aeromonas eucnephila, Clavibacter michiganensis and Pseudomonas aeruginosa immobilized on CA-NFW. This immobilization is quite advantageous when compared to free cells in suspension. For instance, the integration of bacteria with the NFW allows us for ease of handling, reusability and doesn't require a separate growth medium. Additionally, the surface attachment of microorganisms onto nanofibers is advantageous which can lead to higher cell sustainability and activity. Moreover, microbial biofilm formation can be supported by nanofiber structures, and as a result the whole system provides a stable and accelerated biodecolorization/degradation.

At present, there is no single process which is capable of treating wastewater. In the light of literature, it will be smart to combine different techniques such as nanofiber and bacteria to be employed in wastewater treatment. Therefore, in our study, the above mentioned bacteria were individually immobilized onto electrospun porous CA-NFW which were then used for the treatment of aqueous medium containing MB. The results suggested that, electrospun CA-NFW were very effective to immobilize bacteria. To maximize the removal capacity of dye through bacteria immobilized NFW, we have studied decolorization time and different dye concentrations. Finally, the reusability of bacteria immobilized CA-NFW was tested.

Experimental

Electrospinning of cellulose acetate nanofibrous webs (CA-NFW)

The electrospinning of porous CA-NFW was performed as detailed in our previous studies. The chemicals were purchased from Sigma-Aldrich (Germany) and used without any purification (dichloromethane, DCM, ≥99% (GC); acetone, ≥99% (GC); cellulose acetate, (CA, Mw: 30000 g/mol, 39.8wt. % acetyl)). A clear electrospinning solution was prepared by dissolving CA in a DCM/acetone (2/1 (v/v)) binary solvent mixture at 7.5% (w/v) polymer concentration. A clear electrospinning solution was prepared by dissolving CA in a DCM/acetone (2/1 (v/v)) binary solvent mixture at 7.5% (w/v) polymer concentration. Then, this solution was taken in a 3 mL syringe fitted with a metallic needle of 0.6 mm inner diameter. The syringe was located horizontally on a syringe pump (model KDS-101, KD Scientific, USA). The electrode of the high-voltage power supply (Spellman, SL30, USA) was clamped to
the metallic needle, and the plate aluminum collector was grounded. Electrospinning parameters were arranged as follows: feed rate of solutions = 1 mL/h, applied voltage = 15 kV, tip-to-collector distance = 10 cm. Electrospun CA-NFW were deposited on a grounded stationary metal collector covered with an aluminum foil. The electrospinning apparatus was enclosed in a Plexiglas box and electrospinning was carried out at 25 °C at 20% relative humidity. The collected nanofibers/nanoweb were dried over night at room temperature in a fume hood.

**Bacterial strain**

This study was performed using pure cultures of the **Aeromonas eucrenophila** (GenBank ID: GQ466170) and **Clavibacter michiganensis** (GenBank ID: GQ466171) which are isolated from water samples taken from water treatment system. While, **Pseudomonas aeruginosa** (ATCC 47085) is taken from Culture Collection of Gazi University, Life Sciences Application and Research Center. **A. eucrenophila** is a Gram-negative, rod-shaped, mainly motile, facultative anaerobic, oxidase positive and glucose-fermenting bacterium. **C. michiganensis** is a Gram positive, rod-shaped and aerobic bacterium. **P. aeruginosa** is a Gram-negative, aerobic, coccobacillus bacterium with unipolar motility. Pure cultures were kept at 4 °C and transferred them to N. Agar medium every 3 months.

**Contaminant: methylene blue (MB)**

MB (C16H18N3ClS, CAS No. 61-73-4) was purchased from Sigma–Aldrich (Germany). The stock solution of MB was prepared (200 mg L⁻¹) in distilled water. The concentration of MB in each aqueous solution was measured on an UV–vis spectrophotometer while taking the absorption at 660 nm.

**Growth and immobilization of bacterial strain**

Nutrient Broth (peptone from meat 5.0 g, meat extract 3.0 g and sodium chloride 6.0 g in 1 L) medium (pH 7) for immobilization experiments was sterilized and inoculated with 1 mL (~10⁷ CFU/ml) of bacterial culture. 20 mg of sterilized CA-NFW were added to the inoculation flasks and incubated at 30 °C in a rotary shaker at 100 rpm for 7 days.

