

Research Article

Characterization of Corrosive Bacterial Consortia Isolated from Water in a Cooling Tower

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An analysis of a culturable corrosive bacterial community in water samples from a cooling tower was performed using traditional cultivation techniques and its identification based on 16S rRNA gene sequence. Seven aerobic bacterial species were identified: *Pseudomonas putida* ARTYP1, *Pseudomonas aeruginosa* ARTYP2, *Massilia timonae* ARTYP3, *Massilia albidiflava* ARTYP4, *Pseudomonas mosselii* ARTYP5, *Massilia* sp. ARTYP6, and *Pseudomonas* sp. ARTYP7. Although some of these species have commonly been observed and reported in biocorrosion studies, the genus *Massilia* is identified for the first time in water from a cooling tower. The biocorrosion behaviour of copper metal by the new species *Massilia timonae* ARTYP3 was selected for further investigation using a weight loss method, as well as electrochemical and surface analysis techniques (SEM, AFM, and FTIR). In contrast with an uninoculated system, thin bacterial biofilms and pitting corrosion were observed on the copper metal surface in the presence of *M. timonae*. The use of a biocide, bronopol, inhibited the formation of biofilm and pitting corrosion on the copper metal surface.

1. Introduction

In order to implement efficient monitoring and control strategies for the inhibition of biocorrosion, it is important to have knowledge of the microbial population responsible for this phenomenon, as well as interactions of different microorganisms with metallic surfaces [1–8]. In many industries, cooling towers are commonly used for heat transfer from recirculated water to the atmosphere, typically by means of trickling or spraying the water over a material with high surface area [9]. These towers generally have sizable water reservoirs, with temperature typically maintained between 25°C and 35°C. These conditions provide an ideal environment for microbial growth and propagation [10–13]. Both microbes and the substrates for microbial growth can either be present in the incoming water or be introduced from the atmosphere. Copper and copper alloys, which are used in many cooling tower systems, are known to be susceptible to microbiologically influenced corrosion (MIC) [10, 14]. Corrosion and

its products have a negative impact on heat transfer and can cause a decrease in cooling efficiency of the cooling tower. Organisms responsible for MIC, including bacteria, microalgae and fungi readily attach themselves to the copper surface by excreting extracellular polymeric substances (EPS) to form a slime layer [15–18] and thereby initiate corrosion. A multilayer structure of microorganisms and their EPS have been reported to be entrapped between layers of different inorganic corrosion products on copper-based surfaces after exposure to natural seawater environment [19–24].

Interestingly, many traditional chemicals used water treatment, for example, antiscalants and zinc-based corrosion inhibitors, which are a source of nutrients that accelerate the growth of microbes in cooling towers [25]. Nonetheless, the control of corrosive bacterial fouling can be achieved through the application of effective biocides [26], or nitrate, or nitrite [27–30]. Environmental regulations and the development of water reservoirs in environmentally sensitive areas have spurred the development of easily degradable “green”

TABLE 1: Physicochemical characteristics of water from cooling tower.

| Sl. no. | Physicochemical characteristics | Quantity |
|---------|---------------------------------|----------|
| 1 | Temperature (°C) | 25 |
| 2 | pH | 8.1 |
| 3 | Total solids (mg/L) | 341 |
| 4 | Total hardness (mg/L) | 150 |
| 5 | Total alkalinity (mg/L) | 165 |
| 6 | Chloride (mg/L) | 296 |

biocides that are less toxic to higher nontarget organisms, like fish [31]. Nitrite, a specific metabolic inhibitor of corrosive bacteria, especially sulphate reducing bacteria [32, 33], is also relatively nontoxic and inexpensive and has been successfully used to inhibit corrosive bacteria [34]. 2-Bromo-2-nitropropane-1,3-diol (bronopol) has a broad spectrum of antibacterial activity and is widely used as a preservative in pharmaceutical and cosmetic products and many industrial environments such as paper mills, oil exploration, and production facilities, as well as a disinfectant in cooling water [35, 36]. The present investigation describes 16S rRNA gene sequencing and the identification of bacterial communities (at the species level) present in water from a cooling tower. Microbiologically induced corrosion and the use of the biocide bronopol are also examined. As the literature on the identification of bacteria in such a system in tropical countries appears lacking, this work would be useful in the understanding of bacterial groups and biocides in cooling tower.

2. Materials and Methods

2.1. Sample Collection and Isolation of Bacteria. A sample of water was collected using sterilized conical flasks, from a cooling tower site located at the Faculty of Engineering at the National University of Singapore, and was transported in an ice box to the laboratory. The physicochemical parameters of the cooling water are summarized in Table 1. The water sample was serially diluted (10-fold) with sterile saline water (0.85% w/v sodium chloride) and plated using pour plate technique. Winogradsky nutrient medium [37], consisting of (grams per liter): 1.0 K_2HPO_4 , 1.27 $NaNO_3$, 0.1 $CaCl_2$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.05 $FeSO_4 \cdot 7H_2O$, 0.54 NH_4Cl , 1.27 $NaNO_3$, and 10 D-glucose anhydrous, was supplemented with 0.5 yeast extract and used as the medium. Viable bacteria count was enumerated on agar plates containing the supplemented Winogradsky nutrient medium (WNM) with 1.5% agar (pH 7). Experimental cultures were grown in conical flasks at $28 \pm 2^\circ C$ for two days. Bacterial growth was detected by a colour change in the medium (from green to red). Total viable count of bacteria was enumerated by pour plate method using sterile WNM plates and incubated at $28 \pm 2^\circ C$ for two days.

