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Role of Bacterial Plasmid on Biofilm Formation and Its Influence on Corrosion of Engineering Materials

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Abstract Bacterial plasmids are involved in biofilm formation, degradation of hydrocarbons and biosynthesis of antibiotics in extreme environments. A number of intercalating compounds have been reported to be used for curing the plasmids in various bacterial species, since they act as inhibitors during plasmid replication by selectively binding to the plasmid DNA. In this research, we investigated the influence of bacterial plasmid on biofilm formation on metal surface and corrosion of engineering metals, such as mild steel (MS), stainless steel (SS) and brass (BS). The biocorrosion behaviour of *Bacillus thuringiensis* EN2 wild and cured strain was studied using immersion tests based on the weight loss method, electrochemical analysis and surface analysis techniques (FTIR spectroscopy). The plasmid cured strain EN2 was unable to form biofilm, showing significantly less corrosion over all the tested metal surfaces. On the contrary, the presence of plasmid led to higher corrosion rate (0.18, 10.3 and 91.5 mm/year) and surface hydrophobicity for MS, SS and BS. Overall, it can be concluded that the plasmid gene was found to be responsible for the biofilm formation, which determines the corrosion rate of metals.

Keywords Plasmid · Biocorrosion · Biofilm · Metals · *Bacillus thuringiensis*

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1 Introduction

Microbial influenced corrosion (MIC) led to the destruction of metal surface via the alteration of electrochemical reaction. This reaction depends on the physio-chemical characteristics of the environment, such as pH, temperature and conductivity. The effect of metal pipeline corrosion will result in decrease of water supply, continuous microbial growth and "red or black water" environment [1–3] with important and massive economic relevance and health damages. For example, in Italy, the loss of water in the system of distribution is considered as 2.61 billion of m^3, corresponding to 226 million of euro and 30 % of the water supply, with an average of water loss in advanced countries of 15–20 % [4]. New solutions, including technological approaches, are urgently needed. In metallic and non-metallic surface in aquatic phase, the bacterial community adheres on the surface and forms a polymeric matrix of biofilm. Biofilm is a complex of large quantity of EPS which have polysaccharides, proteins and lipids. This biofilm leads to change of the local physical chemical alteration on the metal surface, causing the corrosion of metal [5–7]. A number of organisms, including bacteria, protozoa, fungi and other marine organisms, are capable of forming biofilm on the surface [8]. The formation of

biofilm greatly depends on the nutritional environment and microbial motility structures such as *flagella* and *pili* [9]. The presence of biofilm has been reported for a wide variety of surfaces, including industrial instruments, medical devices, cooling system engineering materials [6].

Industries, with special reference to chemical and extractive activities, commonly use stainless steel, mild steel and carbon steel as construction materials, due to their excellent mechanical properties [10, 11]. The serious problem of bio-fouling on metal surface is due to contact with aquatic system. Biofilm plays a significant role in the corrosion process thus influencing the cathodic/anodic depolarization, hydrogen production, reduction of metal, production of organic acids and exopolymers [12].

Plasmids are double-stranded extra-chromosomal circular DNA which replicates independently in the host of chromosome [13]. Bacterial plasmids have resistance to metal and antibiotics [14, 15]. They are involved in biofilm formation [16, 17], degradation of hydrocarbons [18–20] and biosynthesis of antibiotics in extreme environments [21]. A number of intercalating compounds such as sodium dodecyl sulphate, acriflavine, ethidium bromide and acridine orange were reported to be used for curing the plasmids in various bacterial species [22]. These intercalating compounds act as inhibitors during plasmid replication by selectively binding to the plasmid DNA [23]. Therefore, in this research, the role of the cured bacterial plasmid towards biofilm formation and its influence on corrosion property were investigated. Different characterization assays, i.e. weight loss, crystal violet, Lewis acid–base assay and electrochemical studies were carried out to elucidate the involvement of cured bacterial plasmid towards biofilm and their interaction with different metal surfaces during corrosion process.

