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Biofouling control on ultrafiltration membrane through immobilization of polysaccharide-degrading enzyme: optimization of parameters

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ABSTRACT

Membrane biofouling remains a significant challenge in the application of ultrafiltration (UF) pretreatment systems in desalination and water industries. Bacterial biofilms produce extracellular polymeric substances, which contain alginate as a major component. There has been an ongoing search to look for passive/non-chemical means of mitigating this problem. We present a method based on immobilization of a polysaccharide-degrading enzyme, alginate lyase (Alg L), onto cellulose acetate membrane to control biofilm formation. Various parameters like Alg L concentration, cross-linker concentration and pH were optimized. Two immobilization procedures were adopted and the Alg L immobilization efficiency of each method was compared. Activation of membrane with a cross-linking agent, followed by Alg L immobilization was found to be relatively more effective. Immobilization was confirmed by determining the activity of the immobilized enzyme; viscosity decrease corresponding to enzymatic degradation of the substrate was observed. The immobilization protocol was found to be highly reproducible. The ability of the test membrane to mitigate *Pseudomonas aeruginosa* biofilm formation was confirmed by scanning electron microscopy. The results show that Alg L immobilization on UF membrane can be used for controlling polysaccharide fouling on membrane filters used in advanced water purification techniques.

Keywords: Enzyme immobilization; Alginate lyase; Ultrafiltration; Membrane biofouling; Glutaraldehyde

1. Introduction

Membrane filters are widely used in advanced water purification techniques. Natural water contains organic and inorganic substances that cause membrane fouling. Biofouling, which is often termed as biotic form of organic fouling, contributes to about 45% of fouling occurring on membranes [1]. Formation of biofilm can lead to adverse effects such as decline in membrane flux, requirement of increased differential

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pressure and feed pressure, membrane biodeterioration, increased salt passage and reduced product water quality [2–7].

Conventionally, biofouling control is achieved by: (a) biocide treatment using oxidizing biocides such as chlorine, chlorine dioxide, chloramines, iodine and hydrogen peroxide [8–10] or non-oxidizing biocides like formaldehyde, glutaraldehyde and quaternary ammonium compounds [11] and (b) limiting the supply of nutrients like carbon and phosphorous [12,13] during the pretreatment stage. However, there are drawbacks with these methods. Application of chlorine and chloramines results in the formation of undesirable disinfection by-products (DBPs) and damage to the membrane [1,9]. Use of non-oxidizing biocides, on the other hand, may lead to development of resistance in the microbes present in the water [11]. The disadvantage with nutrient limitation is that it is carried out using chemical precipitants like alum, lime, iron salts and polyelectrolytes, resulting in high maintenance cost, sludge handling and disposal problems [14]. Biological controls methods include use of quorum sensing [15–17], bacteriophages [18,19] and surfactants [20–22]. Surface modification methods have also been attempted to control membrane biofouling. Surface modification by polyethylene glycol and zwitterionic molecules prevented the adsorption of proteins and polysaccharides present in biofilms [23–25], but the total control of adsorption was difficult. The immobilization of antibacterial agents like nano-sized titanium dioxide, silver, etc. on to the membranes is also an effective method of preventing biofouling [26–28]. However, the nanoparticles require light irradiation [27] or elution of metal ions into water to generate antibacterial active species [26,28]. Extracellular polymeric substances (EPS) play a very important role in membrane biofouling. Bacteria present in water accumulate on the membrane surface along with organic and inorganic substances, proliferate and form biofilm by producing the EPS [27]. EPS in either bound or soluble/colloidal form is currently considered as the major cause of membrane fouling in membrane bioreactors [28]. It binds the microbes within a three-dimensional matrix, thereby influencing the physicochemical characteristics of the microbial aggregates, affecting the mass transfer, surface characteristics, adsorption ability and stability [29]. EPS also contributes to the mechanical stability of biofilms, allowing them to withstand considerable shear forces [30]. The main fractions of EPS are polysaccharides, proteins, nucleic acids and humic substances arising from cell lysis, secretion or from sources already present in the influent [31]. Although EPS is an irreversible foulant [32], it can be removed from surfaces by traditional physical and chemical methods. Enzymes can break down EPS and prevent biofilm formation [33]; this treatment strategy can be considered passive as it does not involve use of chemical biocides or produce any harmful by-products. Addition of these enzymes in bulk water may not be economically feasible [34]. Fouling is a problem affecting the surfaces, hence it is imperative that it is mitigated at the very surface. Thus, immobilization of polymer-degrading enzymes on the membrane surface can be a best suited alternative. Proteins and polysaccharides both form integral part of the EPS. Fouling of reverse osmosis (RO) and nanofiltration (NF) membranes with bovine serum albumin, alginate and galacto-oligosaccharides, as model protein and polysaccharide, has been reported [35–37]. Fouling mitigation through EPS degradation using hydrolytic enzymes in free form has been recently reported [38]. Koseoglu-Imer et al. reported the immobilization of savinase enzyme for reducing protein fouling on cellulose acetate ultrafiltration membrane [39]. In another study, Saeki et al. reported the immobilization of lysozyme on to RO membranes to reduce bacterial fouling [40]. Immobilization of laccase enzyme from *Pleurotus sajor-caju* on polyamide membrane was also reported for the treatment of industrial effluents [41]. Although proteases have been immobilized on membrane for biofilm control [39], there are no studies reported on the use of polysaccharide-degrading enzymes in immobilized form in controlling biofilms. In this work, we have attempted to immobilize a polysaccharide-degrading enzyme (alginate lyase from *Sphingomonas* sp.) by covalently linking it onto cellulose acetate membrane surface. We present data on the optimization of various parameters like cross-linker concentration, cross-linker solution pH and enzyme concentration used in the immobilization process. Further, we also present data on biofilm inhibition by the enzyme-immobilized membrane using *Pseudomonas aeruginosa* as a test strain.