**Morphological characterization of NFW, bacteria and bacteria immobilized CA-NFW**

The morphologies of pristine CA-NFW, bacteria (**Aeromonas eucrenophila**, **Clavibacter michiganensis** and **Pseudomonas aeruginosa**), and bacteria immobilized CA-NFW were investigated by using Scanning Electron Microscope (SEM, Quanta 200 FEG, FEI Instruments, USA). Samples were washed twice with PBS buffer and fixed by overnight incubation in 2.5% glutaraldehyde at room temperature. Then the samples were dehydrated by immersing in a series of ethanol-water solutions ranging from 20% to 100%. Prior to SEM imaging, all samples were coated with a 5 nm layer of gold-palladium.

**MB decolorization test: Effect of contact time**

20 mg of NFW were dropped into 50 mL dye solution (ns 20 mg L⁻¹) and placed on a constant temperature shaking incubator at 30 °C for different time (3, 12, 24 and 48 hours). The same dye concentration (20 mg/L) is employed to obtain positive control with bacterial inoculum, negative control with CA NFW and experimental set with bacteria immobilized CA-NFW.

**MB decolorization test: Effect of initial dye concentration**

Textile processing wastewater with dye contents in the range of 10–200 mg L⁻¹ are highly colored. For this reason, initial dye concentrations were adjusted to 20, 100, 250, and 500 mg L⁻¹ to represent low, medium and high concentrations of dye. 20 mg of NFW and 50 mL different concentrations of MB dye solutions (20, 100, 250, and 500 mg L⁻¹) were put into a 100 mL conical flask. Then the conical flasks were placed on a constant temperature shaking incubator at 30 °C. A 5 mL sample was taken daily from each flask. Samples were centrifuged to precipitate suspended biomass at 3421×g for 10 min. Supernatant was analyzed by the UV-vis spectrophotometer to measure the residual concentration of MB. The results are given as reduced dye concentrations. The removal yield is defined as the ratio of reduced concentration of dye to the initial dye concentrations. Removal percentage is calculated from Eq. (1)

\[ R\% = \frac{C_0 - C_{eq}}{C_0} \times 100 \]  

where \( C_0 \) and \( C_{eq} \) are the initial and equilibrium concentrations of MB (mg L⁻¹), respectively. Each experiment was carried out in triplicate. All determinations were made daily during the incubation period.

**Reusability experiments for bacteria immobilized CA-NFW**

MB decolorization studies were performed 4 times to assess the reusability of the bacteria immobilized NFW for an initial concentration of 20 mg L⁻¹. Before each cycle, NFW pieces were washed three times with sterile PBS buffer and incubated overnight in PBS to remove any unattached bacteria. MB decolorization experiments were performed at 100 rpm and 30 °C for 48 h after each washing step, for a total of 4 cycles. Dye concentrations were measured at 0 h and 48 h. Each cycle was terminated after 48 h of total incubation and washing steps were repeated for NFW samples before the initiation of the next cycle. All tests were done in triplicate.
Results and discussion

Immobilization of *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* on CA-NFW

Schematic representation of the electrospinning process for CA nanofibers, attachment of bacterial cells on CA-NFW and photograph of the decolorization test are shown in Fig. 1. Fig. 2 shows the representative SEM images of porous CA-NFW. Generally, electrospun CA nanofibers have smooth fiber morphology. However, we can produce porous CA nanofibers by employing highly volatile solvent mixture (DCM/acetone) as reported in our previous study. CA-NFW is an effective support and has an advantage for facilitating the diffusion of vital nutrients and waste products between the environment and the bacteria. In addition, these webs are biodegradable and biocompatible, which can be rendered for biological applications so CA is quite applicable and favorable especially for the wastewater treatment purposes.

Figure 2. Representative SEM images of electrospun CA nanofibrous web (a) low magnification, and (b) high magnification.

Representative SEM images (after 24 h of incubation) are shown in Fig. 3a, 3b and 3c for *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* cells, respectively. As seen in Fig. 3, *A. eucrenophila* and *C. michiganensis* are rod shape, and *P. aeruginosa* has coccobasil morphology. SEM analysis was performed after 7 days of incubation for the bacterial attachment to NFW and shown in Fig. 4a-c. As seen in the Fig. 4, after 7 days, three types of bacteria were attached and formed biofilm layers on the NFW. Biofilms are communities of microorganisms in a matrix that joins them together and to living or inert substrates. In the light of literature, we know that *P. aeruginosa* has been extensively utilized as a model organism for the biofilm experiments. Moreover, San *et al.* showed that *A. eucrenophila* and *C. michiganensis* produced compact biofilm and caused microbial corrosion.

