Role of calcium-depositing bacteria *Agrobacterium tumefaciens* and its influence on corrosion of different engineering metals used in cooling water system

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Abstract The present investigation deals with the role of calcium-depositing bacterial community on corrosion of various engineering metals, namely, brass alloy (BS), copper (Cu), stainless steel (SS) and mild steel (MS). Based on the corrosion behavior, *Agrobacterium tumefaciens* EN13, an aerobic bacterium is identified as calcium-depositing bacteria on engineering metals. The results of the study are supported with biochemical characterization, 16S rRNA gene sequencing, calcium quantification, weight loss, electrochemical (impedance and polarization) and surface analysis (XRD and FTIR) studies. The calcium quantification study showed carbonate precipitation in abiotic system/biotic system as 50 and 700 ppm, respectively. FTIR results too confirmed the accumulation of calcium deposits from the environment on the metal surface by EN13. Electrochemical studies too supported the corrosion mechanism by showing a significant increase in the charge transfer resistance (R_{ct}) of abiotic system (44, 33.6, 45, 29.6 $\Omega \text{ cm}^2$) than compared to biotic system (41, 10.1 29 and 25 $\Omega \text{ cm}^2$). Hence, the outcome of the present

study confirmed the enhanced bioaccumulation behavior of calcium by the strain, EN13.

Keywords Microbial corrosion · Mild steel · Copper · Calcium deposits

Introduction

Microbial-influenced corrosion (MIC) is one of the well-documented phenomena in corrosion, that causes deleterious effect on petroleum pipelines, storage tanks and, various industries (Rajasekar and Ting 2011; Stapleton 1987). MIC damages the equipments and leads to the reduction performance in cooling water system (Aquaprox 2009; Narenkumar et al. 2017; Machuca et al. 2016). In general, cooling water system (CWS) recirculates the water to transfer the heat, to maintain a proper temperature in the processing industry. But they operate in an open atmosphere, due to which the microbial growth is highly favored. The microbes grown in this environment are reported to cause MIC in CWS (El-Shamy et al. 2009; Rahmani et al. 2016). According to previous reports, about 20% of the metal and building material damages were caused by the microorganism (Ping et al. 2012). In an aqueous system, microbes tend to form a biofilm on the metal surface which leads to a change in the local electrochemical potential, thus accelerating the corrosion process (Costerton et al. 1987, 1999; Landoulsi et al. 2008; Swaroop et al. 2016; Parthipan et al. 2017a).

Copper, brass, stainless steel and mild steel are widely used engineering metals for the construction of processing industries due to its exceptional physical and chemical properties (Audouard et al. 1984; Prabakaran et al. 2014). The salt present in the recirculating water gets deposited on

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the metal surface leading to the accumulation of calcium by calcium-depositing bacteria, namely, *Calcifying bacterial*, *Arthrobacter*, *Bacillus firmus* and, *Xanthomonas* sp. (Cacchio and Ercole 2003; Hammes and Verstraete 2002). They form a layer of calcium hydroxide that reacts with carbonic acid to form an insoluble scaling product, calcium carbonate (CaCO_3) on the metal surfaces (Beech and Gaylarde 1999; Wang et al. 2013; Chafetz 1994). Other group of bacteria, viz., *Escherichia*, *Agromyces*, *Cellulomonas*, *Cupriavidus*, *Microbacterium*, *Ralstonia*, *Sphaerotilus* sp. *Thiobacillus ferrooxidans* are involved in the biotransformation of metal oxides such as manganese and iron (Booth 1971; Starosvetskya et al. 2001; Yang et al. 2013). The main role of bacteria in the accumulation process is ascribed to their ability to produce an alkaline environment through physiological activities (Gollapudi et al. 1995; Douglas and Beveridge 1998; Ehrlich 1990).

The accumulation of minerals/scale formation on equipments and pipes was found to greatly decrease the performance of the equipment, thus by increasing the circulation pressure (Liu et al. 2002; Fujita et al. 2000). Chen et al. (2009) reported that occurrence of bacterial-mediated biomineralization in CWS leads to scale formation on the metal surface. Apart from industrial operations, deposition of CaCO_3 in oral cavity and kidney stone due to bacterial action was also reported (Kajander and Ciftcioglu 1998). Hence, this study is aimed to analyze the role of calcium-depositing bacteria and its influence on scaling formation and corrosion on various engineering metals. The investigation is supported with weight loss method (WL), Fourier Transform Infrared spectroscopy (FTIR), X-ray diffraction spectroscopy (XRD) and electrochemical impedance spectroscopy analysis (EIS) studies.