2.1.1. Partial Biochemical Characterization of the Isolates and Molecular Identification of Bacteria. Dissimilar aerobic bacteria isolated from the agar plates were identified

according to Bergey's Manual of Determinative Bacteriology [38]. The isolated bacterial cultures were identified up to the genus level by their morphological and partial biochemical characterizations using the following: (i) gram staining, (ii) motility test, (iii) indole production, (iv) methyl red test, (v) Voges-Proskauer test, (vi) citrate utilization test, (vii) H_2S production test, (viii) carbohydrate fermentation test, (ix) catalase test, (x) oxidase test, (xi) starch, (xii) gelatin, and (xiii) lipid hydrolysis. In addition, citrate agar was used to detect any iron oxidizing activity of the isolates. Genomic DNA of the bacterial isolates was extracted according to Ausubel et al. [39]. Amplification of gene encoding small subunit ribosomal RNA was carried out using eubacterial 16S rRNA primers (forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' (*E. coli* positions 8 to 27) and reverse primer 5'-ACGGCTACCTTGTTACGACTT-3' (*E. coli* positions 1494 to 1513)) [40]. Polymerase chain reaction (PCR) was performed with a 50 μL reaction mixture containing 2 μL (10 ng) of DNA as the template, with each primer at a concentration of 0.5 μM and 1.5 mM $MgCl_2$ and each dNTP at a concentration of 50 μM as well as 1 μL of *Taq* DNA polymerase and buffer as recommended by the manufacturer (MBI Fermentas). PCR was carried out with a Mastercycler Personal (Eppendorf) with the following program: initial denaturation at $95^\circ C$ for 1 min, 40 cycles of denaturation (3 min at $95^\circ C$), annealing (1 min at $55^\circ C$), and extension (2 min at $72^\circ C$), followed by a final extension (at $72^\circ C$ for 5 min). The amplified product was purified using GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences) and cloned in pTZ57R/T vector according to the manufacturer's instructions (InsT/Aclone PCR Product Cloning Kit, MBI Fermentas) and transformants were selected on LB medium containing ampicillin (100 $\mu g/mL$) and X-gal (80 $\mu g/mL$). DNA sequencing was carried out using ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). For sequencing reaction, Big Dye Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer) was used.

2.1.2. Phylogenetic Analysis of the Bacterial Isolates. The sequences obtained were analysed with BLAST search version 2.2.20 [41] and tools of Ribosomal Database Project II Release 10 (<http://rdp.cme.msu.edu>) for taxonomic hierarchy of the sequences. Multiple sequence alignments were performed using CLUSTAL X2 [42] with a collection of taxonomically related sequences obtained from National Center for Biotechnology Information (NCBI) Taxonomy Homepage (<http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/>) and Ribosomal Database Project-II Release 10 (<http://rdp.cme.msu.edu>). Phylogenetic and similitude analyses were done with the common 16S rRNA gene regions and all alignment gaps were treated as missing data. The paired similitude and pairwise distance calculations using the transversion/transition weighting ($R = s/v$) and the Kimura-2-parameter model [43] were performed with the MEGA version 4.1 program [44]. The phylogenetic trees were constructed (neighbor-joining method) and 1000 bootstrap replications were carried out to validate internal branches [45]. MatGAT v. 2.01 software [46] was used to calculate the similitude percentages among sequences.

2.1.3. Nucleotide Sequence Accession Numbers. The 16S rRNA sequence data reported in this work has been deposited in the GenBank database, under the accession numbers from FJ755909 to FJ755915.

2.2. Biocorrosion Studies and Surface Analysis. Copper metal ($\geq 99\%$ Cu) was used in the study of the MIC behaviour of the bacteria *M. timonae* ARTYP3. Different size coupons (10 mm diameter disc of 2 mm thickness and 50×10 mm rectangular coupons of 2 mm thickness) were sequentially ground with a series of grit silicon carbide papers (grades 180, 500, 800, 1200, and 1500) to a smooth surface and finally polished to a mirror finish surface using 0.3 micron alumina powder. The polished coupons were rinsed with deionized water and surface was sterilized by immersion in an aqueous solution of 70% ethanol for one minute and finally dried in a desiccator. The prepared coupons were used for biocorrosion studies and surface analysis (using weight loss method, EIS, SEM-EDX, and FTIR). The polished coupons of both shapes and sizes were introduced into a 500 mL conical flask containing 250 mL sterilized cooling tower water with 1% of WNM broth as the control system (System 1), while a sterilized cooling tower water with 1% of WNM broth inoculated with 1 mL of *M. timonae* bacterial culture (about 10^5 CFU/mL) was used as the experimental system (System 2). Another experimental system (System 3) consisted of System 2 with the addition of 5 ppm biocide (2-bromo-2-nitropropane-1, 3-diol) (Bronopol). Bronopol is commonly used as a microbiocide or microbiostat for the control of slime-forming bacteria, fungi, and algae [47]. Corrosion experiments were initiated by hanging polished copper coupons on a nylon string in all the systems. After 10 days of incubation, the coupons were removed from each system for electrochemical, FTIR, and SEM-EDX analyses. Three coupons were exposed in each conical flask with duplicate flasks for each system. In total, six coupons were used for the weight loss for each system; the average weight loss and standard deviations (SD) were calculated. Impedance and potentiodynamic polarization data were obtained using an Autolab PGSTAT potentiostat. All experiments were performed in a three-electrode electrochemical cell, with a platinum electrode as the counter electrode and an Ag/AgCl electrode as the reference electrode. The EIS measurements (using duplicate coupons) were performed ex-situ; the coupons that were removed from the appropriate systems served as the working electrode by embedding them in a sample holder of the corrosion cell (purchased from Metrohm Pte, Ltd.). The working electrode had an exposed surface area of 0.785 cm^2 . 500 mL of the media was transferred from each system into the electrochemical cell to serve as the electrolyte for the EIS analysis [6]. EIS were made at open circuit potential using a 10 mV amplitude sinusoidal signal over frequencies ranging from 5 mHz to 100 kHz. Tafel plots were measured with a scan rate of 0.5 mVS^{-1} and were obtained by scanning from the open circuit potential (E_{corr}) towards 200 mV anodically and -200 mV cathodically using duplicate coupons. A scanning electron microscope coupled with energy-dispersive X-ray spectroscopy analysis (SEM-EDX) microscope (JEOL, model JSM-5600) with a beam voltage of 15 kV was used to visualize

the morphology of the biofilm. Surface topography of the coupons and its elemental composition were characterized after removal of corrosion product, using SEM-EDX analyses. The corrosion product on the copper metal was removed by immersion in 50% concentrated hydrochloric acid for 1–3 min (ASTM G1–90). To examine the deposition of EPS on the coupons and its chemical composition, the coupons were analysed using FTIR and SEM as described earlier [48]. An FTIR microscope (Bio-Rad Model: FTS 135) equipped with a narrow band of liquid nitrogen-cooled HgCdTe (MCT) detector was used for collecting the IR spectra. An atomic force microscopy (AFM) analysis was carried out to visualize the biofilm formed on the metal surface after the corrosion experiment. The coupon was removed from the media, lightly rinsed in sterile distilled water, and air-dried. The coupon was examined under an AFM (Bruker Dimension Icon Nanoscope V) in the tapping mode to capture the images of biofilms on the coupon surface. Silicon N-type cantilever nanoprobe with a spring constant of $k = 25\text{--}75 \text{ N/m}$ (App NANO) were used.