Decolorization assay

The effects of experimental parameters such as contact time (0–48 h) and initial dye concentration (20–500 mg L\(^{-1}\)) on the decolorization of MB were studied. The decolorization of dyes by bacteria could be due to adsorption by microbial cells or biodegradation. Dye adsorption can be clearly judged by inspecting the cell mats. Cell mats become deeply colored because of the adsorbed dyes, whereas those retain their original color when biodegradation takes place. In our study, we observed that bacteria decolorized the MB by degradation. In addition, no decolorization activity was detected in the supernatant of culture media after the removal of cells. This implies that no secreted enzyme or any other bioproduct might be involved in decolorization.

Figure 3. General morphology of (a) *Aeromonas eucrenophila* (b) *Clavibacter michiganensis* (c) *Pseudomonas aeruginosa* by SEM.

Figure 4. Representative SEM images of CA nanofibrous web after immobilization of (a-i, a-ii) *Aeromonas eucrenophila*, (b-i, b-ii) *Clavibacter michiganensis*, (c-i, c-ii) *Pseudomonas aeruginosa* after 7 days.
Contact time is one of the important parameters to successfully adsorb the pollutants for practical application. To ensure that adequate time was given for the decolorization of MB, the optimum decolorization time of MB by bacteria immobilized CA-NFW was determined. Free bacteria and pristine CA-NFW were also tested for comparison. The MB decolorization performance of bacteria-free CA-NFW, free bacteria and bacteria immobilized CA-NFW after certain time period (3 h, 12 h, 24 h and 48 h) are shown in Fig 5a, 5b and 5c, respectively. As seen in Fig 5a, bacteria-free CA-NFW was responsible for the initial removal of MB dye by adsorption from the aqueous environment due to its very high surface area. The maximum removal of MB was around 15% after 48 h. On the other hand, the MB decolorization by free bacteria and bacteria immobilized CA-NFW was increased dramatically in the first 24 h in which the MB removal was around 95% and a very slight improvement was observed after 48 h. The highest % removal of MB dye was obtained after 48 h, therefore, 48 h time period was selected as a suitable contact time for all the other decolorization tests we have performed. After 48 h, % removal of MB dye was calculated as 15.8±1.3 % for bacteria-free CA-NFW. Yet, much efficient MB removal was achieved by bacteria immobilized CA-NFW; 96.5±0.4 %, 96.1±0.4 % and 95.6±0.3 % for P. aeruginosa, A. eucrenophila and C. michiganensis immobilized CA-NFW, respectively. Likewise, in the case of free bacteria, P. aeruginosa (97.3±1.4) had slightly better decolorization performance than A. eucrenophila (95.6±1.2) and C. michiganensis (97.1±2.4) (Fig. 5b). Slightly higher efficiency of P. aeruginosa might be originated from rapid growth and dye resistance properties of Pseudomonas which is widely used for decolorization of textile dyestuff in industry wastewater.

Our results showed that decolorization performance of bacteria immobilized CA-NFW are very impressive and quite close to the free bacteria, nevertheless, using bacteria immobilized CA-NFW has certain advantages than using free bacteria. Firstly, bacteria immobilized NFW can be reusable whereas free bacteria cells when dispersed throughout the medium/wastewater, it is quite difficult to harvest and reuse them in another dye-contaminated wastewater. Secondly, bacteria immobilized NFW occupy less space and require smaller volume of growth medium when compared to stock solutions of free bacteria. So, bacteria immobilized NFW are more practical and cost-effective. Finally, biofilm formation in bacteria immobilized NFW bring some advantages such as higher resistance to harsh environmental conditions such as salinity and metal toxicity.

