Influence of Tetracycline on Gram-Negative Strain *Klebsiella oxytoca* in Mild Steel Corrosion

S. Chitra[1,*], B. Anand[2] and V. Balasubramanian[1]

[1]Department of Chemistry, AMET University, Chennai-603 112, India
[2]Department of Chemistry, Sir Theagaraya College of Arts and Science, Chennai-600 021, India

*Corresponding author: E-mail: chitrachem@gmail.com

In this work, the inhibition effect of tetracycline on *Klebsiella oxytoca* on mild steel corrosion has been investigated using weight loss measurement and electrochemical impedance spectroscopy. The results have shown that tetracycline shows better inhibition towards microbe. The agreement with the experimental data was also found to be satisfactory. Further, surface morphological examination through SEM confirms that the inhibitor inhibits the microbes by blocking extracellular polysaccharides secreted by microbial cells.

Keywords: Biocorrosion, Tetracycline, *Klebsiella oxytoca*, Mild steel.

INTRODUCTION

Biofilms could be deleterious to materials, because it affects the kinetics of corrosion processes of metals caused by microorganism adhering to the interface. Microbiologically influenced corrosion (MIC) is a serious problem in a number of industries due to the presence of microbes, adequate nutrients and corrosive byproducts [1]. Microorganisms grow on substratum surfaces to form colonies and they produce various metabolic by-products like sticky polymers, which tend to attract and aggregate other biological and non-biological (metals and chloride, for example) species to the colonization sites [2]. This extracellular polysaccharide (EPS) (sticky polymers) forms the frame work of microbial mats and are typically composed of polysaccharide, lipid and protein in the form of hetero-polymers [3]. They do not form uniform layers, but rather, local "community centers" [4]. During adhesion process, Gram-negative bacterium will be more active in degradation process because of their endowed capacity [5]. Consequently, with this communication extracellular polysaccharide is generally considered to be important in bonding bacterial cells together in biofilm structure [6]. Normally extracellular polysaccharide possess anionic character mainly due to the high content of weak acids. The rate at which corrosion propagate is mainly depends on the weak acid produce by bacteria in aerobic or by anaerobic environment [7,8]. As with the literature review the main type of organism associated with degradation are sulphate reducing and sulphate oxidizing bacteria, iron and CO_2 reducing bacteria, iron and manganese oxidizing bacteria and acid producing fungi [9]. So in the present study,

we aimed at investigating the effect of *Klebsiella oxytoca*, which are present in organic pollutant rich areas like gasoline.

EXPERIMENTAL

Tetracycline was purchased from Sigma Aldrich and used as an inhibitor. Double distilled water was used throughout all the experiments.

Preparation of test panels: The mild steel strips were cut into pieces of 5 cm × 1 cm with the thickness 3 mm having the following composition (in percentage) % C = 0.017; Si = 0.007; Mn = 0.196; S = 0.014; P = 0.009; Ni = 0.013; Mo = 0.015; Cr = 0.043 and Fe = 99.686 was used. The metal samples were scratched with various grades of abrasive paper and cleaned with ethanol solution.

Culture preparation: The bacterial strains were used throughout the investigation. All the bacterial cultures were obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The young bacterial broth cultures were prepared before the screening procedure.

Composition of growth medium: The nutrient agar is the frequent medium used for all bacterial isolation.

Preparation of inoculums: Stock cultures were maintained at 4 °C on slopes of nutrient agar. Active cultures of the experiment were prepared by transferring a loopful of cells from the stock cultures to test tube of Muller-Hinton broth (MHB) for bacteria that were incubated without agitation for 24 h at 37 and 25 °C, respectively. The cultures were diluted with fresh Muller-Hinton broth to achieve optical densities corresponding to 2.0×10^6 colony forming units (CFU/mL) for bacteria [10-12].