Materials and method

Sample collection

The samples of scale deposition were collected from metal surface in cooling water system located at chemical processing industry, Sipcot, Ranipet, Vellore district, Tamilnadu, India (Latitude—12.953671° and Longitude—79.313132°) (Fig. 1). The samples were collected into sterile conical flasks, kept in an ice box and transferred to laboratory for further studies.

Screening and isolation of calcium-depositing bacteria

For identification of calcium-depositing bacteria (CDB), the biofilm samples were subjected to serially dilution (onefold) and plated using a pour plate technique on sterile selective medium B4 medium (g/l) composed of: calcium acetate 2.5 yeast extracts 4.0, and glucose, 10 (Baskar et al. 2006). The plates were incubated for 24–48 h at 37 °C. The total viable counts were enumerated and the CDB bacterial population were articulated as colony-forming units (CFU/ml). Dissimilar morphology colonies were selected randomly and purified by streak plate method on sterile B4 agar plate. The obtained CDB bacterial isolates from the B4 medium were identified according to Bergey's Manual method (1994), (i) Gram staining, (ii) motility test, (iii) indole production, (iv) methyl red test, (v) Voges–Proskauer test, (vi) citrate utilization test, (vii) carbohydrate fermentation test, (viii) catalase test, (ix) oxidase test, (xi) starch content, (xii) gelatin content, and (xiii) lipid hydrolysis test (Holt et al. 1994).

Fig. 1 Map showing the location of sampling site of cooling tower system, Tamilnadu, India

Molecular identification of CDB and phylogenetic analysis

Genomic DNA extraction from the isolated bacteria was adopted from the Weisburg et al. (1991). Genomic DNA was subjected to polymerase chain reaction (PCR) using universal primers 16S r RNA (forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGCTACCTTGTTACGACTT-3') (Altschul et al. 1990). PCR was performed with 50 µl reaction mixture containing 2 µl of template DNA, 1.5 mM MgCl₂, forward and reverse primers concentration at 0.5 µM, dNTP at a concentration at 50 µM and 1 µl of *Taq* DNA polymerase and buffer as recommended by the manufacturer (MBI Fermentas), the PCR reaction was adopted from Rajasekar and Ting (2014). PCR products were purified by Montage PCR Clean up kit (Millipore). Sequencing was carried out by Big Dye terminator cycle sequencing kit (Applied BioSystems, USA), and sequences for the bacterial 16S r RNA were determined using an automated DNA sequencing system (Model 3730XL Applied BioSystems, USA).

The sequences were analyzed by BLAST search version 2.2.20 (Tamura et al. 2007) and accessories of the Ribosomal Database Project II Release 10 (<http://rdp.cme.msu.edu>) to find the taxonomic hierarchy of the sequences. Taxonomically associated sequences were acquired from the National Centre for Biotechnology Information (NCBI) taxonomy data base and Ribosomal Database Project-II (Release 10). Phylogenetic and similarity analysis were performed on frequent 16S rRNA gene regions, and misaligned sequence were treated by the MEGA software program (Version 4.1) (Rajasekar and Ting 2014). Phylogenetic tree was constructed by neighbor-joining method and Mat GAT version 2.01 was used to analyze the similitude percentages between sequences.

Characterization of calcium carbonate precipitation by EN13

The EN13 culture was inoculated into B4 medium and incubated at 37 °C for 2–3 weeks. After incubation, the

cultures were centrifuged at 10,000 rpm for 10 min. The pellets were collected and dried at 60 °C for 3 h. The dried pellet was characterized using FTIR (Bruker's VERTEX 70 series) (Maruthamuthu et al. 2010). The quantification of calcium precipitation by EN13 and pH of the medium were measured. Surface analysis of calcium precipitation by EN13 was carried out in FTIR.

Biocorrosion study

Weight loss study

The weight loss (WL) experiment was performed on brass alloy (BS), copper (Cu), stainless steel (SS 316), and mild steel (MS 1010), to study the biocorrosion behavior of the bacteria. The composition of all the metals is presented in Table 1. Metal coupons of BS, Cu, SS and MS of dimension 2.5 × 2.5 mm and 0.1 cm² were used for weight loss and electrochemical studies analysis. The coupons were allowed to surface sterilize with 70% ethanol. Finally, the coupons were dried and stored in a desiccator for further experiment (Narenkumar et al. 2016). *Agrobacterium tumefaciens* EN13 was selected for biocorrosion studies. The polished BS coupons (i.e., three coupons) were introduced into 500 ml Erlenmeyer flask containing sterile 400 ml of cooling tower water with 1% of sterile B4 medium as BS control system. In BS experiment system BS control with EN13 (1.2×10^4 CUF/ml) was added; likewise, Cu, SS, MS were also introduced in their respective control and experimental systems. The triplicate for each system was carried out and the average corrosion rate was analyzed.

