Evaluation of \textit{E. coli} biofilm as a protective barrier against microbiologically influenced deterioration of concrete (MICD) under mesophilic temperatures

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\textbf{ABSTRACT}

In this study, \textit{Escherichia coli} DH\textsubscript{5}a biofilm was evaluated for its potential to control and minimize microbiologically influenced concrete deterioration (MICD) under mesophilic temperatures (37 °C). \textit{Escherichia coli} DH\textsubscript{5}a biofilm was first grown on Portland cement mortar disks for 8 days. Mortar disks were then exposed to two different types of sulfur oxidizing bacteria (SOB) (\textit{Thiobacillus neapolitanus} and \textit{Thiobacillus thiooxidans}), which use sulfur compounds as substrate and oxidize them to sulfate and sulfuric acid. The effectiveness of the biofilm against MICD was evaluated by measuring pH, sulfate, calcium concentrations in the reactors and surface analysis of the mortar samples using X-ray diffraction and visual inspection. Overall, the results indicate that the \textit{E. coli} DH\textsubscript{5}a biofilm showed good protection against MICD induced by SOB at 37 °C.

\textbf{Key words} | biofilm, cement mortar, concrete, corrosion, deterioration, \textit{E. coli}

\textbf{INTRODUCTION}

Microbiologically influenced concrete deterioration (MICD) is a worldwide problem that causes severe damage to concrete structures of wastewater treatment plants and sewer pipelines (Nica \textit{et al.} \(2000\); O’Dea \(2007\)). Concrete deterioration is particularly severe at elevated temperatures such as those used for mesophilic treatment processes. Sewage contains abundant amounts of sulfate ion which is reduced to hydrogen sulfide in the presence of anaerobic sulfate reducing bacteria. During MICD, sulfur oxidizing bacteria (SOB) convert sulfur compounds to sulfuric acid and accelerate the deterioration process. Concrete deterioration is initiated by the reaction of sulfuric acid with calcium hydroxide in the concrete to produce gypsum and followed by the reaction between gypsum and calcium aluminate hydrate to form ettringite (Monteny \textit{et al.} \(2000\)). Ettringite formation is generally observed in deeper layers of exposed concrete surface where pH is relatively high because ettringite is known to be unstable at low pH (Sand \textit{et al.} \(1994\)). Both gypsum and ettringite are expansive, which leads to the increase of internal pressure and deterioration of concrete matrix. Different methods have been suggested for the mitigation of MICD. Biocide addition is the simplest and most common method of inhibition, but biocides are toxic and may inhibit the growth of the beneficial microorganisms necessary for the treatment processes. Chemical coatings are vulnerable to chemical and biological attack, and can become costly owing to the necessity for the regular monitoring and reapplication. The use of biofilm as a protective layer on the surface of metals to inhibit microbiologically induced corrosion has shown promising results (Jayaraman \textit{et al.} \(1998\); Zuo \(2007\)); however, it has never been used for MICD prevention of concrete surfaces exposed to wastewater. Biofilms offer several advantages, including their ability to repair and replenish themselves naturally. The goal of this research was to investigate whether \textit{Escherichia coli} DH\textsubscript{5}a biofilm could provide protective properties against deterioration of Portland cement mortar, which is the main constituent of concrete induced by SOB, in the mesophilic temperature range at which the majority of the anaerobic digesters are operated. The \textit{E. coli} biofilm is likely to protect mortar by acting as a protective barrier, hence preventing the exposure of mortar surface to an aggressive environment and by reducing the availability of the nutrients and oxygen for the growth of SOB.
MATERIALS AND METHODS

Mortar sample preparation

Mortar samples were prepared using Portland cement, sand and water. The mortar mixture had a water-cement ratio (w/c) of 0.45 and was composed of 540 g Portland cement and 1,360 g sand. After mixing, the cement mortar was cast in cylindrical molds with 10 cm diameter and 15 cm height, and were kept in a curing room with a relative humidity of 95–100% at 30–35 °C for 24 h. After curing of mortar cylinders for at least 28 days, the cylinders were cut into 1-cm-thick mortar disks with 10 cm diameter. The mortar disks were used for this study.

