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Applications of nisin for biofouling mitigation of reverse osmosis membranes

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ABSTRACT

This study addresses the potential of application of nisin, a polycyclic antimicrobial peptide produced by *Lactococcus lactis*, as a novel biological agent for control/mitigation of biofilms formed by three different microorganisms: (i) *Pseudomonas aeruginosa* P60, (ii) *Bacillus* species, and (iii) a mixed culture of the two species. Nisin did not affect the growth rate of either strain, while the viability of *Bacillus* sp. was decreased, as compared to *P. aeruginosa* P60. Nisin was particularly effective for the dislodging of bacterial cells and extracellular polymeric substances (EPS) for *P. aeruginosa* P60. Water permeability of biofouled membranes was recovered by over 92% for all the bacterial strains investigated after nisin cleaning in a dead-end filtration system. Nisin has no detrimental effect on RO membrane and thus, has a potential as a biological agent for the mitigation of membrane biofouling.

1. Introduction

Biofouling is difficult to control. If after membrane cleaning, a few surviving cells are left, this is sufficient for the reestablishment of biofilms once these cells adhere to the membrane surface and multiply in the aqueous phase. This is considered the most challenging issue in membrane filtration for water and wastewater treatment [1].

Thus maintenance of membrane filtration performance requires the application of various physical cleaning methods; however, eventually, membrane cleaning using chemicals such as bases, oxidants, acids, acid chelates, alkaline chelates, and surfactants are required [2–5]. Polymeric membranes currently are the most commonly used in the seawater desalination market; however, the frequent use of chemicals increases costs [6], impair the selectivity of the membrane reducing the quality of the permeate water [7] and shortens the lifetime of polymeric membranes [8]. Moreover, the chemicals employed are potentially toxic to marine ecosystems and humans [9,10].

Recently, biological approaches: quorum sensing regulation, enzyme treatment, and energy uncoupling agents have been investigated as potential alternatives to chemical cleaning methods [11–17]. However, these biological agents could present high costs, low commercial accessibility, increased toxicity, limited suitability for industrial scale application, sensitivity to environmental conditions, and storage difficulties [18–21]. Therefore there is a critical need to develop a cost-effective and eco-friendly anti-biological agent.

As cleaning is an inevitable part of membrane-based systems, the efficiency with which this operation is done greatly affects the operational cost and the final product quality. In this study, nisin ($C_{143}H_{230}N_{42}O_{37}S_7$) was proposed as a cleaning agent for biofilms formed on membrane surfaces because it is considered a safe biodegradable antimicrobial agent, and has been approved as a GRAS (generally recognized as safe) [22], therefore, nisin poses no threat to human health and the environment.

Nisin is an antimicrobial peptide produced by strains of the *Lactococcus lactis* subspecies. Nisin is a 34-amino acid polypeptide that is active over a broad pH range, relatively amphipathic and small (3353 Da), cationic, and effective at low concentrations [23–25]. Nisin has been shown to have strong antimicrobial activity against Gram-positive bacteria by forming pores in cytoplasmic membranes that disrupt the proton-motive force and the pH equilibrium, causing leakage of ions and hydrolysis of adenosine triphosphate (ATP), resulting in cell death [26]. In membrane-based water treatment nisin has been conjugated with graphene oxide (GO) membrane for efficient removal and effective killing of methicillin-resistant *Staphylococcus aureus* (MRSA) for water treatment [27]. Nisin was evaluated to be safe for use in food by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) [22] and because of this, it is used in food science to reduce food-borne bacteria on various surfaces.

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The purpose of this study was to evaluate the potential application of nisin for removing biofilms formed on reverse osmosis (RO) membranes by microorganisms. *Pseudomonas aeruginosa* P60 and/or *Bacillus* sp. were selected as model bacteria for biofilm formation on RO membranes [28,29]. To assess the capabilities of nisin, the following factors were evaluated: (i) its effect on cell growth and viability, (ii) numbers of attached bacteria, and (iii) efficiency in reducing EPS. In addition, the effects of treatment conditions, such as the concentration of nisin and exposure time, on biofouling mitigation were investigated. Finally, permeate flux recovery through the application of nisin on membrane biofouling was determined using a dead-end filtration system.

2. Materials and methods

2.1. Preparation of bacteria and materials

P. aeruginosa P60 (Korean Environmental Microorganisms Bank, KEMB 9006-001), and *Bacillus* sp. G-84 (Korean Collection for Type Cultures, KCTC 3872) were used as model bacteria. Both strains were inoculated and cultured overnight in Luria-Bertani (LB) medium (Becton Dickinson, USA) at 37 °C. The cultured cells were incubated in LB medium for 16 h and adjusted to a final optical density (OD) of 600 nm (OD_{600}) of 1.0 before use for biofilm formation. Nisin solution was prepared by diluting nisin (Sigma-Aldrich, USA) in phosphate-buffered saline (PBS) (Life Technologies, USA). A polyamide thin film composite RO membrane (SHN-RE8040; Toray Chemical Korea Inc., Korea) was used as a model RO membrane.

2.2. Bacterial growth and viability

To determine the effects of nisin (1000 μg/mL) on bacterial growth, *P. aeruginosa* P60 and *Bacillus* sp. were incubated in LB medium for 8 h and bacteria in the absence of nisin were used as a control. To quantify total cell number and viability, bacteria in aqueous suspension were stained using 0.1 mg/mL 4`,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, USA) and LIVE/DEAD *BacLight* bacterial viability kits (Life Technologies, USA), respectively. After staining, fluorescent images were captured using LSM5 inverted confocal laser scanning microscopy (CLSM) (Zeiss, Germany). Quantitative analysis of the fluorescent signals in the CLSM images was performed using image analyzer software (isolution/Lite, iMTechnology, Korea).