2. Methodology

2.1. Materials

Cellulose acetate (CA) ultrafiltration (UF) flat sheet membrane (UC 100T) was supplied by Microdyn Nadir. It is a hydrophilic membrane and has interconnected pores with a molecular weight cut-off of 100 kDa. Alginate lyase (Alg L) (EC 4.2.2.3) from *Sphingomonas* sp. in solid powder form (10,000 U/g solid) and alginate were purchased from Sigma Aldrich (St. Louis, MO, USA). Glutaraldehyde (GTA) (25% v/v; AR grade) was used as a cross-linking agent between

the enzyme and the membrane and was obtained from Merck, Germany and was used without further purification. NaOH, 1 M was used for pH adjustment.

2.2. Optimization of sequence

Immobilization of alginate lyase on CA membrane was approached in two different ways. In method 1, the membrane was treated with GTA, followed by enzyme treatment, while in method 2, the membrane was first treated with enzyme solution, followed by addition of GTA. Both the protocols are summarized in Table 1. As a common step for both processes, the membrane was cut into 2 × 2 cm pieces and immersed in 20 mM phosphate buffer pH 7.0 for 15 min and rinsed thoroughly to remove any impurities present. In method 1, the membrane was activated using 2 ml of glutaraldehyde (0.6% v/v, pH 5.0) for 1 h, followed by addition of 5 ml of Alg L solution (50 μg/ml phosphate buffer, pH 7.0) and the enzyme was allowed to react with the activated membrane for an hour. This was followed by washing the membrane thrice with 20 mM phosphate buffer at pH 7.0. In case of method 2, the membrane was immersed in 20 mM phosphate buffer, pH 7.0 for 15 min. The enzyme Alg L solution (50 μg/ml phosphate buffer, pH 7.0) was allowed to react with the membrane, followed by activation with a cross-linker (0.6% v/v, pH 5.0) for an hour. Finally, the membrane was washed thrice with 20 mM phosphate buffer, pH 7.0, to remove unbound enzyme. After the washing steps, the immobilized membrane was stored in moist condition at 4°C until further use.