At the end of the WL experiment (15th day), the coupons were removed from the respective systems and the collected corrosion products were analyzed by FTIR and XRD (system controlled XRD, Bruker-8030) among 10° to 85° – 2θ (Narenkumar et al. 2016). For weight loss study, the coupons were pickled with respective pickling solution of 1 liter HCl containing 20 g of antimony trioxide and 50 g of stannous chloride at room temperature for 25 min (Rajasekar et al. 2007). The corrosion rate was calculated as suggested by National Association of Corrosion Engineers (NACE) (Mcintyre and Mercer 1994).

Table 1 Elemental composition (wt. %) of different metal coupons (Br, Cu, SS, MS)

Metals	Cr	Mo	Ni	C	Zn	Fe	Pb	Cu	Mn	P	S	Sn	Si
Brass	–	–	–	–	16	0.05	0.05	60.66	–	–	–	–	–
Copper	–	–	6.09	–	0.03	1.41	0.01	–	0.65	–	–	0.01	0.004
Stainless steel 316	14	1	2	1	–	–	–	–	–	–	–	–	–
Mild steel 1010	–	–	–	0.2	–	0.03	–	–	0.2	0.50	0.03	–	–

Table 2 Partial biochemical characterization of calcium-depositing bacteria, *Agrobacterium tumefaciens* EN13

Characteristics	<i>Agrobacterium tumefaciens</i> EN13 NCBI Accession No (KU955329)
Gram stain	Negative
Shape	Rod
Motility	+
Sporulation	+
Growth at (°C)	
20	—
30	+
40	+
Indole test	—
Methyl red test	+
Voges–Proskauer test	+
Citrate utilization test	—
Oxidase test	+
Catalase test	+
Production of acid from	
Glucose	+
Galactose	+
Fructose	+
Sucrose	—
Hydrolysis of	
Starch	+
Cellulose	+
Casein	—
Urea	+

Electrochemical studies

Electrochemical studies such as impedance and polarization were performed through a three electrode system (CH Instrument Inc., USA model CHI- 608E): Ag/AgCl as

reference electrode, Metal (BS, Cu, SS and MS) coupons as working electrode, and platinum wire as counter electrode. Impedance and Tafel polarization measurements procedures were adopted from our earlier studies (Rajasekar et al. 2017; Narenkumar et al. 2016).

Results and discussion

Bacteriological analysis of cooling water system

Preliminary identifications of the isolated bacteria by biochemical test confirmed the Gram negative nature of the bacteria. The results of biochemical characterization of the isolate are presented in Table 2. From the biochemical characterization, EN13 is reported as a facultative an aerobic, rod-shaped, motile, catalase and oxidase positive bacteria. The obtained sequence is aligned and compared with GenBank. The results showed > 99% homology with *Rhizobium* sp. Hence, this organism is named as *Agrobacterium tumefaciens* EN13. The sequence is submitted to BLAST search to repossess the consequent phylogenetic relative. The phylogenetic relationship is confirmed by every related species prediction table by taxonomy and categorization pecking order in accordance with the NCBI tools. The neighbor-joining phylogenetic tree was constructed and presented in Fig. 2. The EN13 gene sequence is submitted to GenBank under the Accession Number of KU955329.

The pH curve of EN13 strain in B4 medium is shown in Fig. 3. In control system, the initial pH of the B4 medium is found to gradually reduce from 5.0 to 4.0 at the end of incubation period. On contrast, the experimental system shows a significant increase from the initial pH of 5.0–9.9 at the end of incubation period. The role of

Fig. 2 Phylogenetic analysis of calcium-depositing bacteria *Agrobacterium tumefaciens* EN13 by 16S rRNA gene sequences