Bacteria immobilized CA-NFW have shown efficient decolorization of MB dye within 48 h. In addition, effect of dye concentration on decolorization using A. eucrenophila, C. michiganensis and P. aeruginosa immobilized CA-NFW were investigated at different initial dye concentrations between 20 mg L⁻¹ and 500 mg L⁻¹ at pH 7 and the results are shown in Fig 6.
At the end of the 48 h incubation period, the maximum % removal for 20 mg L\(^{-1}\) MB solution was 96.1±0.4 %, 95.6±0.3 % and 96.5±0.4 % for *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* immobilized CA-NFW, respectively. However, the % decolorization capacity decreased with an increase in the dye concentration as expected. For 100 ppm dye concentration, the decolorization capacity of MB by *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* immobilized CA-NFW were 69.9±1.8 mg L\(^{-1}\), 65.9±1.6 mg L\(^{-1}\) and 61.9±1.3 mg L\(^{-1}\), respectively. When dye concentration is increased to 250 ppm, the decolorization capacity was 124±1.2 mg L\(^{-1}\), 120.5±1.4 mg L\(^{-1}\) and 108.6±1.4 mg L\(^{-1}\) for *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* immobilized CA-NFW, respectively. In the case of 500 ppm MB concentration, the removal of MB dye was 115.3±0.4 mg L\(^{-1}\), 96.1±0.4 mg L\(^{-1}\) and 95.6±0.3 mg L\(^{-1}\) for *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* immobilized CA-NFW, respectively. It was noted that more or less 100 mg L\(^{-1}\) dye was removed by all three bacteria from the solutions having dye concentrations of 100, 250 and 500 mg L\(^{-1}\). It is anticipated that the dye removal capacity is relevant to amount of bacteria and nanofiber. Hence, decolorization can be improved with the increase of bacteria and nanofiber quantity.

**Reusability results**

Dye decolorization capabilities of reused bacteria immobilized CA-NFW were tested for four cycles of reuse (Fig. 7). It was observed that the MB decolorization efficiency was decreased for higher cycles. The decline in the removal efficiency might be due to detachment of the immobilized bacteria in the washing step. After the three cycles of regeneration, favorable % removal of MB dye was observed as 58.3%, 50.1% and 58.6% for *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* immobilized CA-NFW, respectively. For the 4th regeneration cycle, the MB decolorization dropped to 45.7%, 43.1% and 48.04% for *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* immobilized CA-NFW, respectively. For practical applications, the level of reusability is an important issue. ~45% of the dye decolorization capacity was obtained for the final cycle (4th cycle) which suggests that *A. eucrenophila*, *C. michiganensis* and *P. aeruginosa* immobilized CA-NFW can sustain their decolorization capacity under several cycles of reuse and may be utilized repeatedly for dye decolorization of wastewater in textile and paint industry. After completion of four cycles for reusability test, the morphology of the used bacteria immobilized CA-NFW was studied by SEM, and the images (Fig. 8) confirmed that the fibrous morphology and bacteria was retained.

**Conclusions**

We have chosen electrospun CA-NFW as water-insoluble, non-toxic, and highly porous support for immobilization of three types of bacteria which are capable of dye decolorization in wastewater. MB dye was chosen as the target contaminant since it is extensively used in textile and paint industry. The bacteria immobilized CA-NFW were quite successful for the removal of MB dye from the aqueous environment. Due to its simple, reusable and porous characteristics, this NF bio-composite can be a promising membrane material for industrial wastewater treatment. Moreover, this process can be considered harmless for aquatic life during the dye decolorization process.
To the best of our knowledge, our work presents the first detailed study on dye decolorization by bacteria immobilized electrospun NFW. Here, electrospun CA-NFW was found very effective porous solid support for immobilizing bacterial cells. We have immobilized three types of bacteria (A. eucrenophila, C. michiganensis and P. aeruginosa) on CA-NFW which are capable of decolorization of MB dye solution by degradation. The efficient dye decolorization was achieved within 24 hours and % removal was about 95%. The reusability of bacteria immobilized NFW was determined after four cycles and ~45% of the dye decolorization capacity was obtained at the end of the 4th cycle suggesting that these bacteria immobilized CA-NFW can be reused and may be utilized repeatedly for dye decolorization in industrial wastewater. Our results showed that MB dye decolorization performance of bacteria immobilized CA-NFW was quite close to the free bacteria. In short, our findings may be useful for designing and developing an efficient and cost-effective treatment process for the decolorization of dye from industrial wastewater by using bacteria immobilized NFW.

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