2.3. Alginate lyase activity

Alg L activity was determined by estimating the release of uronic acids by the degradation of alginate (0.1% w/v in 20 mM potassium phosphate buffer pH 6.3). As per the manufacturer's instruction, one unit will produce an increase in absorbance ($A_{235\ nm}$) of 1.0 per minute per ml of sodium alginate solution at pH 6.3 at 37°C. The total enzyme immobilized was calculated as shown in Eq. (1). The immobilized Alg L activity was directly determined using alginate as substrate. In Eq. (1), the initial enzyme activity corresponds to the activity of the enzyme used for immobilization (50 μg/ml), whereas the final enzyme activity corresponds to the activity obtained in wash buffer after the immobilization process. The surface area of the membrane was 4 cm^2.

$$\text{Total enzyme immobilized } \left(\text{U/cm}^2\right) = \frac{\text{Initial enzyme activity} - \text{Final enzyme activity}}{\text{Surface area of the membrane}} \quad (1)$$

2.4. Viscometric analysis

Alginate is a linear copolymer of guluronic acid and mannuronic acid linked by glycosidic bonds and is viscous in nature. Alg L degrades alginate by attacking the glycosidic linkage, resulting in the release of free oligosaccharides, leading to decrease in alginate's viscosity. Therefore, viscometry can be used to correlate with the activity of the Alg L. If the enzyme bound to the test membrane retains its activity, it will react with the substrate, which will lead to decrease in viscosity, thereby indicating that the Alg L immobilized on to the membrane is active. The test membrane and substrate were allowed to react for 5 min, after which the viscosity of the alginate solution was measured using a rotational rheometer (Anton Paar Physica MCR 301) with cone and plate geometry at a shear rate of 63.1 s^{-1}.

2.5. Immobilization procedure

For optimization studies, membrane size of 2 × 2 cm was used. From hereafter, all data presented

Table 1
Shows the schematics of immobilization procedure, method 1 (MGE) and method 2 (MEG)

Method 1: MGE	Method 2: MEG
Step 1: Membrane immersed in 20 mM phosphate buffer pH 7.0 for 15 min	Step 1: Membrane immersed in 20 mM phosphate buffer pH 7.0 for 15 min
Step 2: Membrane activation with 2 ml glutaraldehyde (0.6%, pH 5.0) for an hour	Step 2: Immobilization with 5 ml alginate lyase (50 μg/ml) solution pH 7.0 for an hour
Step 3: Immobilization with 5 ml alginate lyase (50 μg/ml) solution pH 7.0 for an hour	Step 3: Glutaraldehyde (0.6%, pH 5.0) was added for an hour for membrane activation
Step 4: Final wash and immobilized enzyme activity determined	Step 4: Final wash and immobilized enzyme activity determined

are from the procedure discussed in Table 1 in method 1. The experiment was performed thrice in triplicates, every time with a new membrane.

2.6. Optimization of cross-linker concentration and pH

Various concentrations of GTA ranging from 0.1 to 5.0% were used for optimizing the GTA concentration and pH. The experiments were done at pH ranging from 4.0 to 9.0. GTA pH was altered using 1 M NaOH. Two ml of solution of different pH values was added onto the membrane separately in the step 2 of the immobilization procedure. After the enzyme immobilization procedure, the activity of immobilized enzyme was checked to confirm immobilization.

2.7. Optimization of Alg L concentration

Five ml of various concentrations of enzyme solution ranging from 20 to 500 μg/ml were used for optimizing enzyme concentration. The enzyme was treated with the activated membrane and allowed to react as shown in the step 3 of the immobilization procedure.

2.8. Reproducibility of enzyme immobilization method 1 (MGE)

The method was repeated six times to confirm the reproducibility of the method. The activity analysis of the immobilized enzyme confirmed the reproducibility of the method.

2.9. Biofouling with P. aeruginosa

2.9.1. Culture preparation

P. aeruginosa (wild type) was used as the test strain for the biofilm studies [42]. A 24-h-old single colony grown on Luria Bertani agar plate was picked up and inoculated into Luria broth. The culture was incubated overnight at 37°C. The cells were pelleted down, resuspended in PBS and the optical density at 600 nm was adjusted to 0.025, which corresponded to a cell density of 10^6 CFU/ml. This cell suspension was used as inoculum for biofilm studies.