Fig. 3 pH curve of *Agrobacterium tumefaciens* *Agrobacterium tumefaciens* EN13 in B4 medium

increasing pH was increased towards the precipitation of calcium ions on the metal surface in the environmental which was already reported (Knorre and Krumbein 2000). The results of present study too supported this observation of increasing pH in the EN13 strain inoculated culture medium, which may be due to the accumulation of calcium acetate and calcium ions. The mechanism of calcium deposition on materials is based on the metabolic utilization of organic acids by the microbes. Acids commonly involved in the metabolic utilization are citrate, oxalate, glyoxylate and succinate (Braissant et al. 2002). The increased pH at the end of the day was due to the production OH^- ions. The accumulation of calcium carbonate and calcium phosphate by bacterial metabolism was due to high pH of the cell wall (Maruthamuthu et al. 2010). This shows the quantification of calcium carbonate precipitation which is due to the presence of EN13. A high amount of calcium carbonate of 700 ppm is observed than the control system (50 ppm). The quantification results of calcium concluded that EN13 has the ability to deposit calcium carbonate in the environment (Boquet et al. 1973).

Figure 4 shows the FTIR spectrum of the calcium deposit which was collected from B4 culture medium. A peak at 3444 and 2922 cm^{-1} are assigned to the stretching vibrations of NH and C=C group (amine and aromatic). The corresponding peak observed at 1724 cm^{-1} indicates the stretching vibration of CO_4 (metallic carbonate). A peak at 1400 cm^{-1} was corresponds to Ca (CO_4) and the other peaks observed at 1282, 984 and 623 cm^{-1} are assigned to SO_4 , CaCO_3 and CO_4 , respectively. These results confirmed the calcium carbonate deposition by the strain EN13.

Fig. 4 FTIR spectrum of B4 medium in the presence/absence of *Agrobacterium tumefaciens* EN13

Weight loss experiment

The corrosion rate of the different metal coupons in the presence/absence of EN13 strain is presented in Table 3. The corrosion rates (CR) of control system for BS, Cu, SS and MS are noted as 2.0, 0.1, 0.02 and 2.1 mm/year, respectively. In the presence of EN13, the CR of BS, Cu, SS and MS are observed as 2.6, 0.2, 0.03 and 3.2 mm/year, respectively. The WL results confirmed on the calcium accumulation behavior of EN13 that have accelerated the corrosion process on all the metal surfaces. This was supported by other studies too (Maruthamuthu et al. 2010; McConnaughey and Whelan 1997). Microbes associated with carbonate deposits have been described wildly in human kidney stone formation (Kramer et al. 2000), saline lakes (Boquet et al. 1973) and sea water environment (Morita 1980). The calcium deposition by EN13 can be explained by the following equations given below.

The strain EN13 continuously produces CO_2 and OH^- ions (Eq. 1) which will determine the pH of the solution. The formation of OH^- enhances the pH and favors calcium accumulation on the metal surface. Further, this reaction has encouraged a shift in the bicarbonate equilibrium and a subsequent pH rise in the medium (Maruthamuthu et al. 2010). As the reaction proceeds, HCO_3^- (Eq. 2) combines with Ca^{2+} ions and forms calcium carbonate (Eq. 3).

Table 3 Weight loss and corrosion rate of different metals in the presence/absence of *Agrobacterium tumefaciens* EN13

S. no	Metals	Systems	Weight loss (g)	Corrosion rate (mm/year)
1	Brass	Control	0.021 ± 1	2.0
		Experimental	0.027 ± 2	2.6
2	Copper alloy	Control	0.014 ± 1	0.1
		Experimental	0.019 ± 2	0.2
3	Stainless steel	Control	0.0002 ± 1	0.02
		Experimental	0.0004 ± 1	0.03
4	Mild steel	Control	0.17 ± 1	2.1
		Experimental	0.26 ± 2	3.2

Fig. 5 XRD spectrum of surface film formed on various engineering metal surface after immersion of various biocorrosion systems: (A) BS control, (B) BS experimental, (C) Cu control, (D) Cu experimental, (E) SS control, (F) SS experimental (G) MS control, (H) SS experimental

θ - CaCO_3 , α - MgCO_3 , β - Fe_2O_3 , θ - FeOCl , ϕ - FeOOH , * - $\text{Ca}_3(\text{PO}_4)$.

