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CASE REPORT

Quantitative study on the effect of calcium and magnesium palmitate on the formation of *Pseudomonas aeruginosa* biofilm

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Abstract Calcium palmitate and magnesium palmitate (which are major constituents of waste water) are insoluble precipitates that accumulate in bodies of water. This leads to the formation of biofilms because bacterial cells can use these fatty acid salts as a carbon source. It is important to study the formation of biofilms because they cause corrosion of pipelines and water contamination. In this study, the effect of calcium palmitate and magnesium palmitate on *Pseudomonas aeruginosa* biofilm formation has been evaluated. In the presence of calcium palmitate, the biofilm biomass, extracellular polysaccharide, and adhesion force were 3.45 ± 0.06 (A_{590}), 1810 ± 47 μg, and 14.5 ± 0.9 nN, respectively. In the presence of magnesium palmitate, the biofilm biomass, extracellular polysaccharide, and adhesion force were 2.72 ± 0.03 (A_{590}), 1370 ± 56 μg, and 8.0 ± 0.2 nN, respectively. The results suggest that biofilm biomass, extracellular polysaccharide, and adhesion force were higher in the presence of calcium palmitate.

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Introduction

Calcium palmitate or magnesium palmitate are formed when calcium or magnesium ions (present in hard water) react with the palmitate anion (sodium palmitate is the main constituent of soaps) to form insoluble calcium palmitate or magnesium palmitate. This insoluble precipitate accumulates in pipelines thereby resulting in the formation of biofilms as bacterial cells use these salts as a carbon source for their growth. Calcium palmitate and magnesium palmitate are also constituents of gallstones. This could lead to the growth of microorganisms and subsequently result in the formation of biofilm. This causes drug resistant infections in humans.

Biofilms consist of bacterial cells immobilized in a matrix of extracellular polymeric substances.[1] This matrix helps in immobilization of the cells and also functions as a protective barrier against antibiotics and disinfectants. The resulting biofilms may increase pipe and machine corrosion leading to economic losses, and some biofilm material may also be released into flowing water, thereby resulting in its contamination. This contaminated water could cause infections in humans.

Hence, there is a need to study the factors that are crucial for biofilm formation because they have been reported to cause losses in industry and also cause infections in humans. In this study, the effect of calcium palmitate and magnesium palmitate on the formation of *Pseudomonas aeruginosa* biofilm has been examined.

Materials and methods

Culture media

The organism *P. aeruginosa* was chosen for this study on biofilm formation because it is a model organism used in biofilm research. The strain (MTCC 2297) was cultured in nutrient broth (Hi Media, Mumbai, India) at 37°C prior to inoculation.

Biofilm production media

M9 minimal media with 1% (w/w) calcium palmitate (Media M1) or 1% (w/w) magnesium palmitate (Media M2) was used for the growth of biofilms. It was found that there was no formation of biofilm in M9 minimal media (without calcium and magnesium palmitate). Bacterial cells can use magnesium palmitate and calcium palmitate as a carbon source (for their growth). Hence they were used in the biofilm production media. The pH of the mineral medium was adjusted to 7.2. After inoculation (with *P. aeruginosa* cells), samples were incubated at 37°C for 5 days.

Synthesis of magnesium and calcium palmitate

Magnesium palmitate and calcium palmitate were prepared according to the procedure given by Quraishi et al.[2] Equimolar concentrations of palmitic acid and potassium hydroxide were mixed. The suspension was heated and stirred continuously to yield potassium palmitate. Magnesium chloride, in slight excess of what was required for the stoichiometric reaction, was added dropwise to the solution. The product was then cooled and the precipitate was filtered and washed with water and acetone to remove any unreacted fatty acids or reactants used. Magnesium palmitate thus obtained was dried to constant weight. Calcium palmitate was prepared in a similar manner.

Analysis using an atomic force microscope

The atomic force microscope (AFM) image of polished silicon substrate was resolved in intermittent contact mode using JPK Instruments AG, Berlin, Germany Nanowizard II and the surface roughness parameters were evaluated. The cantilevers used for this purpose were purchased from Applied Nanostructures Inc., Mountain View, CA, USA. The biofilm samples (for measurement of adhesion force) were prepared according to the procedure given by Oh et al.[3] Silicon nitride probes were used for this study and were calibrated before use. A total of 64 force—distance curves were generated to evaluate the adhesion force of the biofilm.

Sample preparation for inductively coupled plasma analysis

Inductively coupled plasma (ICP) analysis is used to determine the concentration of metal ions (which are present at very low concentrations) with high sensitivity. In this study, this method was used to analyze the concentration of calcium and magnesium ions in the biofilm matrix. The samples for ICP analysis were prepared according to the protocol given by Cruz et al.[4] Cells were collected by centrifugation and washed in double distilled water. They were then acid digested in 2 mL of nitric acid. The digested samples were then centrifuged and the supernatant diluted prior to ICP analysis.