2.9.2. Biofilm experiment

Both test and control membranes were allowed to foul with 10^6 CFU/ml of test strain in half strength Luria Bertani broth and incubated for 24 h at 37°C. The membranes were then rinsed by gentle swirling with 1X PBS (pH 7.2) for a few minutes and processed for SEM analysis.

2.10. SEM analysis

SEM was used to examine the surface of the membrane after it underwent Alg L immobilization and fouling with *P. aeruginosa*. The membranes were fixed in 2% GTA (v/v) for 15 min followed by air-drying for 5 min. This was followed by dehydration of the samples in 30–100% ethanol gradient for 10 min each. The air-dried samples were mounted on aluminium stubs, sputter-coated with gold and imaged with a VEGA 3 TESCAN scanning electron microscope.

3. Results and discussion

3.1. Optimization of immobilization procedure

To optimize the immobilization procedure, both methods MGE and MEG reported in the literature were adopted and the activities of the respective immobilized Alg L were compared. Higher activity of Alg L was observed when Alg L was immobilized by MGE method, as shown in Fig. 1. One of the major possibilities of cross-linking of Alg L to CA membrane is as follows. In the first step at pH 5.0, the aldehyde groups react with the hydroxyl group of the cellulose via condensation reaction. In the next step, the nucleophilic amine of Alg L attacks the carbon on the GTA backbone leading to cross-linking of the Alg L onto the cellulose. The schematic of the MGE method is illustrated in Fig. 2. At low pH, GTA is present as monomer in its free aldehyde form, as hydrate or hemiacetal [43]. According to the authors, the

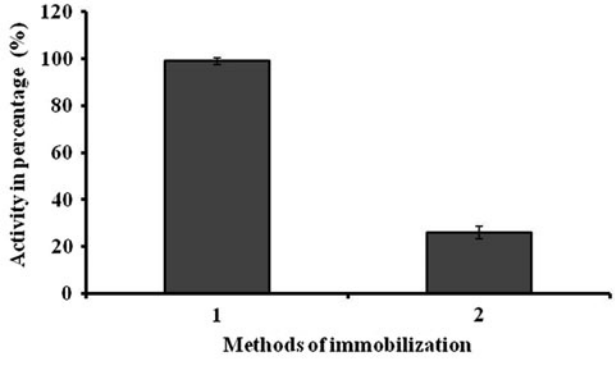

Fig. 1. Enzyme activity following two methods of immobilization: (1) MGE and (2) MEG. Amongst the two methods, the one yielding better activity was taken as 100 ± 2%. The experiment was performed five times, each time in triplicates. Error bar indicates standard deviation.

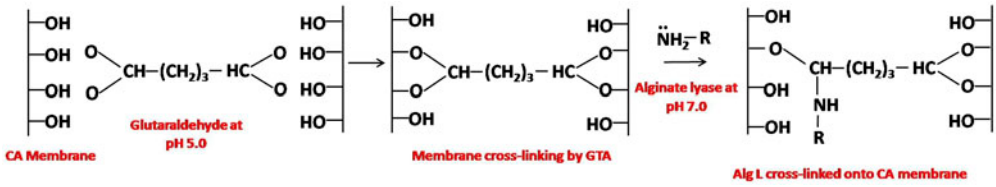

Fig. 2. Probable mechanism of alginate lyase immobilization onto cellulose acetate membrane by the MGE method.

activation of support base is carried out at low pH to catalyse acetal formation, while the coupling of biomolecule is conducted at higher pH to promote nucleophilic attack onto the carbonyl group, resulting in better immobilization, corroborating our results. Alternatively, when immobilization is carried out by the MEG method, cross-linking among the amino acids in the protein molecule by GTA may change the conformational configuration of the polypeptide chain, affecting the active site of the enzyme, thereby leading to enzyme activity loss [44,45]. Since in the MGE method, the Alg L immobilization is carried out after preactivating the membrane with GTA. The lysine groups in the Alg L are involved in binding to GTA; therefore, few structural changes occur in the protein, ensuring higher activity.