Biofilm growth on mortar disks

Escherichia coli DH5α (pKMY319), which is a facultative aerobic bacterium, was used based on its ability to form biofilm with protective properties (Jayaraman et al. 1997a, b). A continuous-flow experimental set-up was designed and built for growing biofilm on mortar disks. The set-up consisted of a reactor where biofilm growth was achieved and a reservoir for feeding nutrients required for bacterial growth (Figure 1). A mortar disk was placed at the bottom of the reactor, which was made of a glass cylinder covered on top and bottom with two 1-cm-thick Teflon® plates. The reactor was filled with 150 mL of Luria-Bertani (LB) broth supplemented with 25 μg/mL tetracycline to prevent contamination from other bacteria. A 0.1% v/v of the E. coli inoculum from the overnight grown culture was injected into the medium. The mixing reservoir was also filled with 150 mL of fresh LB broth supplemented with tetracycline. Bacteria were allowed to grow for 12 h in batch mode inside the biofilm reactor; then, the growth medium plus the bacteria were circulated at a flow rate of 12 mL/h using a peristaltic pump. From the 3rd day of growth, 5 mL of 4× concentrated LB broth was added to the reservoir on a daily basis as the nutrient source for biofilm growth. Both the reactor and the reservoir were placed on a rotary shaker at 60 rpm. The biofilm growth was continued for 8 days under these conditions.

Growth of SOB

Pure cultures of two types of sulfur oxidizers, Thiobacillus neapolitanus and Thiobacillus thiooxidans, were purchased from American Type Culture Collection (ATCC). Thiobacillus neapolitanus (ATCC 23641) is a neutrophilic sulfur oxidizer that grows well at pH 6–7 and can reduce the pH to around 4. Thiobacillus neapolitanus was grown in the medium suggested by Vishniac & Santer (1957). Thiobacillus thiooxidans (ATCC 19703) is an acidophilic sulfur oxidizer that reduces pH from 4–5 to 1–2, and was cultivated in the medium suggested by Starkey (1925). Pure cultures of both bacteria were grown until they reached their exponential growth phase and used as a seed for the biogenic acidification experiments. A modified broth (MB) with a neutral pH containing 0.1 g/L (NH4)2SO4, 0.8 g/L MgCl2.6H2O, 0.25 g/L CaCl2, 3 g/L KH2PO4, 3 g/L K2HPO4, 5 mg/L of FeSO4 and 10 g/L Na2S2O3.5H2O, 1.25 g/L LB broth was used for the biogenic acidification experiments. The MB was selected based on its ability to promote the growth of both SOB.

Biogenic acidification of mortar disks with SOB bacteria

The reactor and experimental set-up shown in Figure 1 was also used for biogenic acidification of the mortar disks. The experimental set-up was kept inside an environmental incubator to keep the temperature at 37 ± 1 °C during the experiments. Two mortar disks were used, one covered with biofilm and one without biofilm (control). Each mortar disk was placed at the bottom of the reactor and the reactor was filled with 200 mL of the MB, inoculated with 5% v/v T. neapolitanus and T. thiooxidans at their exponential growth phase, and placed on a rotary shaker operated at 60 rpm. Biogenic acidification was performed in two cycles of SOB growth owing to the need to replace the MB medium, and each cycle was continued as long as SOB could sustain the low pH of the medium against the buffering capacity caused by calcium hydroxide release from the mortar. After the first cycle of 24 days, the reactor was emptied and then filled with fresh MB, inoculated with T. thiooxidans and T. neapolitanus, and operated for
another 20 days. During the experiments, pH, calcium and sulfate concentrations inside the reactor were monitored.

**Calcium, sulfate and pH analysis**

A calcium ion selective electrode (ISE) with a reference electrode incorporated in the body of the electrode (PY-1071S Combination Electrode, Sartorius Mechatronics) connected to a VWR ISE reader (SP90M5, VWR Symphony) was used for calcium concentration measurement. The calcium ISE was calibrated once before each measurement. It was immersed in the solution containing the calcium ions for about 3–5 minutes for measurement. For measuring sulfate concentration, the Hach spectrophotometric method (Method No. 8051, concentration range of 2–70 mg/L, DR2800 Spectrophotometer, Hach), which was adopted from *Standard Methods for the Examination of Water and Wastewater* (APHA *et al.* 2005), was used. The samples were diluted 100 times to be in the range of the acceptable concentration for the measurement.