2.3. Biofilm formation and detachment

The effect of nisin on mitigation of biofouling was evaluated by using nisin in the post-biofilm formation stage to confirm biofilm detachment (Fig. 1). To verify the detachment effect of nisin on biofilms, 20 μL microbial suspension (OD_{600} = 1.0) *i.e.*, (i) *Pseudomonas aeruginosa* P60, (ii) *Bacillus* species, and (iii) a mixed culture (1:1 ratio of the two microorganisms) was inoculated into a 24-well plate containing 2 mL of PBS, 20 μL of LB medium and an RO membrane (1 cm × 1 cm) and incubated for 4 days. These biofouled membranes were washed three times with PBS and treated with 1000 μg/mL of nisin solution, except for control samples which received no nisin treatment. After 2 h, membranes were washed three times with PBS to remove residual nisin, and then transferred to 1.5 mL micro centrifuge tubes containing 1 mL of PBS. Bacteria were detached from membranes by vortexing for 2 min followed by sonicating for 5 min. Total cell number and viability of detached bacteria were measured as described above.

2.4. Bacteria attachment according to various concentrations and exposure times of nisin

To determine the effects of nisin on membrane biofilms, each well within the 24-well plates had an RO membrane (1 cm × 1 cm) attached and was filled with 2 mL of LB medium and 20 μL of bacteria (OD_{600} 1.0). The plates were incubated for 4 days at 37 °C in a continuously shaking incubator (70 rpm). The content of each well was aspirated, and each biofouled membrane was washed three times with PBS. To determine the concentration effects of nisin on biofilm detachment, various amounts of nisin (*i.e.*, 300, 500, and 1000 μg/mL) were added to each well and incubated for 2 h at room temperature. To study the effects of exposure time on biofilm detachment, 1000 μg/mL of nisin was added and incubated for various time periods (*i.e.*, 1, 2, 4, 6, and 12 h) at room temperature. After the incubation, each membrane was washed three times with PBS to remove residual nisin, and then transferred to 1.5 mL micro centrifuge tubes containing 1 mL of PBS. Bacteria were detached from membranes by vortexing for 2 min followed by sonicating for 5 min. Total cell numbers were measured as described above.

2.5. EPS extraction and quantification

To quantify EPS in biofilms on the membrane surface, cell culture flasks (SPL, Korea) containing RO membranes (3 cm × 3 cm) were filled with 18 mL of LB medium and 180 μL of each microbial suspension (OD_{600} = 1.0) (*i.e.*, (1) *Pseudomonas aeruginosa* P60, (2) *Bacillus* species, and (3) a mixed culture of the two species). The flasks were incubated for 4 days at 37 °C and 70 rpm. The suspended bacteria were discarded, and each biofouled membrane was washed three times with PBS. Then 1000 μg/mL of nisin solution was added to each flask and incubated for 2 h at room temperature. Biofilms on the membrane were detached by vortexing for 2 min followed by sonicating for 5 min. The EPS were extracted by chemical and physical methods as previously described [30]. In brief, each 10 mL of detached sample was treated with 0.06 mL formaldehyde (36.5%) at 4 °C for 3 h. Next, samples were centrifuged (14,000 rpm, 20 min), and the supernatant was filtered through a 0.2 μm cellulose acetate membrane (ADVANTEC, Japan). The filtered samples were dialyzed using 3500 Da molecular weight cut-off dialysis tubing (Thermo Scientific, Waltham, USA) and lyophilized. The lyophilized samples were re-suspended in 10 mL of deionized water and the concentrations of carbohydrates and proteins were analyzed. Carbohydrate concentration was assessed following the method established by Wu and Xi [31]. Protein concentration was analyzed using a BCA assay kit following the manufacturer's protocol (Thermo Scientific, USA).

2.6. Excitation-emission matrix of EPS

Characteristics of EPS extracted from biofilms were identified using a fluorescence spectrophotometer (Hitachi, Japan). A three-dimensional excitation-emission matrix (EEM) was collected every 5 nm over an excitation range from 220 nm to 450 nm, with an emission range of 250 nm to 600 nm. Scan speed was set at 300 nm/min. The fluorescence spectra of deionized water were subtracted in order to remove Raman scattering and to reduce additional background noise. EEM peaks were classified as A (E_x/E_m = 330 nm to 350 nm/420 nm to 480 nm; visible humic-like), B (E_x/E_m = 270 nm to 280 nm/300 nm to 350 nm; tyrosine- like, tryptophan- like, and protein-like), C (E_x/E_m = 220 nm to 250 nm/430 nm to 450 nm; fulvic-acid like) [32].

2.7. Biofouling and cleaning experiments

To determine the effects of nisin cleaning on biofouled membranes, three different microbial suspensions (*i.e.*, (i) *Pseudomonas aeruginosa* P60, (ii) *Bacillus* species, and (iii) a mixed culture of these two species) were individually filtrated through RO membrane in a dead-end (for accelerated biofilm formation) and a cross-flow filtration systems (Fig. 2). In a dead-end operation mode, firstly, the membrane was compacted using deionized water for 12 h at 10 bar. Secondly, the membrane was conditioned by filtering the feed water with electrolyte