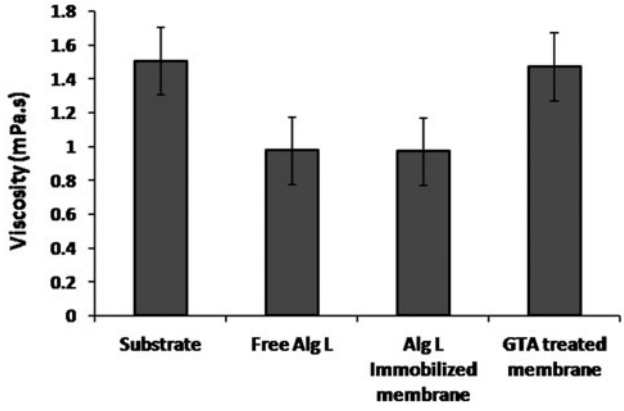

Fig. 3. Change in substrate (alginate) viscosity after treatment with free Alg L (normalized to loading on membrane), Alg L immobilized membrane and glutaraldehyde (GTA)-treated membrane.

3.2. Alg L activity in immobilized form

The Alg L activity of the test membrane was determined in terms of degradation of alginate to uronic acids, which is measured spectrophotometrically at 235 nm. Alg L degrades alginate into smaller molecules, thereby reducing the viscosity of the solution. Viscometry analysis of the alginate solution exposed to test membrane was carried out and the values were compared with the viscosity of alginate solution left in contact with raw membrane, GTA-treated membrane and free enzyme (normalized to the enzyme loading on the membrane). As shown in Fig. 3, the results showed that the alginate solution (0.1%) had an inherent viscosity of 1.505 mPa s. Both free and immobilized Alg L-treated alginate showed similar values of viscosity. The GTA-treated control membrane showed no decrease in viscosity in the alginate solution, indicating that it does not degrade alginate. Thus, the result clearly showed that the observed decrease in viscosity was due to alginate degradation by Alg L, which was not rendered inactive by the immobilization process.

3.3. Optimization of GTA concentration and pH

Membrane activation was carried out using GTA as a cross-linker. GTA is used as an activator in the immobilization procedure due to its high reactivity with amino group [46]. Optimization of GTA was performed by altering (a) concentration of GTA and (b) pH of the GTA solution. Fig. 4(A) shows the concentration of GTA ranging from 0.1 to 5% used for membrane activation. The activity in percentage was determined after the final step of the immobilization procedure, which showed that there was no significant difference in activity at all the concentrations tested. A slight increase in activity was seen at 0.6%, v/v GTA.

The pH of GTA solution is as shown in Fig. 4(B), the highest activity was observed at pH 5.0. We found that the pH played a significant role in Alg L immobilization; the maximum activity was observed when coupling was done at pH 5.0. At high pH values, the reactivity of ε-amino acids of Lys groups is expected to be quite low and the strength of the multipoint covalent attachments is not very high [44].

3.4. Optimization of Alg L concentration

Alg L concentrations ranging from 20 to 500 μg/ml were used for immobilization as shown in Fig. 5.