2.3. Bacterial Adhesion to Hydrocarbons (BATH) and Salt Aggregation Test (SAT). BATH assay was used to assess the hydrophobicity of the bacteria ARTYP3, with hexadecane being used as the carbon source. *Massilia timonae* was harvested from growing cultures by centrifugation at 8000 g for 10 min at 4°C , washed twice, and suspended in PBS buffer (pH 7.1; containing (g/L) K_2HPO_4 , 22.2; KH_2PO_4 , 7.26; urea, 1.8; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2) at an initial absorbance of 0.12–0.14 (at 400 nm). The detailed procedure for the BATH test and the calculation of the emulsifying capacity as evaluated by an emulsification index (E_{24}) has been described earlier [2]. SAT test was used to measure the relative cell surface hydrophobicity of the bacteria. Sodium phosphate (0.002 M, pH 6.8) was used to dilute a solution of 4 M $(\text{NH}_4)_2\text{SO}_4$ at pH 6.8. Serial dilutions were made giving $(\text{NH}_4)_2\text{SO}_4$ concentration ranging from 4.0 to 0.2 M differing by 0.2 M per dilution and ranging from 0.20 to 0.02 M differing by 0.02 M [49]. A bacterial suspension of 25 μL in 0.002 M sodium phosphate buffer (pH 6.8) was mixed with an equal volume of salt solution into 24-well tissue culture tray. The bacterial/salt mixture was gently rocked for 2 min at 25°C and visual observation was made against a dark background. The results were expressed as the lowest molarity of ammonium sulphate that caused bacterial aggregation.

3. Results

3.1. Physicochemical Characterization and Bacteriological Analysis of Cooling Tower Water. Table 1 shows the physicochemical parameters of the water sample collected from the cooling tower. The concentration of chloride and total hardness in the cooling water sample were 296 mg/L and 150 mg/L, respectively. The total viable count in the supplemented WNM was 6.1×10^5 CFU/mL. Preliminary identification of the bacteria by biochemical test indicated that the isolates belong to the genera *Massilia* sp. and *Pseudomonas* sp. The phenotypic profiles of the species isolated from cooling

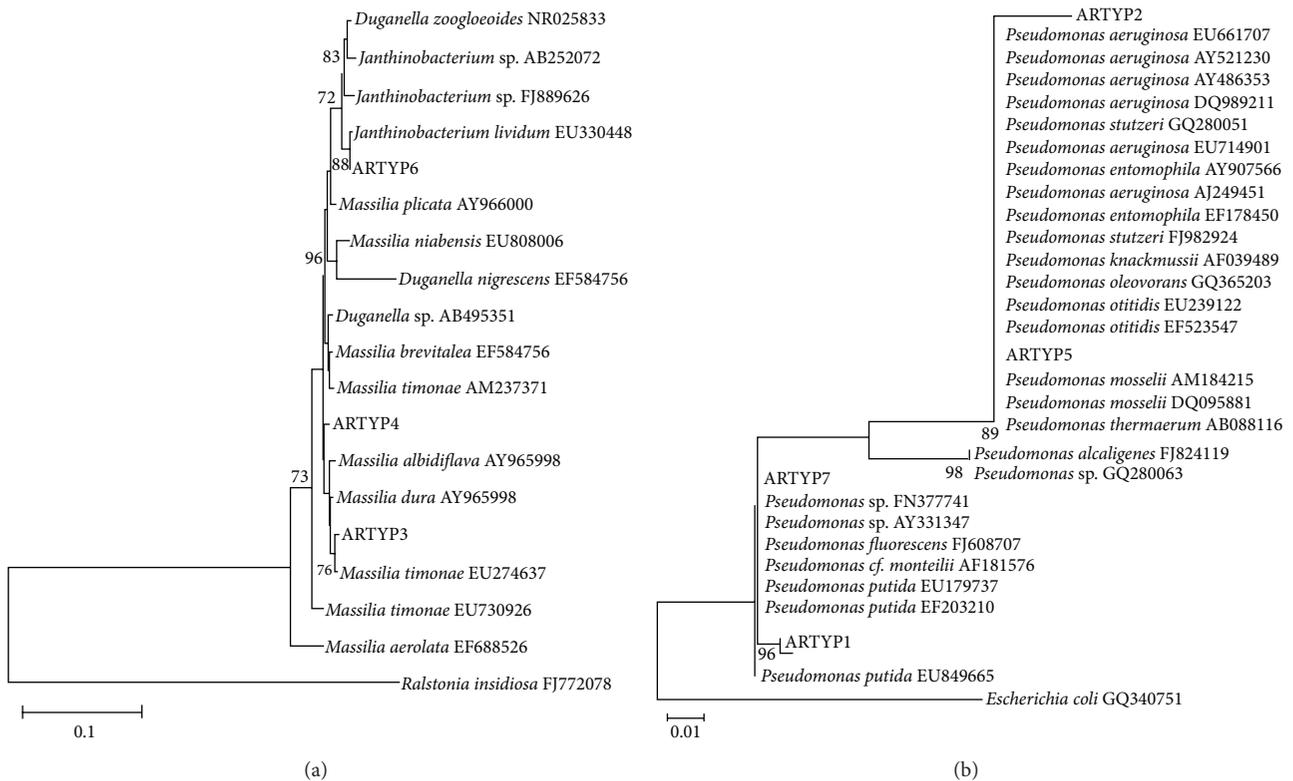


FIGURE 1: (a) Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the *Massilia* related species. *Ralstonia insidiosa* was used as a bacterial out-group. Numbers at nodes indicate bootstrap values > 50% from 1,000 replicates. GenBank accession numbers are given in parentheses. The scale bar indicates sequence divergence. (b) Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the *Pseudomonas* related species. *Escherichia coli* was used as a bacterial out-group. Numbers at nodes indicate bootstrap values > 50% from 1,000 replicates. GenBank accession numbers are given in parentheses. The scale bar indicates sequence divergence.