The pH was measured using a Thermo Scientific Orion 5 Star pH meter.

**X-ray diffraction**

X-ray diffraction (XRD) was employed to assess the formation of ettringite and gypsum crystals as these crystals are the expansive sulfate crystals that are formed during the biodeterioration of concrete and mortar (*Monteny et al.* 2000, 2001). Using a profile grinder (Metabo Ge700, ACE Tool), the acidified area of the mortar disks were ground in two layers of 0.5 mm thickness. The collected powder from each layer was used for XRD analysis. The XRD measurements were performed using a Scintag XDS 2000 diffractometer and CuKα radiation. Spectra were obtained in the range of $4^\circ<2\theta<65^\circ$ using a step size of 0.03° at 5 s intervals.

**RESULTS AND DISCUSSION**

**Chemical analysis**

Figures 2, 3 and 4 show the pH, sulfate and calcium concentrations measured in the reactor media that contained the mortar disks with biofilm (referred to as biofilm reactor) and without biofilm (referred to as control reactor). During the first cycle of biogenic acidification, the activity of SOB was able to reduce the pH of the medium in the control reactor to 4.1 after 16 days of growth compared with the biofilm reactor where the lowest pH was 5.5 after 12 days (Figure 2). The medium in the biofilm reactor had a higher and sustainable pH likely due to the buffering capacity of the *E. coli* biofilm. The bacteria that grow in an acidic environment have a tendency to keep their internal pH at a more alkaline pH than that of the environment by controlling the movements of cations through their...
membrane and increasing the extracellular buffering capacity (Booth 1999). Finally, calcium hydroxide that is present in the mortar helps to gradually buffer the pH, which explains the eventual pH increase in the control reactors and the need for replacing the medium for the second cycle. Based on these results, it was concluded that the presence of biofilm on the mortar surface avoided a substantial drop in the pH, which would help reduce MICD.

SOB bacteria use sulfur compounds as the substrate and oxidize them to sulfate, which is then converted to sulfuric acid. Therefore, an increase in sulfate concentration and a decrease in pH are expected during the SOB growth (Kuenen, et al. 1992; Bielefeldt et al. 2010). Sulfate concentrations measured in the control reactor were approximately three times higher than the sulfate concentrations measured in the biofilm reactor, indicating a higher SOB activity in the control reactor (Figure 3). After 23 days of exposure to SOB during the first cycle of acidification, the highest sulfate concentration was 6,350 and 3,550 mg/L in the control and biofilm reactors, respectively. Higher sulfate concentrations result in higher sulfuric acid concentrations, which also explain the difference in the pH of the control and biofilm reactors (Figure 2).

In the second cycle of acidification, after refreshing the broth containing SOB, sulfate concentration increased rapidly in the control reactor resulting in a sudden decrease in the pH (Figures 2 and 3). The sulfate concentration reached 4,650 mg/L in 1 day, causing the pH to drop to 3.5. The sulfate increase in the biofilm reactor was only a third of that. Overall, the rate of SOB growth in the

![Figure 5](https://iwaponline.com/wst/article-pdf/68/2/303/439830/303.pdf)

**Figure 5** | XRD pattern of mortar powder taken from the top 0.5 mm surface after biogenic acidification with SOB at 37 ± 1°C; (a) control mortar, (b) biofilm-covered mortar; dolomite (D), gypsum (G), quartz (Q), magnesium calcite (C), magnesium sulfate hydroxide (MH), winchite potassium (W), aluminate (A).
second cycle of acidification was less than the first cycle in both reactors and the maximum sulfate concentrations in the control and biofilm reactors were 5,300 and 2,400 mg/L, respectively (Figure 3). Having a lower SOB activity in the second cycle of acidification resulted in a step-wise increase in the pH of the control reactor, but the biofilm reactor was able to buffer the pH changes and maintain a stable pH value (Figure 2).