2 Materials and Methods

2.1 Bacteria

Bacillus thuringiensis EN2 strain was isolated from biofilm sample of cooling water system and the 16S rDNA gene sequence was submitted under the accession number of KR183873. This EN2 strain has endospore-forming ability and identified as dominant in the cooling water system. The biochemical characteristics of the isolate EN2 are presented in Table 1.

Table 1 Partial biochemical characterization of *B. thuringiensis* EN2

Characteristics	*B. thuringiensis* EN2 (KR183873)
Gram stain	Positive
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	+

2.2 Plasmid Extraction and Curing of *B. thuringiensis* EN2

Plasmid DNA extraction from *B. thuringiensis* EN2 was done following the adapted method of Birnboim et al. (1979) and using Medox plasmid kit [24]. The extracted plasmid DNA was dissolved in 20 µl of tris ethylenediaminetetraacetic acid (TE) buffer and was run on agarose (0.8 %) gel electrophoresis, which was observed under the UV transilluminator.

For plasmid curing, overnight grown *B. thuringiensis* culture (1.2×10^{-4}) was inoculated in a series of sterile test tubes containing nutrient broth (NB) with Ethidium bromide (ETBR) at a concentration of 0.5, 1 and 1.5 %. The inoculated tubes were incubated overnight with shaking at 37 °C. Plasmid loss was confirmed by agarose gel electrophoresis [25].

2.3 Biocorrosion Study

The widely used metals in cooling tower systems are stainless steel (SS 316), brass alloy (BS) and mild steel (MS 1010), and these have been selected for the biocorrosion assays and the composition of each metals is detailed in Table 2. Metals coupons of SS, BS and MS of dimension 2.5×2.5 mm and 0.1 cm^2 were used for weight loss method and electrochemical impedance spectroscopy (EIS) analysis. The coupons were rinsed with deionized water and

Table 2 Elemental composition (wt%) of various engineering metal coupons

Metals	Cr	Mo	Ni	C	Zn	Fe	Pb	Cu	Mn	P	S
Stainless steel 316	14	1	2	1	–	–	–	–	–	–	–
Brass alloy	–	–	–	–	16	0.05	0.05	60.66	–	–	–
Mild steel 1010				0.2		0.03			0.2	0.50	0.03

surface sterilized with 70 % ethanol. Finally, the coupons were dried and stored in a desiccator. These coupons were used for biocorrosion studies, surface analysis and weight loss method [26]. *B. thuringiensis* EN2 (wild and cured) were selected for biocorrosion study. The polished stainless steel (SS) coupons (i.e. three coupons) were introduced into a 500-mL Erlenmeyer flask containing 400 mL of cooling tower water with 1 % of sterile nutrient broth (NB) inoculated with 1 mL of wild/cured strain of *B. thuringiensis* EN2 (1.2×10^{-4} CFU/mL) as the system I and II. Instead of SS, BS was used for the system III and IV and instead of BS, MS was used for the system V and VI with wild/cured EN2 strains, respectively. The triplicate for each system was carried out for corrosion rate analysis.

After 14th day of biocorrosion experiment, the coupons were separated from the respective systems and the corrosion products were scrapped from the each metal for analysis by Fourier Transform Infrared Spectroscopy (FTIR) [26]. For weight loss measurement, the coupons were pickled with respective pickling solution (1 L HCl containing 20 g of antimony trioxide and 50 g of stannous chloride at room temperature for 25 min) [27]. Weight loss, standard deviation (SD) and ANOVA results are calculated and presented in Tables 3 and 4. The corrosion rate was calculated as suggested by the National Association of Corrosion Engineers (NACE) [28]. Collected corrosion products were crushed into a fine powder and used for FTIR analysis. For bacterial curve, the biofilm formed on coupons from each system was scraped and the total viable count was measured by standard plate count method [29].