Quantification of biofilm formation

The formation of biofilm was estimated using the protocol of Merritt et al.[5] The biofilm samples were first rinsed with ethanol to remove the loosely bound planktonic cells. They were then incubated in 0.1% crystal violet solution for 10 minutes before rinsing with water to remove any unbound crystal violet. The bound crystal violet from the samples was removed using 95% ethanol (solubilizing buffer). The absorbance of the solubilizing buffer was measured at 590 nm. All experiments were conducted in triplicate.

Isolation and quantification of extracellular polysaccharides

Isolation of extracellular polysaccharides was based on the procedure given by Forde and Fitzgerald.[6] Cells were harvested by centrifuging the sample at 12,500*g* for 15 minutes. They were then suspended in 5 mL of sodium hydroxide and boiled for 15 minutes. After boiling, the samples were centrifuged at 12,500*g* for 30 minutes to remove the cells. Ethanol, 99.9% (v/v), was added to the

Table 1 Effect of calcium and magnesium palmitate on biofilm formation

Media	Carbon source	Biofilm biomass (A590)	Adhesion force (nN)	Extracellular polysaccharide (μg)	Concentration of metal ions in biofilm (mg/L)
M1	Calcium palmitate	3.45 ± 0.06	14.5 ± 0.9	1810 ± 47	Ca: 2.2 Mg: 1.7
M2	Magnesium palmitate	2.72 ± 0.03	8.0 ± 0.2	1370 ± 56	Ca: 0.08 Mg: 2.1

A = Absorbance

supernatant and the sample was incubated at 4°C to precipitate the polysaccharides. The precipitated polysaccharides were then resuspended in 10 mL of sterile water.

The polysaccharides were quantified using the acid hydrolysis method of Parkar et al.[7] To 1 mL of sample, 8 mL of 98% (v/v) sulphuric acid was added and cooled for 10 minutes. One milliliter of 1% (w/v) cold tryptophan was added to this sample and heated for 15 minutes to affect hydrolysis. The amount of polysaccharide was then estimated by measuring absorbance at 500 nm. All experiments were conducted in triplicate.

Results and discussion

Polished silicon wafers were used as the solid support for the growth of biofilms from *P. aeruginosa*. AFM images of these wafers were resolved over an area of 10 μm × 10 μm (Figure S2) and surface roughness was evaluated. The surface roughness of these wafers was found to be 1 nm (indicating that the surface was smooth). A typical image of biofilm formed on polished silicon wafers (taken using scanning electron microscopy for the purpose of visual confirmation) is shown in Figure S1.

The effect of calcium palmitate and magnesium palmitate on biofilm formation was compared with respect to three parameters: extracellular polysaccharide production, biofilm biomass, and adhesion force. Quantification of biofilm formation (to evaluate biofilm biomass) was done using the dye crystal violet.[8] Biofilm biomass was evaluated as a function of optical density (of crystal violet) at 590 nm. Biofilm biomass formed when Media M1 (M9 + calcium palmitate) and Media M2 (M9 + magnesium palmitate) were used were 3.45 (A_{590}) and 2.72 (A_{590}), respectively (Table 1). The higher biofilm biomass formed when Media M1 was used is due to the increased production of extracellular matrix material when grown in increased Ca^{2+} conditions.[9] The amount of extracellular polysaccharide produced when Media M1 and M2 were used were 1810 μg and 1370 μg, respectively (Table 1). Extracellular polysaccharides play an important role in the formation of biofilms and it is only when extracellular polysaccharides are produced that cell aggregation and biofilm formation occur. The higher amount of extracellular polysaccharide produced when Media M1 was used could also have been due to the aforementioned reason.

The adhesion force when grown in Media M1 and Media M2 was 14.5 nN and 8.0 nN, respectively (Table 1). The distribution of adhesion force is given in Figure S3. The adhesion force (or tip-biofilm interaction force) is a measure of the adhesiveness of biofilm. The results suggest that biofilms formed in Media M1 (containing calcium palmitate) are more adhesive than biofilms formed in Media M2 (containing magnesium palmitate). One of the reasons for the higher adhesion force of biofilm grown in Media M1 could have been the presence of a higher amount of extracellular matrix material.[3] The higher concentration of Ca^{2+} ions incorporated in the biofilm matrix in biofilm grown in Media M1 (Table 1) suggests that these biofilms would have a higher mechanical stability than biofilms grown in Media M2. This could be because incorporation of Ca^{2+} ions in biofilm structures results in stronger and more specific binding with the negatively charged polysaccharides when compared with the weak and nonspecific interactions of magnesium ions.[9,10] Furthermore, Ca^{2+} has been implicated in cell—cell binding mechanisms which could also have a positive effect on the mechanical stability of biofilms. These results suggest that calcium palmitate has a greater effect on biofilm formation than magnesium palmitate.

In conclusion, the effect of magnesium palmitate and calcium palmitate (as a carbon source) on biofilm formation was studied. The results suggest that biofilm biomass, extracellular polysaccharides, and adhesion force were higher in the presence of calcium palmitate.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jmii.2015.06.001.