SELECTION OF BACTERIAL POOL FOR STUDYING BIOFILM FORMATION

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ABSTRACT

Multispecies biofilms formed on reverse osmosis membranes for water treatment generate operational problems that directly affect the quality/quantity of the water produced. Studies of the effectiveness of cleaning these membranes reinforce the importance of a good biofilm-producing inoculum for obtaining a robust analysis. The standardization of biofilm formation is essential for the subsequent inactivation and cleaning processes. This work aimed to evaluate the influence of factors such as the type of inoculum (bacterial pool) and nitrogen/carbon source on the structure and chemical composition of the multispecies biofilm on a polystyrene microplate. Four bacterial pools from two oil-refining water treatment units, three nitrogen, and two carbon sources were tested. The quantitative response variables studied were planktonic growth, the medium's final pH, and carbon source consumption. Biofilm formation was also assessed using quantitative (crystal violet) and qualitative methods (image analysis). The results showed that the type of inoculum and nutrient composition are essential for the selection of biofilm-forming bacteria.

Keywords: Multispecies biofilm. Biofouling. Cleaning processes. Enzymes.

1 INTRODUCTION

The main problem with water purification using membrane processes is biofouling[1]. Biofouling reduces membrane performance, as reflected by declining permeate flux, reduced selectivity, membrane biodegradation, and increased energy consumption[2]. Biofouling studies have focused on identifying microbial communities (biofilm), excretion of extracellular polymeric substances (EPS), and their combined role in reducing membrane performance and lifetime[3].

Biofilm consists of sessile microorganisms that produce a matrix with organic and hydrated polymers. Its purpose is to defend the microorganism against the elements of the external environment (variation in pH, temperature, nutrients, action of chemical and physical agents), innate and acquired responses, and the action of antimicrobials[4].

Selecting a biofilm-forming bacteria pool for subsequent cleaning and inactivation assays depends on nutrient[5] and cultivation conditions. The present work aimed to evaluate the influence of the nitrogen source (urea, ammonium phosphate, and ammonium sulfate) and the carbon source (glucose and glycerol) for the selection of a biofilm-forming bacterial pool (A-A, A-S, B-A, and B-S).

2 MATERIAL & METHODS

The biofilm formation on the membrane surfaces was studied using water samples collected from feed and concentrate streams of reverse osmosis units of two oil refineries (A and B). Samples were identified as A-A and B-A (feed water) and A-S and B-S (wastewater). Bacterial isolation was performed by filtering raw water samples through a sterile 0.22 µm membrane. The retained material was resuspended in 20 mL of peptone water (0.1% m/v) containing glycerol (20% v/v) and stored in a freezer at -80 ºC. The inoculum was reactivated in nutrient broth and cultivated in BOD for 12 hours, 30 °C at 150 rpm.

To select the biofilm-forming inoculum, eight different media with different compositions were tested (Table 1), varying 200 ppm carbon source (glucose and glycerol) and three 1 mmol/L nitrogen sources (urea, ammonium sulfate, and ammonium phosphate). The osmolarity (100-300 µS), pH (5.0-5.5), and bacterial concentration (10^6) were kept fixed. The biofilm was grown at the bottom of the 96-well microplate, 200 µL/well, and incubated in BOD at 30 °C, 150 rpm for 1, 3, and 5 days of evaluation. Table 1 presents the composition of each medium.

After 1, 3, and 5 days of incubation, planktonic bacterial growth was characterized by reading the optical density (600 nm). Furthermore, the pH reading was taken at the end of 5 days of the experiment. Finally, glucose and glycerol consumption were measured by HPLC using an HPX-87H column and refractive index detector based on glucose and glycerol curves from 0 to 600 mg/L at the end of five days

Table 1 Composition of the culture media.

Identification	Carbon source (200 mg/L)	Nitrogen source (1 mmol/L)
M1	Glucose	-
M2	Glycerol	-
M3	Glucose	Ammonium phosphate
M4	Glycerol	Ammonium phosphate
M5	Glucose	Urea
M6	Glycerol	Urea
M7	Glucose	Ammonium sulfate
M8	Glycerol	Ammonium sulfate

The biofilm was characterized by evaluating viability using crystal violet and morphology using a bright field. The medium was removed, and the plate was washed twice with 200 µL of 0.1 mol/L phosphate buffer (PBS). After removing the buffer, the plate was dried in the flow chamber. Next, 150 µL of crystal violet dye (0.2% w/v) was added and allowed to react for 15 min. The dye was removed, washed thrice with 150 µL of PBS, and dried. Finally, 150 µL of the solubilizing solution (alcohol: acetone (80:20) (v/v)) was added and left to act for 15 min. The reading was carried out at OD 580 nm. An inverted microscope was used to visualize the morphology of the biofilm.