EIS was carried out in a three-electrode system (CH Instrument Inc., USA model CHI-608E): metal (SS, BS and MS) coupons were selected as working electrode, Ag/AgCl as reference electrode and a platinum wire as counter electrode. Impedance and Tafel polarization measurements were carried out using the procedure described in our earlier studies [30].

2.4 Biofilm, Motility Assay and Surface Characteristics of Bacteria

Biofilm assay was carried out using a 96-well microtiter plates as described earlier by O' Toole et al. (1999) [16]. Overnight grown wild/cured (*B. thuringiensis* EN2) cultures were diluted in 1:100 in ratio with fresh nutrient broth and then 100 µL of EN2 culture was transferred into

Table 3 Weight loss data for wild and cured *B. thuringiensis* EN2 in 1 % sodium chloride biocorrosive system

Metals	*B. thuringiensis EN2*		*B. thuringiensis EN2*	
	Wild strain		Cured strain	
	Weight loss (g)	corrosion rate (mm/year)	Weight loss (g)	Corrosion rate (mm/year)
Stainless steel	0.0005	0.18 ± 2	0.0004	0.12 ± 2
Brass	0.0367	10.3 ± 2	0.0201	5.6 ± 2
Mild steel	0.2860	91.5 ± 2	0.0216	69.1 ± 2

± Standard deviation

Table 4 ANOVA analysis for weight loss measurements

	B. thuringiensis EN2					*B. thuringiensis* EN2				
	Wild strain					Cured strain				
	SS	*df*	MS	*F*	*P*	SS	*df*	MS	*F*	*P*
Between	0.145	2	0.072	3.624	0.093	0.001	2	0.000	0.021	0.979
Within	0.120	6	0.020			0.120	6	0.020		
Total	0.265	8				0.121	8			

P statistically significant difference, *SS* sum of squares, *MS* mean squares, *F* ratio

96-well titer plates and incubated for 4–24 h at 37 °C. After the incubation period, the wells are washed with sterile distilled water and 125 µL of crystal violet was added and hold for 20 min. Finally, 125 µL of acetic acid was added and incubated at room temperature for about 4–15 min [16]. These products were analysed by UV–Visible spectrophotometer at 600 nm. The characterization of wild/cured bacterial motility in nutrient agar plates was examined by motility assays. Overnight culture of EN2 was streaked onto nutrient agar plates and incubated for 16–24 h at 37 °C. Migration was assessed qualitatively by examining the circular zone from the point of inoculation (streaking point) formed on the plates.

Surface hydrophobicity of the EN2 was assessed by microbial adhesion to solvents (MATS) method following the protocol of Bellone-Fontaine et al. [29]. This method is based on the comparison of microbial cell affinity to polar and non-polar solvents (chloroform, hexadecane, ethyl acetate and decane). For MATH experiments, 2.4 mL of bacterial suspension was added with 0.5 mL of solvent and vortexed for 30 s. Then, this mixture was allowed to stand for 30 min until the separation of the two phases. Water phase was analysed for bacterial growth by spectrophotometer (600 nm). The percentage of bacterial cell viability in solvent was calculated using the following formula:

$$\text{Affinity}\ (\%) = 100 \times (1 - A/A_0),$$

where A_0 and A are the optical densities of the bacterial suspension before and after mixing of the solvent.