Inhibitor susceptibility test: Antimicrobial activity *in vitro* was screened by using Muller Hinton Agar (MHA) obtained from Hi-media (Mumbai) by using Bauer *et al.* [13] method. The MHA plates were prepared by pouring 15 mL of molten media into sterile petri-plates. The plates were allowed to solidify for 5 min and 0.1 % inoculums suspension was swabbed uniformly and the inoculums were allowed to dry for 5 min. The metals were placed on the surface of the medium and the extract was allowed to diffuse for 5 min and the plates were kept in incubation at 37 °C for 24 h. At the end of incubation, inhibition zones formed around the disc were measured with a transparent ruler in millimeter. The standard disc is chloramphenicol.

Sampling and biofilm: The Gram-negative strain *Klebsiella oxytoca* was used for this study. To obtain robust layer of mucilage adhering to a metal sample, the metal samples were placed in a Petri dish containing the culture with the exposure of 10 days.

The metal samples were taken away from the culture media and the biofilm on the surface were removed by razor blade [14]. After the removal, the metals were washed with acetone and the final weight was used to calculate the corrosion rate.

$$\text{Corrosion rate} = \frac{87.6 \times W}{D \times A \times T}$$

where, W = weight loss (mg); D = metal density (g/cm^3); A = area of sample (cm^2); T = time of exposure of metal sample (h).

Electrochemical impedance spectroscopy: The electrochemical experiments were carried in a conventional three-electrode cell assembly. In three-electrode system test coupons of 1.0 cm^2 areas exposed as working electrode, a high purity platinum sheet as a counter electrode and saturated calomel electrode (SCE) was used as a reference electrode [15]. Experiments were carried out in Electrochemical Workstation Model 600 D/E Series in without and with the addition of an inhibitor, a stabilization period of 30 min was allowed, which is enough to attain stable OCP value.

Surface analysis: In order to analysis the surface of test panel, first the test panels were immersed in 2 h in gluteraldehyde solution at 4 °C in order to stick the bacterial culture to surface. Second the test panels were rinsed and dehydrated using acetone [16-19]. Then the test panels were coated gold/platinum and studied with the SEM.

RESULTS AND DISCUSSION

Weight loss of test panels: After 10 days of exposure the test panels were weighed after taking out the corrosion products. Mass loss of the test panels exposed to bacterial strain with and without the addition of inhibitor is given in Table-1.

It is obvious that the inhibition efficiency of inhibitor at a concentration of 7×10^{-5} M was found to be 67.5 %. Further-

TABLE-1
DATA OF WEIGHT LOSS MEASURE IN STERILE AND INOCULATED MEDIUM

Medium	Exposure of 10 days		
	Corrosion rate	θ	Inhibition efficiency
Strain + Test coupon	89.671	–	–
Strain + Test coupon + Inhibitor	20.457	0.7320	73.2

more the inhibitor could significantly reduce the mass loss in the presence of bacterial culture [20]. The mass loss of mild steel exposed to bacterial strain in the presence of inhibitor is lower as compared to the specimen without inhibitor [21].

From Table-2, it is clear that the corrosion rate was decreased with increasing concentration of inhibitor and inhibition efficiency, increased with increasing the concentration of inhibitor. But when comparing the inhibition efficiency of tetracycline in general corrosion and microbial growth inhibition, it was found that the inhibition capability of tetracycline is more in general corrosion.

TABLE-2
WEIGHT LOSS OF VARIOUS CONCENTRATIONS OF INHIBITOR IN 1 M NaCl AT ROOM TEMPERATURE

S. No.	Inhibitor concentration	Corrosion rate	IE (%)
1	Blank	86.187	–
2	1×10^{-5}	37.437	47.29
3	2×10^{-5}	28.023	49.32
4	3×10^{-5}	20.482	52.70
5	4×10^{-5}	16.147	56.75
6	5×10^{-5}	12.286	60.13
7	6×10^{-5}	10.203	64.18
8	7×10^{-5}	9.576	67.56

Electrochemical impedance spectroscopy: Electrochemical impedance of mild steel specimen exposed to bacterial strain in the absence and presence of tetracycline is depicted in the Nyquist plot with the concentration of 7×10^{-5} M (Fig. 1). Similarly, electrochemical impedance (Nyquist) spectra of mild steel in 1 M NaCl solution with and without various concentrations of inhibitor are shown in Fig. 2.