water strains are shown in Tables 2(a) and 2(b). The sequences obtained were submitted to a BLAST search to retrieve the corresponding phylogenetic relatives. The phylogenetic affiliations (Gammaproteobacteria and Betaproteobacteria) were confirmed by analyses of all related species recognized by the taxonomic and classification hierarchy done with the NCBI Taxonomy Homepage and Ribosomal Database Project-II Release 10. Two neighbor-joining phylogenetic trees were constructed for the *Massilia* related species (Figure 1(a)) and *Pseudomonas* related species (Figure 1(b)), to analyse the relationships among the sequences of the ribosomal library and related organisms from the GenBank database. The similarity and species identified with the phylogenetic analysis are given in Table 3. In the *Pseudomonas* genera tree, all the isolates belonging to *Pseudomonas* genus exhibited a high nucleotide sequence similarity of more than 99%; the corresponding bacterial isolates are, namely, *Pseudomonas putida* (99.2%), *Pseudomonas aeruginosa* (99%), *Pseudomonas mosselii* (99.5%), and *Pseudomonas* sp. (98.6%). In the class of *Massilia*, several isolates of the genus *Massilia* exhibited high sequence similarity (99%): *Massilia timonae* (99.5%) and *Massilia plicata* (99%), and one isolate, *Massilia albidiflava* had 98.5% similarity.

Phenotypic profile of strain ARTYP3 was shown in Table 2(a). Amplification of the gene encoding for small sub-unit ribosomal RNA of ARTYP3 was done using eubacterial 16S rDNA primers. The 16S rDNA amplicons derived from ARTYP3 are cloned in pTZ57R/T vectors. The recombinant plasmid (pARTYP3, harboring 16S rDNA insert) is partially sequenced. The sequence obtained is matched with the previously published sequences available in NCBI using BLAST. Sequence alignment and comparison revealed more than 99% similarity with *M. timonae*. *Massilia timonae* ARTYP3 has been deposited in GenBank with the accession number FJ755911 and also been submitted to the German Collection of Microorganisms and Cell Cultures (DSMZ).

3.2. Biocorrosion Studies and Surface Analysis

3.2.1. Weight Loss Study and Electrochemical Analysis. In the control system, the average weight loss of the metal coupons (0.0073 mg) was significantly less than in the presence of *M. timonae* ARTYP3 (0.065 mg). The average weight loss was lowest (0.0045 mg) in the presence of the biocide, that is, System 3. Figure 2 shows the Tafel polarization curves for copper in a cooling water system in the absence (System 1)

TABLE 2: (a) Biochemical characterization of *Massilia* related species isolates from cooling tower. (b) Biochemical characterization of *Pseudomonas* related species isolates from cooling tower.

| (a) | | | |
|--------------------------|----------|----------|----------|
| Characteristics | FJ755911 | FJ755912 | FJ755914 |
| Cell morphology | | | |
| Gram stain | Negative | Negative | Negative |
| Shape | Rod | Rod | Rod |
| Motility | + | + | + |
| Sporulation | - | - | - |
| Growth at | | | |
| 20°C | - | - | - |
| 30°C | + | + | + |
| 40°C | + | + | + |
| Biochemical reaction | | | |
| Indole production test | - | - | + |
| Methyl red test | + | - | - |
| Voges-Proskauer test | - | - | - |
| Citrate utilization test | + | - | - |
| Oxidase test | + | + | + |
| Catalase test | + | + | + |
| Production of acid from | | | |
| Glucose | + | + | + |
| Galactose | - | - | - |
| Fructose | + | + | + |
| Sucrose | + | + | - |
| Mannitol | - | - | - |
| Lactose | + | + | - |
| Cellobiose | + | - | - |
| Adonitol | - | - | - |
| Arabinose | - | - | - |
| Raffinose | - | - | - |
| Inositol | - | - | - |
| Hydrolysis of | | | |
| Starch | - | + | - |
| Cellulose | - | + | - |
| Casein | - | + | - |
| Gelatin | - | - | - |
| Urea | + | - | + |
| Tween 80 | - | + | - |

(b)

| Characteristics | FJ755909 | FJ755910 | FJ755913 | FJ755915 |
|-----------------|----------|----------|----------|----------|
| Cell morphology | | | | |
| Gram stain | Negative | Negative | Negative | Negative |
| Shape | Rod | Rod | Rod | Rod |
| Motility | + | + | - | + |
| Sporulation | - | - | - | - |
| Growth at | | | | |
| 20°C | + | + | + | + |
| 30°C | + | + | + | + |
| 40°C | + | + | + | + |

(b) Continued.