3 Results

The presence and absence of plasmid DNA were investigated running the samples on agarose (0.8 %) gel electrophoresis. Figure 1 shows the electrophoresis images of

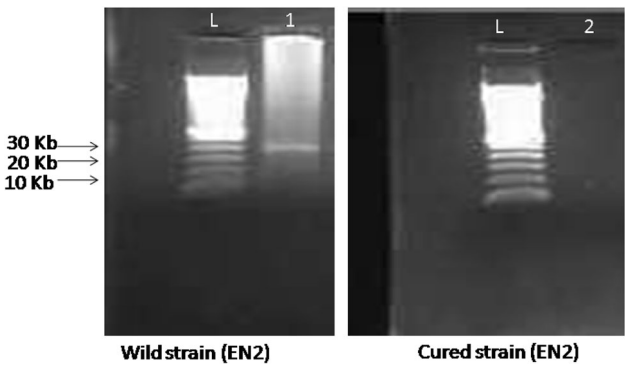

Fig. 1 Electrophoresis images of plasmid isolation and curing of *Bacillus thuringiensis* EN2 strain. *L* 100 bp DNA ladder, *1* plasmid; *2* completely cured plasmid

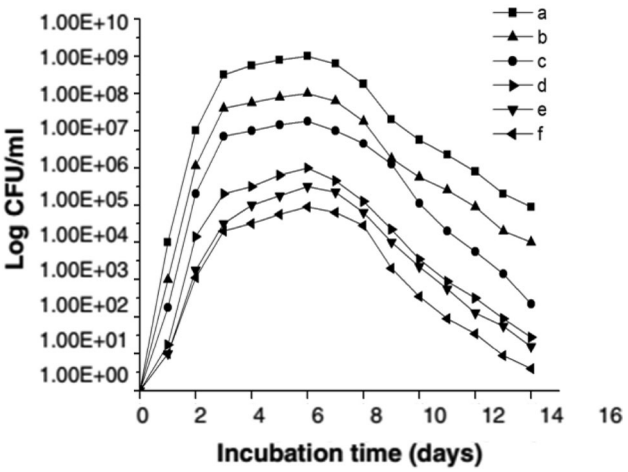

Fig. 2 Growth pattern of wild and cured strains of *Bacillus thuringiensis* EN2 on metal surface. **a** MS wild coupons, **b** BS wild strain, **c** SS wild strain, **d** MS cured strain, **e** BS cured strain, **f** SS cured strain

the plasmid of the cured/wild strain EN2. The growth pattern of the wild/cured strain of EN2 on metal surface is shown in Fig. 2. The growth curve revealed the increasing growth trend (10^9) of the wild strain than the cured strain EN2 (10^5). This may be due to the reduced production of EPS on the metal surfaces by cured strain. The corrosion rate of the metal coupons in cured/wild bacterial strains is presented in Table 3. The corrosion rates of wild strain (SS, BS and MS) were observed as 0.18, 10.3 and 91.5 mm/year, respectively. Variance was analysed by ANOVA, as reported in Table 4. Significantly wild strain variance was about 0.093, and cured strain variance was about 0.979. This indicates that when compared to cured strain, wild strain showed higher variance.

Figure 3 shows the Nyquist plot for engineering metal coupons (SS, BS and MS) in wild and cured strain of EN2. The results of electrochemical studies are presented in Table 5. The wild strain showed the charge transfer resistance (R_{ct}) values of SS, BS and MS as 59, 48 and 31 Ω cm^2, respectively, whereas the cured strain showed 93, 84 and 51 Ω cm^2 for SS, BS and MS, respectively. Figure 4 shows the Tafel polarization curves of the metal coupons (SS, BS and MS) in wild and cured strain of *B. thuringiensis*. The corrosion current (I_{corr}) of wild strain for SS, BS and MS were calculated as 3.1×10^{-6}, 3.2×10^{-6} and 3.1×10^{-6} A/cm^2, respectively, whereas the cured strain showed less corrosion current values of 2.7×10^{-6}, 2.9×10^{-6} and 2.8×10^{-6} A/cm^2 for all the three metals.