Impedance parameters obtained from Nyquist plot are given in the Tables 3 and 4. It was observed that the Nyquist plot shows capacitive loop, the semicircle with high frequency owing to charge transfer resistance for relaxation of electrical double layer and the diameter of semicircle increases in the presence of inhibitor [22-24].
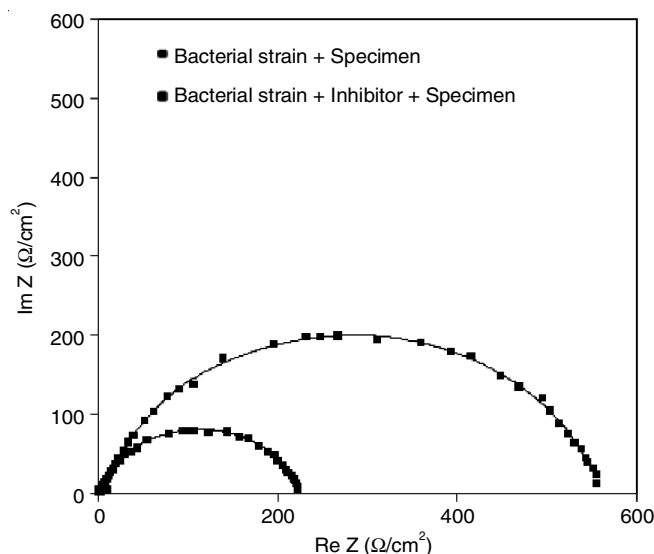

Fig. 1. Nyquist plots for mild steel specimen expose to bacterial strain with and without the inhibitor
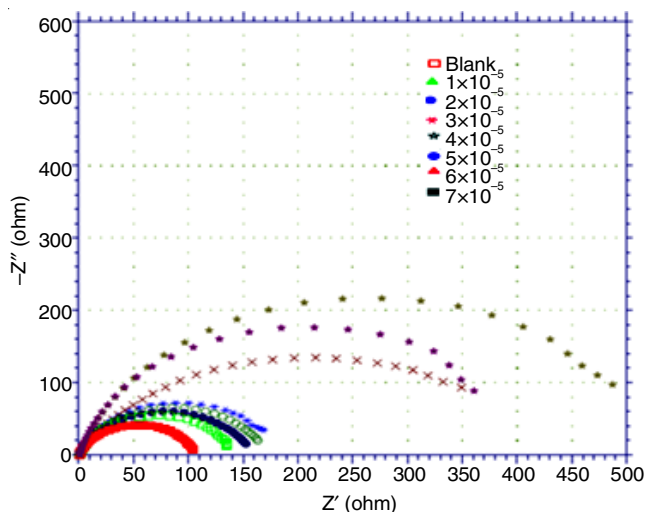
Fig. 2. Electrochemical impedance (Nyquist) spectra of mild steel in 1 M NaCl solution with and without various concentrations of inhibitor

TABLE-3
AC IMPEDANCE PARAMETERS FOR CORROSION OF MILD STEEL IN STERILE AND INOCULATED MEDIUM

Medium	R_{ct} (ohm cm^2)	C_{dl} (ohm µF/cm^2)	Inhibition efficiency (%)
Strain + Test coupon	17.0	220.52	–
Strain + Test coupon + Inhibitor	55.65	96.82	69.45