| Characteristics | FJ755909 | FJ755910 | FJ755913 | FJ755915 |
|--------------------------|----------|----------|----------|----------|
| Biochemical reaction | | | | |
| Indole production test | - | + | + | + |
| Methyl red test | + | - | + | + |
| Voges-Proskauer test | - | - | - | - |
| Citrate utilization test | + | - | - | - |
| Oxidase test | + | - | - | - |
| Catalase test | + | + | + | + |
| Production of acid from | | | | |
| Glucose | + | + | + | + |
| Galactose | - | - | - | - |
| Fructose | + | + | + | + |
| Sucrose | - | + | + | + |
| Mannitol | - | - | - | - |
| Lactose | + | - | - | - |
| Cellobiose | + | - | - | - |
| Adonitol | - | - | - | - |
| Arabinose | + | + | + | + |
| Raffinose | - | - | - | - |
| Inositol | - | - | - | - |
| Hydrolysis of | | | | |
| Starch | + | + | + | + |
| Cellulose | - | + | + | + |
| Casein | - | + | + | + |
| Gelatin | + | - | - | - |
| Urea | - | + | + | + |
| Tween 80 | - | + | + | + |

and in the presence (System 2) of ARTYP3 and in the biocide system (System 3). The corresponding electrochemical data are presented in Table 4. The corrosion currents for System 1 and System 2 were 3.9×10^{-9} A/cm² and 5.5×10^{-7} A/cm², respectively, while, in presence of the biocide (System 3), the current was 1×10^{-9} A/cm². The open circuit potential (OCP) for the control system and in the presence of ARTYP3 and in the biocide systems were -255 mV, -225 mV, and -275 mV, respectively. Nyquist plots for the control and inoculated systems are given in Figures 3(a) and 3(b). The solution resistance (R_s) and the charge transfer resistance (R_{ct}) were derived from impedance measurements. In the control and in the presence of ARTYP3, the charge transfer resistance R_{ct} values were 12.7 Ω ·cm² and 0.01 Ω ·cm², respectively; R_{ct} increased significantly to 163 Ω ·cm² in the presence of the biocide (Table 4).

3.2.2. Surface Analysis. Biofilm formed on the copper metal surface in System 2 and System 3 was observed under SEM and is presented in Figure 4. ARTYP3 colonized the metal surface and formed a thick biofilm (Figure 4(a)) while significantly less biofilm was formed when the biocide was present (Figure 4(b)). The cell density of ARTYP3 at the end of the biocorrosion study was about 2.3×10^7 CFU/mL and 1.2×10^2 CFU/mL in the absence and presence of

TABLE 3: 16S rRNA sequence analysis of the bacterial isolates.

| Name of the isolates | Taxonomic phylum | Closest relationship in GenBank | Similarity (%) | Microbial group identified |
|----------------------|---------------------|--|----------------|--|
| ARTYP1 | Gammaproteobacteria | <i>Pseudomonas putida</i> (EU849665) | 99.2 | <i>Pseudomonas putida</i> (FJ755909) |
| ARTYP2 | | <i>Pseudomonas aeruginosa</i> (EU661707) | 99.0 | <i>Pseudomonas aeruginosa</i> (FJ755910) |
| ARTYP5 | | <i>Pseudomonas mosselii</i> (AM184215) | 99.5 | <i>Pseudomonas mosselii</i> (FJ755913) |
| ARTYP7 | | <i>Pseudomonas</i> sp. (FN377741) | 98.6 | <i>Pseudomonas</i> sp. (FJ755915) |
| ARTYP3 | Betaproteobacteria | <i>Massilia timonae</i> (EU274637) | 99.5 | <i>Massilia timonae</i> (FJ755911) |
| ARTYP4 | | <i>Massilia albidiflava</i> (AY965999) | 98.5 | <i>Massilia albidiflava</i> (FJ755912) |
| ARTYP6 | | <i>Massilia plicata</i> (AY966000) | 99.0 | <i>Massilia plicata</i> (FJ755914) |

TABLE 4: Polarization and impedance parameters for copper in the presence/absence of bacterial isolates and biocide.

| Systems | Polarization | | | Impedance data | | |
|----------|------------------------|--|-----------------------|----------------|--------------------------|-------------------|
| | E_{corr} (mV) | i_{corr} (A/cm ²) | Corrosion rate (mm/y) | R_s Ω | R_{ct} Ω | R_p Ω |
| System 1 | -255 | 3.9×10^{-9} | 4.5×10^{-5} | 100 | 12.7 | 2×10^6 |
| System 2 | -225 | 5.5×10^{-7} | 6.5×10^{-3} | 148 | 0.01 | 1.4×10^3 |
| System 3 | -275 | 1×10^{-9} | 1.1×10^{-5} | 181 | 163 | 1.8×10^7 |

System 1: 250 mL sterilized cooling tower water with 1% of supplemented WNM medium. System 2: System 1 with 1 mL of *M. timonae* bacterial culture (about 10^5 CFU/mL). System 3: Systems 1 and 2 with the addition of 5 ppm biocide (2-bromo-2-nitropropane-1, 3-diol) (bronopol).

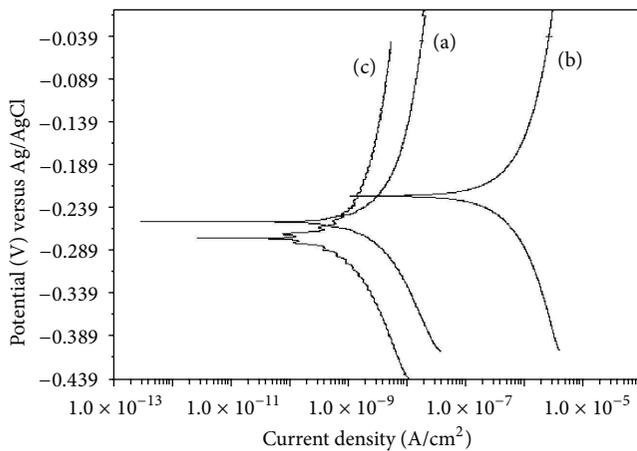


FIGURE 2: Tafel polarization curves of copper metal coupon in various systems after 10-day immersion in cooling water at 28°C: (a) control, (b) *M. timonae* ARTYP3, and (c) *M. timonae* ARTYP3 inoculated with biocide.

the biocide. AFM images (two- and three-dimensional view) of the ARTYP3 biofilm after exposure to the supplemented WNM medium are shown in Figure 4(c) where the thick biofilm on the copper metal surface is evident (Figure 4(c)). Figure 5 shows the SEM topography of the copper coupons after 10 days of exposure in the uninoculated system (Figure 5(a)), in the presence of the isolate (Figure 5(b)), and in the system with the isolate and the biocide (Figure 5(c)), after the removal of corrosion products and biofilm. Pitting corrosion was evident on the surface of the copper metal in the bacteria inoculated system but, in presence of the biocide, significantly less corrosion was observed. FTIR spectra of the ARTYP3 biofilm on the copper metal coupons exposed to the media with and without bronopol are shown in