FTIR spectrum of the corrosion products scrapped in the presence of wild/cured strain of various metals is shown in Fig. 5. The IR spectrum in Fig. 5(SS (a), BS (a) and MS (a)) showed broad peaks at 3500–3000 cm^{-1} and

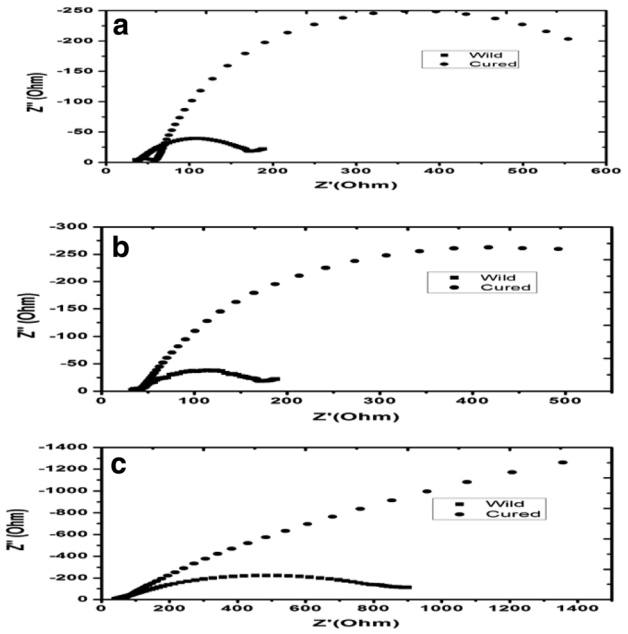

Fig. 3 Impedance curve for wild and cured strains of *Bacillus thuringiensis* EN2 in various metal systems: **a** stainless steel, **b** brass and **c** mild steel

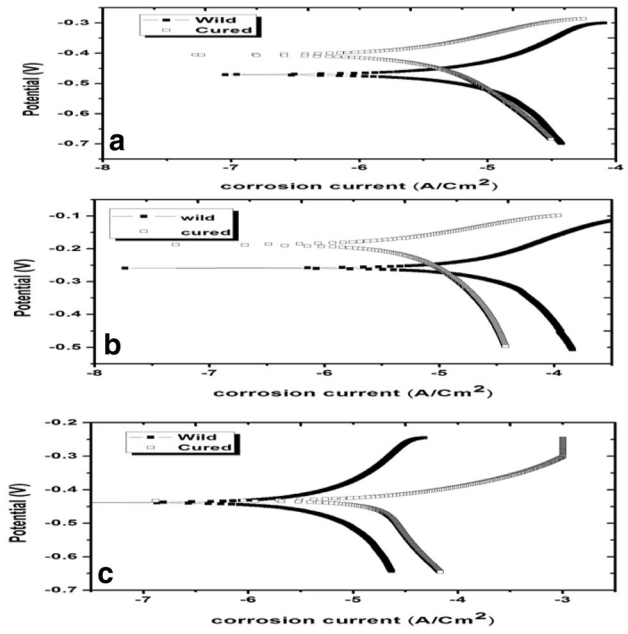

Fig. 4 Polarization curve for wild and cured strains of *Bacillus thuringiensis* EN2 in various metal systems: **a** stainless steel, **b** brass and **c** mild steel

2997 cm^{-1} which indicated the presence of –OH group and –CH aliphatic groups. The peaks at 2880 and 2872 cm^{-1} indicated the presence of –CH aliphatic group and peaks at 1770 and 1660 cm^{-1} attributed the presence of C–O stretch and C–N stretch, respectively. The presence of peaks in the range between 1700 and 1500 cm^{-1} indicates the C–O and C–N stretch. Another peak at 1067 cm^{-1} indicates the C–O alkoxy group. IR spectrum in Fig. 5SS (b), BS (b) and MS (b) showed broad peaks at 3000–3500 cm^{-1} and 1600 cm^{-1} which indicated the presence of –OH group and C–O stretch. The peaks at 2086 and 1632 cm^{-1} indicated the presence of C–H bend and C–N stretch.