TABLE-4
AC IMPEDANCE PARAMETERS FOR CORROSION OF MILD STEEL IN VARIOUS CONCENTRATIONS OF INHIBITOR

Inhibitor conc. (M)	R_{ct} (ohm cm^2)	C_{dl} (ohm µF/cm^2)	IE (%)
Blank	69.5	231.52	–
1×10^{-5}	161.0	287.70	56.83
2×10^{-5}	177.2	271.50	60.77
3×10^{-5}	182.3	219.80	61.87
4×10^{-5}	193.2	202.40	64.02
5×10^{-5}	213.8	160.60	67.49
6×10^{-5}	241.6	134.00	71.23
7×10^{-5}	253.1	112.70	72.54

In Tables 3 and 4, the C_{dl} values were reduced with an increase in the concentrations of inhibitor. This is due to the formation of defending film on the surface of mild steel by the addition of inhibitor, resulting in raising the inhibition efficiency. The EIS parameters such as R_{ct} and C_{dl}, are given in Tables 3 and 4. The values of IE % acquired from the charge transfer resistances are calculated according to eqn. 1:

$$IE\,(\%) = \left(\frac{R_{ct}^{i} - R_{ct}^{o}}{R_{ct}^{i}} \right) \quad (1)$$

where R_{ct}^{i} and R_{ct}^{o} indicate the charge transfer resistance values in the presence and absence of inhibitor, respectively.

Bacterial morphology: SEM analysis was carried out to validate the bond of *Klebsiella oxytoca* to the test specimen. SEM morphology (Fig. 3) indicates that biofilm forming micro-organism may cause corrosion or protective effects on the metal surface [23-25].
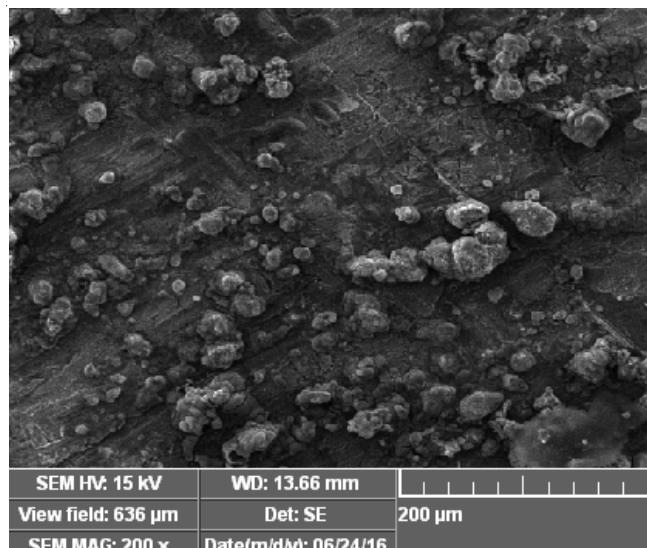

Fig. 3a. Test panel after 10 days exposure in the presence of bacterial strain with the inhibitor
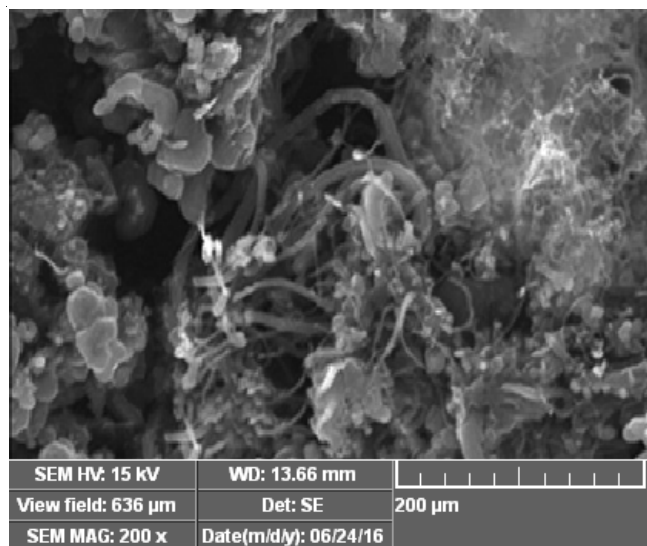

Fig. 3b. Test panel after 10 days exposure in the presence of bacterial strain without the inhibitor