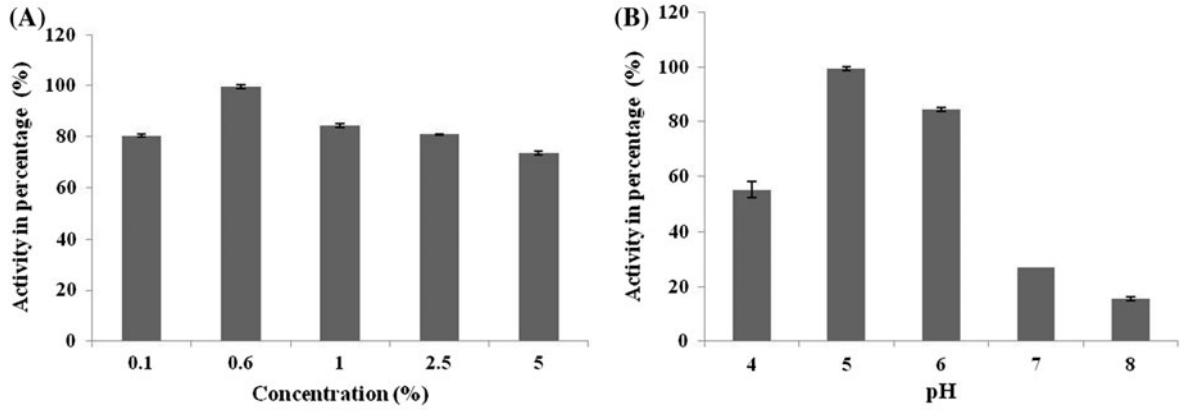

Fig. 4. Optimization of GTA concentration (A) and pH (B) used in the immobilization process. The experiment was performed five times each time in triplicates. The error bar indicates less than 1% standard deviation.

At Alg L concentration of 20 μg/ml, the membrane displayed 45% activity, whereas at 50 μg/ml the membrane showed the highest activity. Beyond 50 μg/ml, the activity steadily decreased, which was likely to be due to lack of reactive groups present on the membrane surface for enzyme attachment [39]. This phenomenon can also be caused by the overcrowding of the enzyme on the surface, thereby masking the active sites and resulting in decreased activity [47]. Excess of enzyme loading may cause protein–protein interaction, which can pose constraints on flexible stretching of the enzyme conformation, resulting in steric hindrance and, thereby, reduced enzyme activity [48]. The loss of enzyme activity due to increased enzyme loading has been widely reported [49–51].

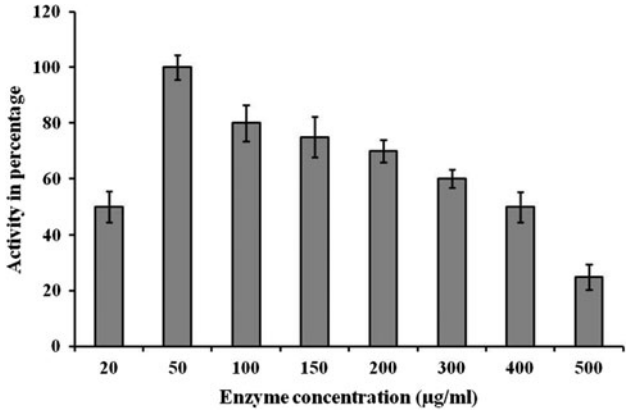

Fig. 5. Optimization of enzyme concentration. The experiment was performed five times, each time in triplicates and the means are plotted. The error bars represent standard deviation.

3.5. Reproducibility of enzyme immobilization method 1 (MGE)

The reproducibility of the immobilization procedure was examined by repeating the procedure under identical conditions, as shown in method 1 of Table 1. As shown in Fig. 6, the immobilization procedure was performed six times, each time in triplicates. These results showed that the immobilization procedure was reproducible (Fig. 6).

3.6. Microscopic study of Alg L immobilized membrane

The SEM images of the CA membrane before and after immobilization show the presence of particulates on GTA and test membrane (Fig. 7), which are suspected to have accumulated during the activation and immobilization steps. However, the results clearly

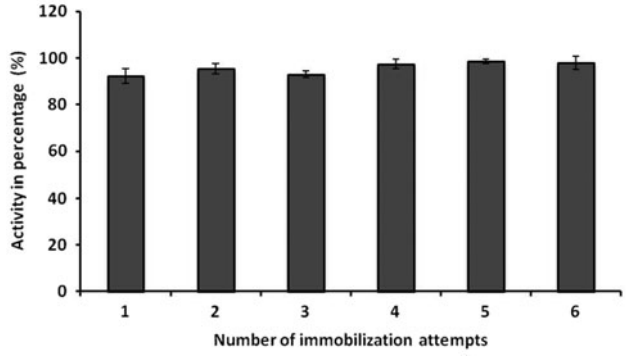

Fig. 6. Reproducibility of the immobilization procedure. The error bars (SD) indicated that the method was highly reproducible (CV < 3%).