Fig. 6 Impedance spectrum of various biocorrosion system (A) BS control (B) BS experiment (C) Cu control (D) Cu experiment (E) SS control (F) SS experiment (G) MS control (H) MS experiment

Figure 5 presents the XRD data of the surface film collected from control and experimental system of various engineering metals. Presence of low intensity peaks corresponding to calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$), copper (II) hydroxide ($\text{Cu}(\text{OH})_2$), magnesium carbon (MgCO_3) are noted in the control system (Fig. 5a, c, e, g). Whereas the experimental system showed high intensity peaks of all corrosion products same as control. In addition, other new peaks such as calcium carbonate (CaCO_3) and Iron hydroxide (FeOOH) are also observed in the experimental system (Fig. 5b, d, f, h). The occurrence of CaCO_3 , $\text{Ca}_3(\text{PO}_4)_2$ and FeOOH indicated that *Agrobacterium tumefaciens* EN13 had accumulation of calcium which led to the corrosion of metal surface.

Electrochemical studies

Figure 6 shows the impedance spectrum for various metal coupons (BS, Cu, SS and MS) in presence/absence of EN13. The results of the electrochemical studies are presented in Table 4. The charge transfer resistance (R_{ct}) of

control (BS, Cu, SS and MS) is about 41, 10.1, 29 and $25 \Omega \text{ cm}^2$, respectively, whereas the experimental system shows 41, 10.129 and $25 \Omega \text{ cm}^2$ for BS, Cu, SS and MS, respectively. Figure 7 shows the Tafel polarization plot of the different metal coupons of control and experimental system with their respective values presented in Table 4. The corrosion current (I_{corr}) of the control for BS, Cu, SS and MS was obtained as 3.2×10^{-5} , 5.0×10^{-5} , 7.0×10^{-5} and $7.2 \times 10^{-4} \text{ A/cm}^2$, respectively, whereas the experimental system of BS, Cu, SS and MS showed the 9.3×10^{-4} , 2.6×10^{-4} , 2.3×10^{-4} and $4.8 \times 10^{-4} \text{ A/cm}^2$, respectively.

From the impedance study, the lower R_{ct} values of the experimental system (EN13) inferred the formation of heterogeneous film (calcium carbonate) on the metal surfaces, which indicates the occurrence of severe corrosion on the metal surface. Thus, the EIS results too supported the WL studies and conformed on the higher corrosion rate in the experimental system than the control system. The higher solution resistance (R_s) and charge transfer resistance (R_{ct}) values of the experimental system have evidenced the possible calcium precipitation by EN13 on all the metal surfaces. The polarization studies too inferred on the accumulation of calcium that might have led both anodic/cathodic reactions on the metal surface (McConaughy and Whelan 1997; Parthipan et al. 2017b; Elumalai et al. 2017). Hence, it can be concluded that the calcium deposition behavior of EN13 strain has played a key role in scale formation and corrosion of CWS.

Conclusion

In this present study, the role of calcium-depositing bacteria, EN13 on the corrosion of different metal surfaces was investigated. *Agrobacterium tumefaciens* EN13 was identified as a CDB on the metal in the CWS which was evaluated by FTIR and EIS. FTIR confirmed

Table 4 Electrochemical data for various metals in the presence and absence of calcium-depositing bacteria *Agrobacterium tumefaciens* EN13 in various biocorrosion system

Metals	System	Polarization data				Impedance data	
		E_{corr} (mV)	I_{corr} ($\mu\text{A/cm}^2$)	β_a (mV/dec)	β_c (mV/dec)	$R_s \Omega \text{ cm}^2$	$R_{ct} \Omega \text{ cm}^2$
Brass	Control	− 270	3.2×10^{-5}	11.46	3.52	3.7	44
	Experiment	− 244	9.3×10^{-4}	0.1	4.0	3.3	41
Copper	Control	− 209	5.0×10^{-5}	0.2	2.5	7.4	33.6
	Experiment	− 216	2.6×10^{-4}	6.9	4.6	3.36	10.1
Stainless steel	Control	− 451	7.0×10^{-5}	6.17	2.95	7.1	45
	Experiment	− 437	2.3×10^{-4}	7.1	4.54	6.1	29
Mild steel	Control	− 603	7.2×10^{-4}	5.72	3.51	3.1	29.6
	Experiment	− 507	4.8×10^{-4}	6.82	4.13	2.6	25

Fig. 7 Polarization spectrum of various biocorrosion system (A) BS control (B) BS experiment (C) Cu control (D) Cu experiment (E) SS control (F) SS experiment (G) MS control (H) MS experiment

a thick film of calcium carbonate formed by EN13 on the metal surface and thus accelerated the corrosion on various engineering metals. Overall, this study proved the role of strain EN13 in accumulation of calcium carbonate leading to severe corrosion on metal surfaces. This study also revealed the presence of calcium carbonate as an important component of scaling and its influence on corrosion of engineering metals used in CWS.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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