When mortar deteriorates, calcium is released from the matrix; therefore, calcium concentrations were measured in the biofilm and control reactors during biogenic acidification to compare the severity of MICD. The decalcification of mortar was observed in both reactors by an increase in the calcium concentrations of the media; however, the amount of calcium leach-out was 4.9 times higher in the control reactor than the biofilm reactor (Figure 4). After the first cycle of acidification, the calcium concentration was 136 ppm in the control reactor compared with 28 ppm in the biofilm reactor. The results indicated that the biofilm was effective in substantially reducing the calcium leach out from the concrete surface, thus minimizing concrete deterioration after biogenic acidification with SOB.

Crystal formation in mortar samples

The main purpose of XRD analysis was to confirm the presence of gypsum and ettringite crystals known to be the indicators of sulfuric acid attack in concrete. The XRD analysis was performed on the powder sample taken from

Figure 6 | XRD pattern of second layer of mortar powder after biogenic acidification with SOB at 37 ± 1 °C; at elevated temperature; (a) control mortar, (b) biofilm-covered mortar; dolomite (D), ettringite (E), gypsum (G), quartz (Q), magnesium calcite (C), magnesium sulfate hydroxide (MH), winchitte potassium (W), aluminate (A).
the control and the biofilm-covered mortar surfaces in two consecutive 0.5-mm-thick layers (0–0.5 and 0.5–1 mm). Comparison of the XRD patterns of the top 0.5-mm layer of the control and the biofilm-covered mortars showed very similar crystal formation, which mainly consisted of quartz, calcite, dolomite and gypsum (Figure 5(a) and (b)). Aluminate formation was also observed in the control mortar. The XRD analysis of the second layer (0.5–1 mm)
of the control mortar showed the presence of ettringite in addition to gypsum, and the ettringite formation was confirmed by the presence of all three main peaks of ettringite (i.e., $2\theta = 9.1^\circ$, $15.8^\circ$ and $22.9^\circ$) (Figure 6(a)). As ettringite is not stable at low pH and is converted to gypsum (Wang 1994), its presence was only observed at deeper layers with higher alkalinity. In the top layer where the pH was lower, ettringite was most likely converted to gypsum and some aluminate phases as both gypsum and aluminates were present in the XRD spectra (Figure 5(a)). As observed in Figure 6(a), the intensity of the aluminate peak decreased as the ettringite was more stable in the deeper layer with higher alkalinity. The XRD analysis of the second layer of the biofilm-covered mortar did not show any presence of gypsum or ettringite (Figure 6(b)). This observation supports the hypothesis that even though gypsum formation was observed in the top surface layer, deterioration in the biofilm-covered mortar was not as substantial as the control mortar sample in which the gypsum and ettringite formation was observed in deeper layers of the sample, indicating deeper mortar deterioration.

**Visual inspection of mortar samples**

Figure 7(a) and (b) show the photographs of two control mortar samples (without biofilm) after biogenic acidification. For both samples, the surface became rougher and exhibited large pores and dark spots likely caused by the changes in the microstructure of the mortar. The photograph of a mortar sample with a non-uniform biofilm coverage which was exposed to biogenic acidification is shown in Figure 7(c). When the biofilm layer was brushed off to see the changes on the mortar surface, spalling and surface roughness was observed on some sections (Figure 7(d)). Comparison of Figure 7(c) and 7(d) shows that the sections with no biofilm coverage were the ones that spalled away during the brushing of the sample. Figure 7(e) shows the photograph of a mortar sample that was fully covered with a uniform biofilm layer. When the biofilm layer was removed after biogenic acidification, no sign of damage or spalling was observed on the surface (Figure 7(f)).

**CONCLUSIONS**

Performance of *E. coli* DH5α biofilm as a protective barrier against Portland cement mortar deterioration induced by SOB *T. neapolitanus* and *T. thiooxidans* was evaluated under mesophilic temperatures (37 ± 1°C). The effectiveness of the biofilm was evaluated by measuring pH, sulfate, and calcium concentrations in the reactors, as well as surface analysis of the mortar samples using XRD and visual inspection. Overall, the results indicate that the *E. coli* DH5α biofilm showed good protection at 37 ± 1°C against MICD induced by SOB. Deterioration is more severe at elevated temperatures and the use of biofilms may offer a simple, inexpensive and environmentally friendly protection mechanism for wastewater treatment structures operated at mesophilic temperatures.

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