Figure 6. The distinct narrow peaks (1140 cm^{-1}) observed in the spectrum (Figure 6(a)) may be ascribed to the C–O–C group of polysaccharides. A broad band was noted in the range of $3000\text{--}3500 \text{ cm}^{-1}$, which may be assigned to adsorbed water molecule OH/NH group. The protein amide I (C=O stretch weakly coupled with C–N stretch and N–H bending) and amide II (C–N stretch strongly coupled with N–H bending) regions are found at approximately 1650 cm^{-1} and 1550 cm^{-1} , respectively. The covalent property of the C–O–Cu bond results in a strong band due to the C–O–C stretching vibration for ester in the range of $1675\text{--}1620 \text{ cm}^{-1}$. This region reflects vibrational modes of carbohydrate C–O–C and the broad phosphate-oxygen stretch. The band at $1242\text{--}1254 \text{ cm}^{-1}$ is due to the –OH group. In the presence of bronopol, no significant peaks were observed on the copper metal surface in the FTIR spectrum (Figure 6(b)).

The bacteria cell surface hydrophobicity was assessed using BATH and SAT assays. Grown in *n*-hexadecane containing medium, ARTYP3 was found to be hydrophobic. The emulsifying capacity of ARTYP3 evaluated using the emulsification index (E) at different time periods, 24, 48, and 72 h, was about E_{24} 18%, E_{48} 22%, and E_{72} 22% [50]. Aggregation of *M. timonae* ARTYP3 with $(\text{NH}_4)_2\text{SO}_4$ was noted visually (against a dark background) even at the lowest salt concentration (0.002 M). This observation confirms the hydrophobic nature of the cell surface.

4. Discussion

As reported by Gallego et al. [51] *Massilia timonae* is one of five species under the genus *Massilia* belonging to the family of Oxalobacteraceae (Betaproteobacteria). Subsequently, other species of the genus *Massilia* were also successfully isolated from blood and environmental samples [52, 53]. *M. timonae* is identified and reported for the first time in water

from a cooling tower. The bacteria *M. timonae* are Gram-negative, motile, and rod-shaped. Catalase and oxidase were present in all the *Massilia* sp. strains (Table 2(a)). The isolates ARTYP1–ARTYP7 from the cooling water deposited ferric hydroxide precipitate around the colony and formed as rust-red colour. As shown in the *Massilia* genera phylogenetic tree (Figure 1(a)), the isolates exhibited high sequence similarity with *M. timonae*, *M. albidiflava*, and *M. plicata* (>98.5%). The involvement of *M. timonae* is unexpected as this species has not previously been reported to be associated with MIC in cooling tower systems.

Pseudomonas species, the most prevalent in industrial water and seawater, have been reported in the corrosion of mild steel, stainless steel, and aluminum alloys in marine and industrial habitats [54, 55]. Aerobic *Pseudomonas* species are recognized to be the pioneer colonizer in biofilm formation; their primary role appears to be to create an oxygen-free environment to harbor other corrosive bacteria (such as the sulphate reducing bacteria). It has been reported that these aerobic slime-formers often grow in a patchy distribution over the metal surface and exclude oxygen via respiration, thereby creating oxygen concentration cells or ion concentration cells [56]. *Pseudomonas* has also been shown to enhance the corrosion of metals and cause pitting corrosion on the metal surface. Pedersen et al. [56] reported that *Pseudomonas* sp. facilitated the breakdown of passivity by excreting organic acids, thus resulting in an increase in the corrosion rates of metals. Busalmen et al. [57] attributed the acceleration in the corrosion rate to the cathodic reduction by the catalase secreted by *Pseudomonas*. The *Pseudomonas* species isolated from the cooling tower also produce the catalase enzyme. In the *Pseudomonas* phylogenetic tree, the isolates exhibited a high nucleotide sequence similarity with *P. aeruginosa*, *P. mosselii*, *P. putida*, and *Pseudomonas* sp. with high similarity values (>98%). The phylogenetic analysis of the isolates showed the dominance of *Pseudomonas* sp. among Gammaproteobacteria, and *Massilia* sp. among Betaproteobacteria (Table 3).

The effect of the bacteria on the metal surface and its induced corrosion process is manifested through electrochemical analysis. The data obtained from the polarization study in the presence/absence of ARTY3 and the biocide are presented in Table 4. The open circuit potential (OCP) in the presence of the biocide was negative (−275 mV) when compared to control (−225 mV), thus indicating that the biocide bronopol acts as a mixed inhibitor and suppresses both anodic and cathodic current significantly. The corrosion rate was higher in the presence of ARTYP3 (6.5×10^{-3} mm/y), when compared to the control (4.5×10^{-5} mm/y). In the presence of the biocide, however, the corrosion rate and current density were reduced (to about 1.1×10^{-5} mm/y and 1×10^{-9} A/cm²). In the presence of ARTYP3, the corrosion current was the highest, thus indicating that ARTYP3 accelerates the corrosion of copper. Figure 2(b) shows that, in the presence of ARTYP3, both the anodic and cathodic curves shifted to the right when compared to the control, which indicated enhanced (anodic and cathodic) reactions, with the uptake of hydrogen and dissolution of copper on