3 RESULTS & DISCUSSION

Understanding the factors that influence bacterial colonization on these surfaces is necessary to understand the mechanism of inactivation/cleaning of a biofilm formed on membranes. We aimed to select an ideal inoculum and compositional medium that would provide biofilm growth and development for subsequent inactivation analyses. The biofilm formation analysis process was evaluated on polystyrene microplates over 1, 3 and 5 days. The growth of planktonic bacteria for each medium can be seen in Figure 1.

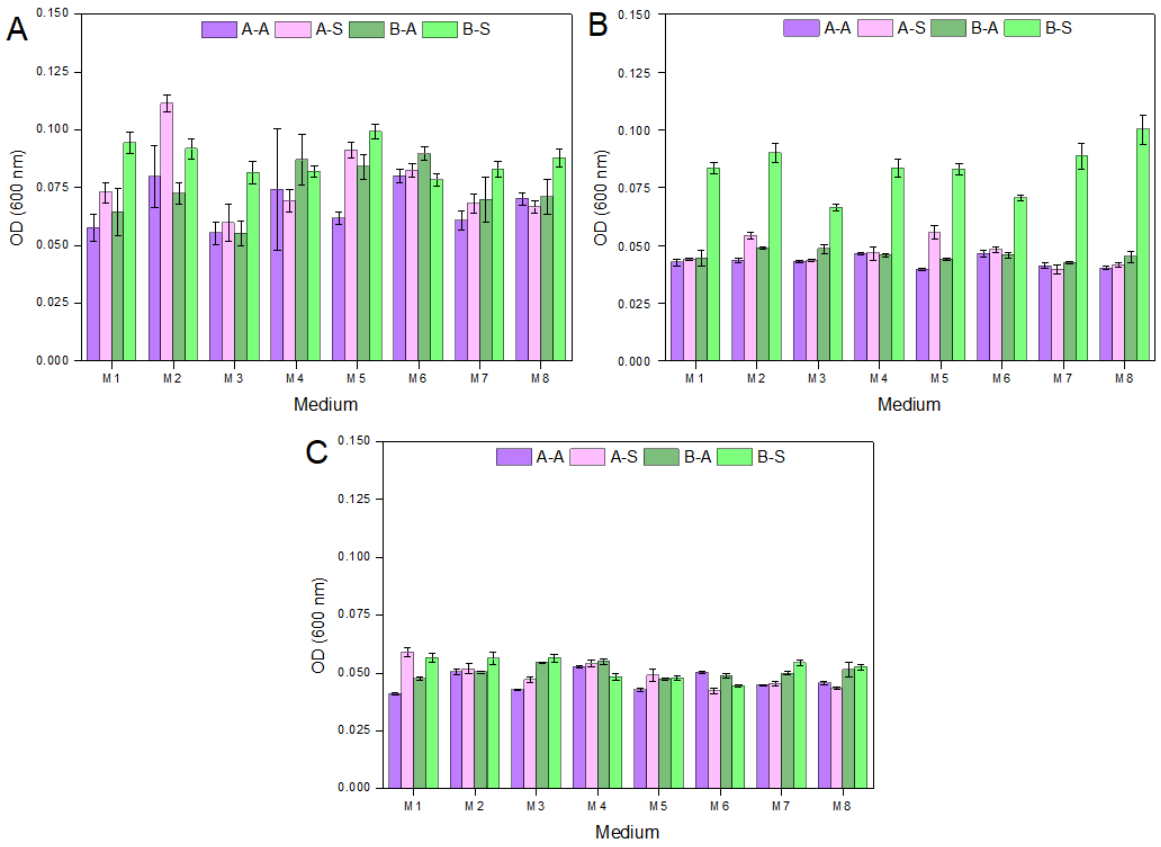

Figure 1 Absorbance of planktonic bacteria read at 600 nm for times of 1, 3 and 5 days for the 4 bacterial pools (A-A, A-S, B-A and B-S).

The behavior of planktonic bacteria followed as expected. Most inoculum showed a reduction in absorbance over time, except inoculum B-S, which remained stable on day 3 and then reduced on day 5. At the end of 5 days of cultivation, no change in pH was observed for all compositions, remaining between 4.5 and 6. Glucose and glycerol were at zero at the end of 5 days of cultivation.

Biofilm growth, on the other hand, is much more complex and occurs as a form of defense. The selection of the inoculum capable of forming biofilm was evaluated using the violet crystal microplate technique. Among the inoculum tested, we observed that the best results in terms of adhesion are related to the bacteria present in the concentrate stream of the B-S units when cultivated in a medium containing glucose and urea/ammonium sulfate (Fig.2A) and to the bacteria present in the concentrate stream of the

A-S unit when cultivated with glycerol and urea in its composition (Fig. 2B). One explanation for wastewater containing more biofilm-forming bacteria could be related to the stages of biofilm formation (reversible adhesion, irreversible adhesion, formation of microcolonies and mature biofilm). During the water filtration membrane process and after the biofilm matures on the membrane's surface, some biofilm is released to colonize other parts of the membrane. In most cases, the detached biofilm is eliminated in the wastewater.