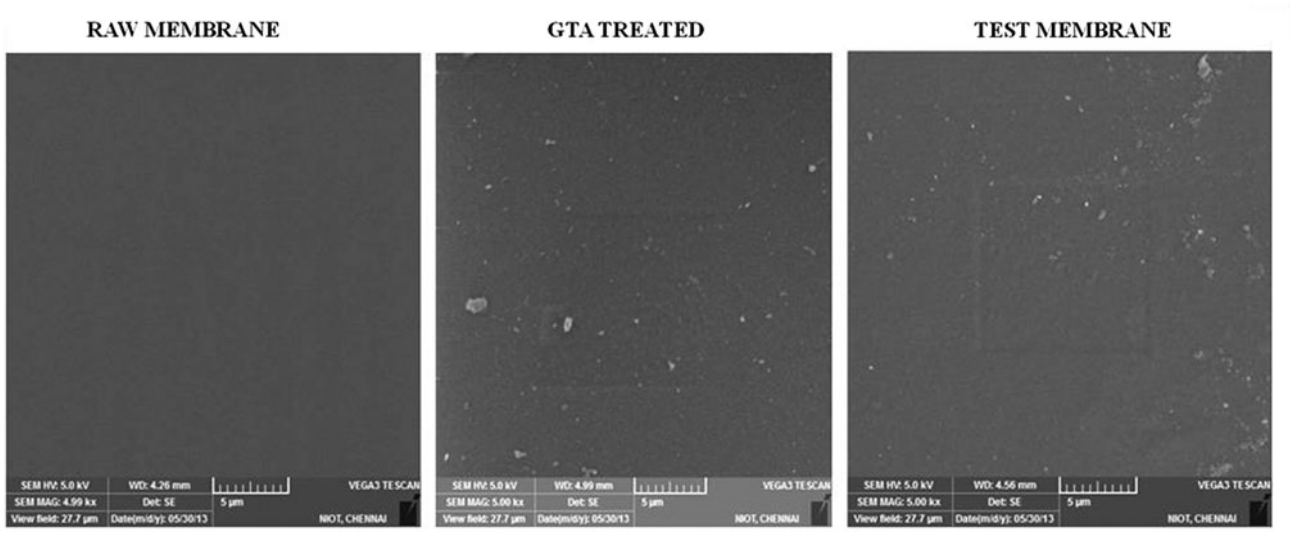

Fig. 7. SEM images of cellulose acetate UF membrane before and after immobilization. Glutaraldehyde (GTA)-treated membrane and test (enzyme immobilized) membranes are compared with untreated membrane.

indicate that the membrane integrity and functionality are maintained.

3.7. Structural evidence of biofouling inhibition

The SEM images of membranes fouled with *P. aeruginosa* (Fig. 8) confirm our observation on the ability of the test membrane to resist biofouling. The raw membrane shows colonization by *P. aeruginosa* within 16 h of incubation (Fig. 8(A)). GTA, used as a cross-linker between membrane and Alg L, is known for its bactericidal properties [52] and therefore, it was ascertained whether GTA treatment alone had any effect on biofilm formation. The result (Fig. 8(B)) clearly showed attachment of *P. aeruginosa* on the GTA-activated membrane. The morphology of the organisms showed signatures of cell damage. On the test membrane (Fig. 8(C)), Alg L clearly prevented biofilm formation, probably by degrading the bacterial EPS so that the cells could not anchor themselves to the membrane surface and develop into microcolonies, which are a distinctive feature of biofilms [53].