FTIR: FTIR spectra is vital technique to identify biofilms and their chemical changes in the presence of inhibitor. Through the balanced growth, amide I and amide II band of C-O stretch at 1650, 1540 and 1084 cm^{-1} is due to the formation of protein films on test coupon as shown in Fig. 4. It is evident from Fig. 4(a) that the most susceptible spectral region to the protein secondary structural components is the amide I band at 1625 cm^{-1}, which is due roughly to C=O stretch vibrations of the peptide linkages. The amide II band, in contrast, derives mainly from in-plane NH bending and from the -CN stretching vibration, showed much less protein conformational sensitivity. The signal range from 1200-900 cm^{-1} is due to polysaccharide production. The band at 759.09 cm^{-1} indicates the presence aromatic methane (strong) in the inhibitors.

Conclusion

It is concluded that inhibition efficiency of antibiotic tetracyclines was found to show a good trend with weight-loss method and electrochemical impedance spectroscopy studies.
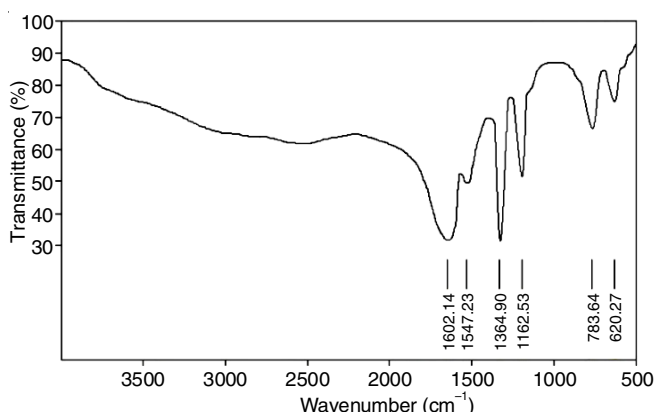
Fig. 4a. FTIR spectra of corrosion product of test coupon in the presence of sterile medium and *Klebsiella oxytoca*
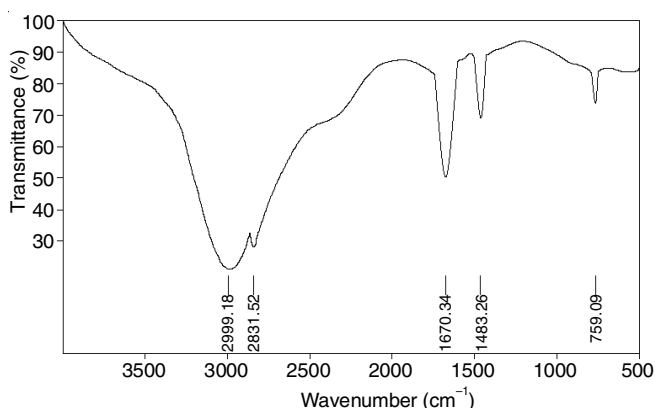

Fig. 4b. FTIR spectra of corrosion product of test coupon in the presence of *Klebsiella oxytoca* and inhibitor

The morphology results also confirmed that the inhibitor inhibits the mild steel corrosion of *Klebsiella oxytoca* only by blocking extracellular polysaccharides secreted by microbial cells.