Consequently, more biofilm-forming bacteria are expected in the inoculum isolated from the concentrate stream of the reverse osmosis water treatment unit. Concomitantly with the quantification of the biofilm by crystal violet, records of this formation were obtained using an optical microscope. The images record changes in biofilm formation (Figure 2C-D) throughout the process.

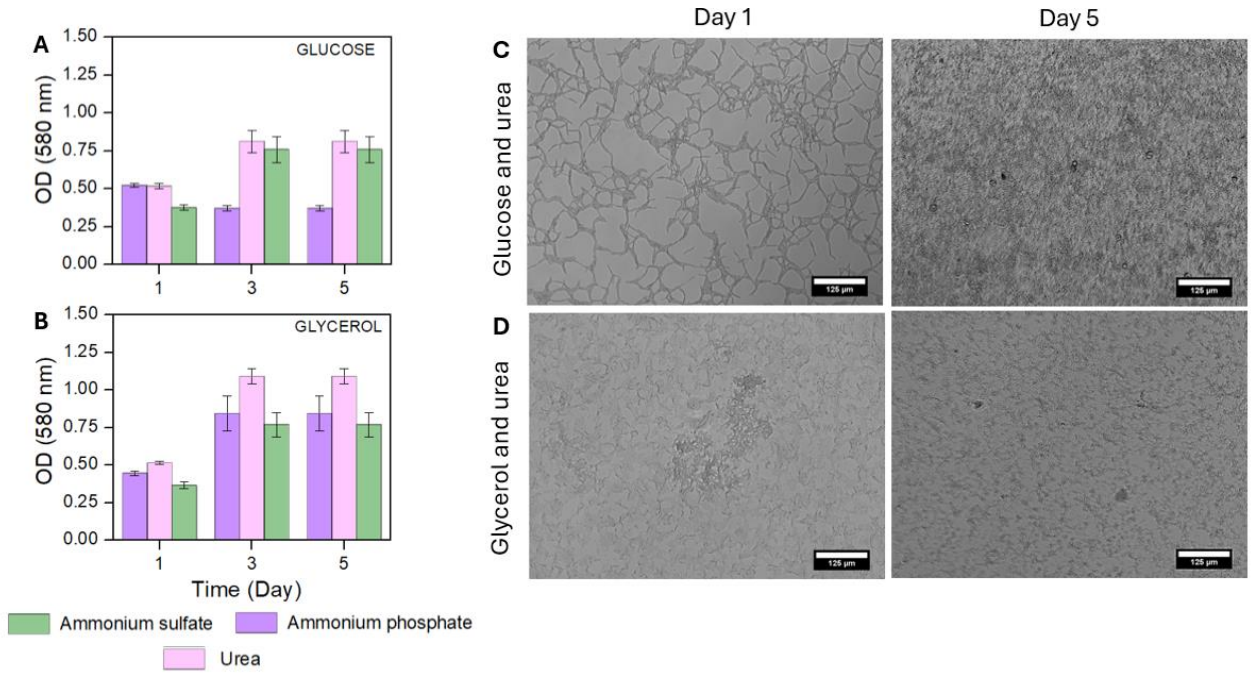

Figure 2 Influence of nitrogen source on biofilm formation on polystyrene plate by Crystal violet method. (A) glucose and (B) glycerol. Biofilm morphology on the polystyrene plate in the best condition (C) glucose and urea and (D) glycerol and urea after 1 and 5 days.

The ability of bacteria to form biofilms was influenced by the available source of carbon and nitrogen, which is crucial for optimizing processes. Different behaviors were observed for both pools in terms of morphology despite the feasibility of presenting similar results for days 3 and 5. For the B-S inoculum (Fig. 2C) on day 1, we observed a more uniform bacterial distribution in the lower part of the sample. For A-S (Fig. 2D) the behavior of a more mature and agglomerated biofilm was observed. After 5 days, the bottom of the wells was already covered, and it was impossible to observe any difference between the inoculations.

4 CONCLUSION

The results obtained in this study demonstrate the complexity and dynamics of microbial communities and the formation of biofilms to mitigate problems associated with membrane performance in water purification processes. Identifying and selecting bacterial pools in different culture media allowed us to evaluate which conditions favor biofilm growth, providing valuable insights for developing control strategies. Different pools and nutrient sources significantly affected biofilm formation and maturation. The best conditions for biofilm formation found in this work, among those tested, were glycerol-containing urea for the A-S pool and glucose-containing urea and ammonium sulfate for the B-S pool. Further studies are being carried out to characterize the diversity of both biofilms, aiming at identifying biofilm-forming microorganisms.

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