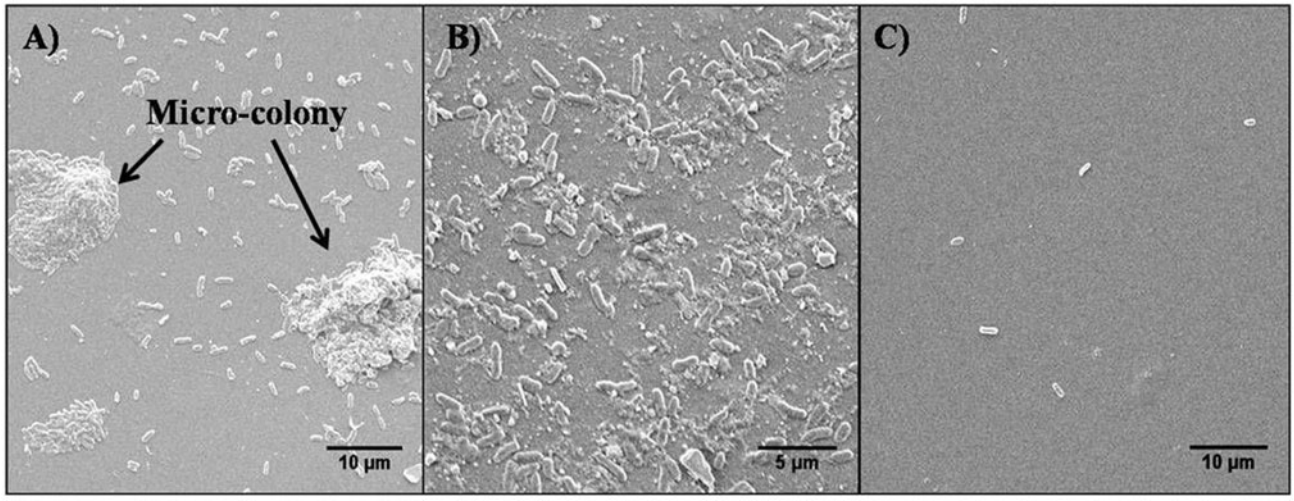

Fig. 8. SEM images of biofilm formation on untreated (raw) (A), GTA activated (B) and Alg L-immobilized (C) membranes after 16 h exposure to suspension of *P. aeruginosa*.

Considering the fact that alginate plays a major role in the formation and maintenance of biofilm architecture, Alg L can potentially destroy most types of biofilms—both single species and multispecies. However, alginate is only one of the polymers that constitute the EPS matrix. EPS produced by complex biofilms would normally consist of a mixture of macromolecules and therefore, their mitigation would require other types of enzymes, which also need to be considered. Biofilm EPS is known to harbour other types of polymers such as proteins and eDNA. One may need to incorporate additional enzymes (e.g. proteases and DNases) to effectively destabilize complex multispecies biofilms. Experiments also need to be carried out with surface water and wastewater, which may incorporate components other than alginate such as organics and inorganics into the biofilm matrix. Furthermore, alginate lyase in the membrane may undergo denaturation after exposure to the DBPs or biocide residues. This would necessitate removal of residual biocides used in pretreatment and the DBPs produced thereof. It is expected that the enzyme-immobilized membrane may not require any chemical cleaning, as the immobilized enzyme would not allow biofilm to adhere tightly to the membrane. The fouling can be easily removed by incorporating a backwash step in the membrane cleaning procedure. The enzyme immobilization method described here can be employed for mitigation of biofouling on membrane filters used in water treatment, without using harmful chemicals that can potentially damage the membrane and reduce its service life. However, its large-scale application in water and wastewater treatment requires further investigations.

4. Conclusion

In this study, Alg L was successfully immobilized onto cellulose acetate UF membrane by means of covalent bonding using GTA as a cross-linking agent. The optimum conditions of the immobilized enzyme were GTA concentration of 0.6%, v/v, and pH of 5.0. The optimum enzyme concentration was found to be 50 μg/ml for a membrane dimension of 2 × 2 cm. The immobilization procedure sequence of membrane activation by GTA, followed by enzyme addition, gave excellent results in terms of enzyme activity and reproducibility. SEM images revealed that the membrane retained its integrity in spite of the immobilization process. Biofouling experiment indicated that the Alg L immobilized membrane could inhibit biofilm formation, indicating potential for industrial application. The method can be employed for biofouling mitigation of membrane filters used in water treatment, without the use of chemicals that can potentially damage the membrane.

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