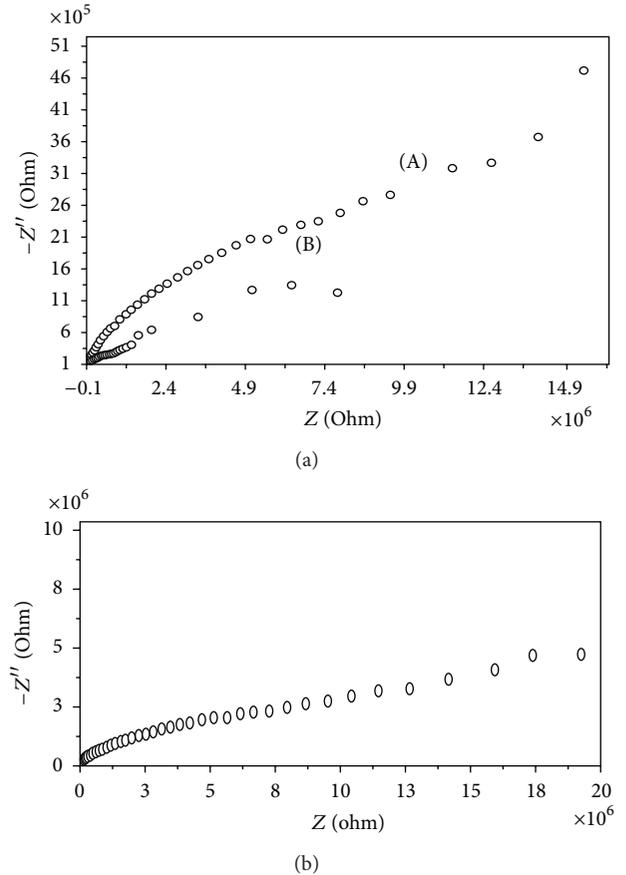


FIGURE 3: (a) Impedance curves of copper metal coupon in various systems after 10-day immersion in cooling water at 28°C: (A) control system and (B) *M. timonae* ARTYP3. (b) Impedance curves of copper metal coupon in *M. timonae* ARTYP3 inoculated with biocide after 10-day immersion in cooling water at 28°C.

the metal surface. This led to pitting corrosion [58]. However, in the presence of the biocide (System 3), both anodic and cathodic reactions were suppressed significantly (i.e., Figure 2(c)). The suppression of corrosion current was due to the inhibition of the biofilm formation on the copper surface. Compared to the control, the resistances of the solution, R_s , in the bacterial cultures were higher, possibly due to bacterial exopolysaccharides in the medium. In the presence of the biocide (System 3), the charge transfer resistance, R_{ct} , was significantly higher (163 Ω) when compared to the control (12.7 Ω) and System 2 (0.01 Ω), possibly due to the compact nitrogen band with copper oxide layer on the metal surface which inhibits biocorrosion. In the presence of ARTYP3, the impedance curve (Figure 3) shows an activation control process due to defects and pores in the adsorbed film on the metal surface [59]. However, in the presence of the biocide, the curve shows a large and well-defined semicircle capacitive loop (Figure 3(b)). The capacitive loop shows the formation of the intact part of the adsorbed film on the metal surface and is large compared to the control. This observation corroborates the polarization studies.

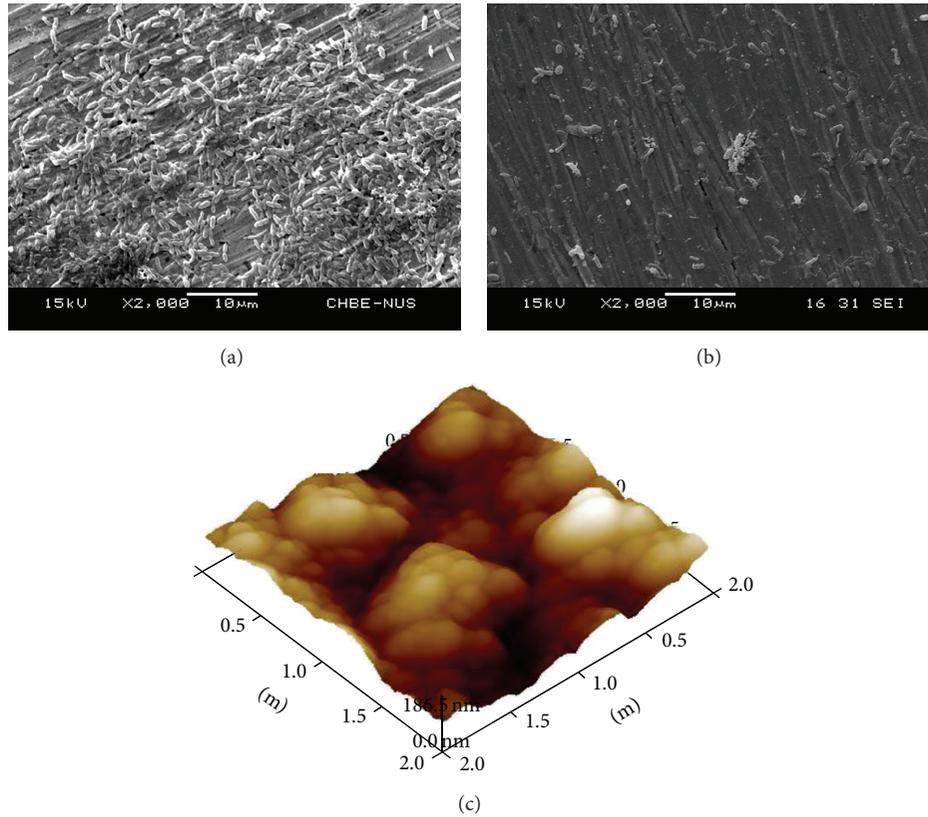


FIGURE 4: SEM and AFM micrographs of the copper surface coupon after immersion in cooling water systems (a) SEM: *M. timonae* ARTYP3 and (b) SEM: *M. timonae* ARTYP3 inoculated with biocide (c) AFM (three-dimensional view) *M. timonae* biofilm.

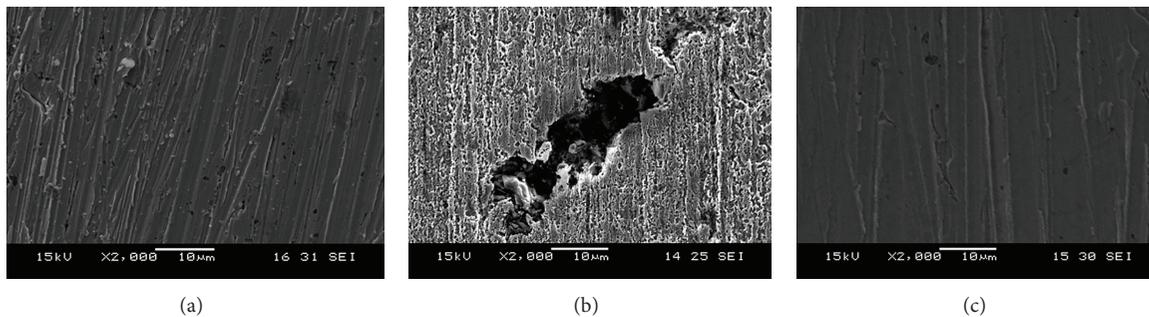


FIGURE 5: SEM micrograph of the copper surface coupon after immersion in cooling water systems (a). Copper surface exposed to the medium without bacteria and biocide. (b) Copper surface exposed to *M. Timonae*. (c) Copper surface exposed to *M. timonae* with the biocide bronopol.