The biofilm formation of wild/cured strain of EN2 was analysed by microscopic staining with crystal violet [31]. The results of the study showed a detectable biofilm formation by the wild strain than the cured strain, in which the biofilm formation was not observed. Lewis acid–base properties of different solvents (i.e. chloroform and hexadecane) were studied to estimate the nature of hydrophobicity in wild/cured strain and the results are presented in Table 6. The results showed that the cell affinity of the cured strain (chloroform 71.4 % and hexadecane 41.6 %) was found to be comparatively lower than the wild strain (chloroform 80.1 % and hexadecane 52.1 %) in non-polar solvents as well as polar solvents (ethyl acetate) wild 38.2 % and cured 27.4 %. The results of the motility test showed the migration of the wild strain was away from the point of the inoculation on the agar

Table 5 Electrochemical impedance spectroscopy (EIS) data for wild/cured strain *B. thuringiensis* EN2 in 1 % sodium chloride biocorrosive system

Metals	EN2 strain	Polarization data		Impedance data	
		E_{corr} (mV) Corrosion potential	I_{corr} (A/Cm^{-2}) Corrosion current	R_s Ω cm^2 Solution resistance	R_{ct} Ω cm^2 Charge transfer resistance
Stainless steel	Wild	−363	3.1×10^{-6}	134	59
	Cured	−478	2.7×10^{-6}	162	93
Brass	Wild	−602	3.2×10^{-6}	112	48
	Cured	−707	2.9×10^{-6}	155	84
Mild steel	Wild	−207	3.1×10^{-6}	92	31
	Cured	−404	2.8×10^{-6}	121	51

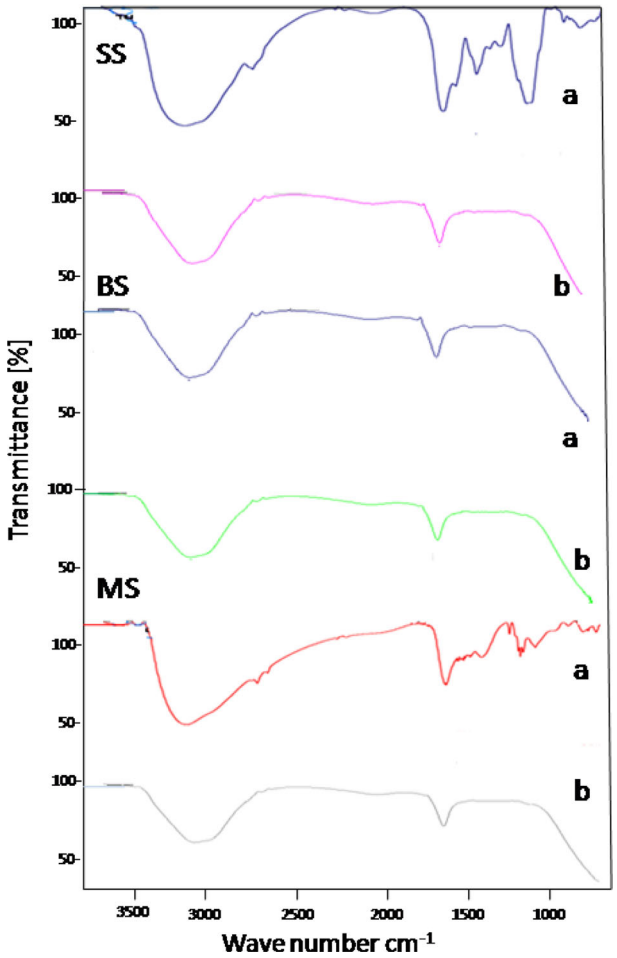

Fig. 5 FTIR spectrum of surface film on the various metal **a** *Bacillus thuringiensis* EN2 wild strain and **b** *B. thuringiensis* EN2 cured strain. *SS* stainless steel, *BS* brass, *MS* mild steel

Table 6 Lewis acid–base properties of the cell surface hydrophobicity of *B. thuringiensis* EN2 on different hydrocarbon

Bt strain	Affinity in hydrocarbon (%)		
	Chloroform	Hexadecane	Acetate
Wild	80	52	38
Cured	72	42	28

plates, whereas the cured strain was found near the point of inoculation and the zone of spreading in the agar Petri dish was observed to be limited.