REFERENCES

1. B.J. Little and J.S. Lee, Microbiologically Influenced Corrosion, John Wiley & Sons, Inc., Hoboken, New Jersey (2007).
2. X. Shi, N. Xie and J. Gong, *Recent Patents Corros. Sci.*, **1**, 118 (2011).
3. K.D. Demadis, C. Mantzaridis and P. Lykoudis, *Ind. Eng. Chem.*, **45**, 7795 (2006); https://doi.org/10.1021/ie0607898.
4. C.M. Hansson, *Metallurgical Mater. Trans.*, **42**, 2952 (2011); https://doi.org/10.1007/s11661-011-0703-2.
5. Trine H. Mogensen, *Clin. Microbiol. Rev.*, **22**, 240 (2009); https://doi.org/10.1128/CMR.00046-08.
6. H.A. Videla, L.K. Herrera and R.G. Edyvean, Paper No. 05488, NACE International, Houston, TX, p. 3 (2007).
7. G.A. O'toole, B. Kaplan and R. Kolter, *Annu. Rev. Microbiol.*, **54**, 49 (2000); https://doi.org/10.1146/annurev.micro.54.1.49.
8. W.B. Beech, eds.: P. Lens, A.P. Moran, T. Mahony, P. Stoodly and V. O'Flaherty, Biofilms on Corroding Materials, In: Biofilms in Medicine, Industry and Environmental Biotechnology-Characteristics, Analysis and Control, IWA Publishing Alliance House: London, p. 115 (2003).
9. W.B. Beech, *Int. Biodeterior. Biodegrad.*, **53**, 177 (2004); https://doi.org/10.1016/S0964-8305(03)00092-1.
10. I.B. Beech, J.A. Sunner and K. Hiraoka, *Int. Microbiol.*, **8**, 157 (2005).
11. J. de Bivar Xavier, C. Picioreanu and M.C.M. van Loosdrecht, *Biotechnol. Bioeng.*, **91**, 651 (2005); https://doi.org/10.1002/bit.20544.
12. C. Hubert, M. Nemati, G. Jenneman and G. Voordouw, *Appl. Microbiol. Biotechnol.*, **68**, 272 (2005); https://doi.org/10.1007/s00253-005-1897-2.
13. A.W. Bauer, W.M. Kirby, J.C. Sherries and M. Turck, *Am. J. Clin. Pathnol.*, **45**, 493 (1966).
14. I. Neria-González, E.T. Wang, F.R. Rez, J.M. Romero and C. Hernández-Rodríguez, **12**, 122 (2006); https://doi.org/10.1016/j.anaerobe.2006.02.001.
15. H.A. Videla, *Int. Biodeterior. Biodegrad.*, **49**, 259 (2002); https://doi.org/10.1016/S0964-8305(02)00053-7.
16. H.A. Videla and L.K. Herrera, *Int. Microbiol.*, **8**, 69 (2005).
17. A. Rajasekar, S. Maruthamuthu, N. Palaniswamy and A. Rajendran, *Microbiol. Res.*, **162**, 355 (2007b); https://doi.org/10.1016/j.micres.2006.02.002.
18. I.B. Beech and J. Sunner, *Curr. Opin. Biotechnol.*, **15**, 181 (2004); https://doi.org/10.1016/j.copbio.2004.05.001.
19. J. Kielemoes, I. Bultinck, H. Storms, N. Boon and W. Verstraete, *FEMS Microbiol. Ecol.*, **39**, 41 (2002); https://doi.org/10.1111/j.1574-6941.2002.tb00905.x.
20. S. Chongdar, G. Gunasekaran and P. Kumar, *Electrochim. Acta*, **50**, 4655 (2005); https://doi.org/10.1016/j.electacta.2005.02.017.
21. R. Zuo, E. Kus, F. Mansfeld and T.K. Wood, *Corros. Sci.*, **47**, 279 (2005); https://doi.org/10.1016/j.corsci.2004.09.006.
22. S. Acosta-Díaz, Y. Barrios-San Martín, F. González-Hernández and J.E. Tacoronte-Morales, *Revista CENIC Ciências Biol.*, **42**, 119 (2011).
23. S. Maruthamuthu, S. Mohanan, N. Muthukumar, S. Ponmarippan, A. Rajasekar, P. Subramanian and N. Palaniswamy, *Indian J. Chem. Technol.*, **12**, 567 (2005).
24. A. Kumar, J. Fernandes and P. Kumar, *Int. J. Pharm. Pharm. Sci.*, **6**, 518 (2014).
25. K.R. Ansari, D.K. Yadav, E.E. Ebenso and M.A. Quraishi, *Int. J. Electrochem. Sci.*, **7**, 4780 (2012).