Weight loss and corrosion rate were also monitored in the presence or absence of *M. timonae* and the biocide. Weight loss increased in the presence of the ARTYP3 (0.065 mg) compared to the uninoculated system (0.0073 mg) but was significantly reduced to about 0.0045 mg in the presence of the biocide. The corrosion rate, at 0.0177, 0.1573, and 0.0109 mm/year in Systems 1, 2, and 3, respectively, showed that bronopol inhibits the pitting corrosion of copper metal. Unlike the control system where uniform corrosion was noted, severe pitting attacks were observed over the surface of

steel in the presence of ARTYP (Figure 5). In the presence of the biocide (System 3), no obvious corrosion pit was observed (Figure 5), thus showing that the biocide significantly inhibits biofilm formation (Figure 4) by ARTYP3 on the metal surface and minimizes corrosion. *M. timonae* and *Pseudomonas* sp. contribute to biofilm formation by producing exopolysaccharides and facilitating the attachment of other microorganisms, hence accelerating the corrosion process [60]. *Massilia* sp. and *Pseudomonas* sp. are capable of oxidizing copper ions as electron donors and gaining energy from the oxidation of

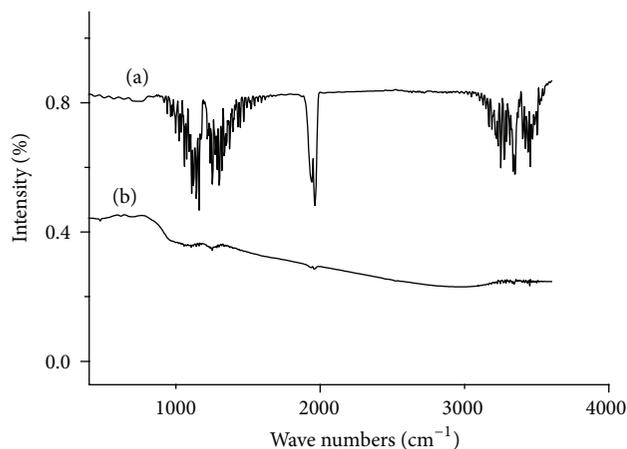


FIGURE 6: FTIR spectrum of copper surface coupon after immersion of cooling water systems. (a) *M. timonae* ARTYP3 and (b) *M. timonae* ARTYP3 inoculated with biocide.

copper to copper oxides. The bacteria promote the formation of copper oxides at low pH (5.0) which leads to pitting corrosion.

Surface hydrophobicity of microorganisms is recognized as a dominant factor that influences its adhesion on surfaces and hence surface corrosion. In biological systems, hydrophobic interactions are usually the strongest of all long-range noncovalent interactions and can be defined as the attraction between apolar or slightly polar molecules, particles, or cells, when immersed in water [61]. The hydrophobic nature of ARTYP3 has been established, and the emulsification index (E) was found to increase with the time of exposure [50]. This suggests the production of biosurfactant (rhamnolipid) by ARTYP3 and thus leads to higher uptake of hexadecane as the carbon source. In addition, aggregates of ARTYP3 were formed, even at the lowest dilution of ammonium sulphate, due to the hydrophobic nature of the cells. A higher hydrophobic behavior of the bacterial isolate induces greater adhesion on the metal surface [50, 62].

EPS comprises macromolecules such as proteins, polysaccharides, nucleic acids, and phospholipids. The capacity of EPS to bind metal ions is an important factor in determining the MIC behavior of the bacterial isolates [48, 60, 63]. Figure 4(a) shows typical rod-shaped cells (about $1\ \mu\text{m}$) on the metal surface (Figure 4(a)). In the presence of the biocide, significantly less bacteria were observed (Figure 4(b)). A thick layer of biofilm with clusters of microbial cells and EPS was also noted on the metal surface (Figure 4(c)). FTIR (Figure 6(b)) confirms the adhesion of EPS (COO^- , amide groups) secreted by the bacteria on the metal surface [63]. The addition of bronopol resulted in significant reduction in all the peak intensity, due to the inhibition of biofilm formation. The antibacterial mechanism of bronopol has been attributed to its interaction with essential thiols within the cell [32, 33]. Shepherd et al. [36] have also reported that bronopol catalyses the oxidation of thiol groups to disulfides with the consumption of oxygen. Such catalytic reaction also results

in the generation of free radicals which subsequently inhibits the growth of the bacteria.

5. Conclusion

In this study, a corrosive bacterial community in water samples collected from a cooling tower was cultivated using traditional cultivation techniques and identified using 16S rRNA gene sequence. *M. timonae* was found to be the dominant corrosive bacteria amongst the culturable organisms and accelerated severe pitting corrosion in copper metal. The genus *Massilia* is identified for the first time in water from a cooling tower. The biosurfactant produced by the ARTYP3 contributes to an increase in the cell surface hydrophobicity of the bacteria and enhanced the bacterial adhesion on the copper metal surface. SEM-EDAX, AFM, and FTIR analyses of the copper metal coupons with bacterial biofilm developed after exposure to cooling water confirmed that EPS accumulate with exposure time and revealed that biofilms were formed as microcolonies, which subsequently caused pitting corrosion. Bronopol was shown to be an effective biocide in inhibiting both biofilm formation on the copper metal surface and the subsequent biocorrosion.

Conflict of Interests

The authors declare that there is no conflict of interests.

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