4 Discussion

Our results showed that the cured bacterial strain of EN2 lost its plasmid after the chemical curing process. During the course of growth, the plasmid loss may arise in response to the changes in environmental parameters such as pH, chemical and temperature [32, 33], whereas in case of the cured strain EN2 of each metal (SS, BS and MS) showed a corrosion rate of about 0.12, 5.6 and 69.1 mm/year, respectively. From the weight loss data, the lesser corrosion rate was observed in cured strain system than the wild strain system. Therefore, it can be concluded that the plasmid played an important role in the corrosion process and may be responsible for the biofilm formation on the metal surfaces [17, 34].

The low R_{ct} values of the wild strain inferred the formation of heterogeneous biofilm on the metal surfaces which leads to severe corrosion on the metal (Table 5). Thus, the EIS results supported the lower corrosion rate of the cured strain if compared to the wild strain. However, the cured strain corrosion current was reduced significantly and this may be due to the slow down of both anodic and cathodic reaction during the corrosion process. The solution resistance (R_s) and charge transfer resistance (R_{ct}) were higher for the cured strain when compared to the wild strain, thus enabling the wild strain to possibly form a thick biofilm on all the metal surfaces (Table 5). On contrary, the absence of plasmid gene in EN2 leads to the inability to form biofilm on the metal surface which results in reduced corrosion rate. Hence, it can be concluded that the plasmid gene has a significant role in the formation of biofilm on the tested metal surfaces [16, 18].

When compared with cured strain, additional peaks appeared in wild strain FTIR and it is due to the more secretion of EPS in wild strain and vice versa (Fig. 5). Hence, wild strain of MS and SS shows additional peaks which indicated the presence of EPS [35]. Biofilm assays highlight higher biofilm formation by the wild strain over the cured strain, allowing us to argue that the bacterial plasmid was mainly responsible for the biofilm formation. As Ghigo [36] reported, a conjugative plasmid of both groups of bacteria was found to be responsible for the formation of biofilm.

Hydrophobicity of wild was higher compared to cured strain (Table 6). These results indicated that the wild strain was an electron donor and the cured strain acted as an electron acceptor. In this scenario, the tested wild strain had higher hydrophobicity and might have helped in the higher production of EPS component to form a thick biofilm on the metal surfaces. The cell surface hydrophobicity of the bacterial strains was reported to play an important role in the communication between polar or non-polar molecules in aqueous environments [37, 38]. In the presence of the wild strain, the spreading zone was bigger if compared to cured strain. The motility of bacterial community in host cell surface or abiotic surface was reported to play a pivotal role in the biofilm formation [39]. The attachment of bacterial cells followed by their surface migration and differentiation into the mature biofilm [31] confirmed the flagella-mediated motility.

5 Conclusions

Overall, in this research, the role of plasmid on biofilm formation on different engineering metal surfaces was evaluated by weight loss method, surface hydrophobicity and electrochemical impedance spectroscopy method. The cured *B. thuringiensis* EN2 strain had lesser biofilm capability on all the metal surfaces. The corrosion rate of wild strain was found to be higher than compared to cured strain. The degree of corrosion rate in the presence and absence of plasmid was in the order of MS (91.5 mm/year) >BS (10.3 mm/year) >SS (0.18 mm/year) and MS (69.1 mm/year) >BS (5.6 mm/year) >SS (0.12 mm/year). Thus, the study demonstrated the importance of the bacterial plasmid towards corrosion of metals and thus the inhibition of corrosion was achieved through the addition of chemicals (bromine, amine and nitrogen).

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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