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Shielding for Microbiologically Influenced Corrosion of Mild Steel by Antagonistic Bacterial Cultural Fluids

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Abstract.- Antagonistic activity of non-corrosive *Bacillus* isolates designated as SN-8 and SS-14 was evaluated against mild steel corrosion influencing, *Bacillus cereus*-SNB-4 and *Bacillus coagulans*-SS-5, respectively, by cross streak, disc diffusion and deep-well methods. Then corrosive bacteria were cultivated in cell-free cultural fluids of their respective antagonists supplemented with nutrient broth in the presence of mild steel coupons (MSC). After 90 days, corrosion rates of MSC exposed to *B. cereus*-SNB-4 and *B. coagulans*-SS-5 were found to be 39.5 and 37 $mg.dm^{-2}.d^{-1}$, respectively. When these bacteria were cultivated in the presence of cell free cultural fluids of their respective antagonists the corresponding values were found to be 9.53 and 9.44 $mg.dm^{-2}.d^{-1}$ as compared to the corresponding values of 24.99 and 22.2 $mg.dm^{-2}.d^{-1}$ of control (un-inoculated) MSC. These vivid decreases in corrosion rates of the experimental microbiologically influenced corrosion (MIC) demonstrated that the presence of cell free cultural fluids of bacterial antagonists not only reduced the intensity of MIC but they protected the mild steel from abiotic corrosion too. Thus select microbial activities have potential to serve as biocontrol strategies for protecting metals from corrosion.

Key words: Corrosion inhibition, Bacterial antagonism and corrosion, Bacterially influenced corrosion.

INTRODUCTION

Corrosion is physicochemical deterioration of a metal due to its interaction with environment. When metals are exposed to water and oxygen, they corrode. Under aerobic conditions, the process proceeds when a flow of electrons is continuously provided at the cathode following reduction of oxygen resulting in oxidation of metal leading to removal of insoluble corrosion products. Iron is first reduced to ferrous iron product ($Fe_{(0)} + 1/2H_2O \rightarrow Fe^{+2} + 2OH^-$). The ferrous iron is further oxidizes to ferric iron which typically forms amorphous solid $Fe(OH)_3$ that may change to other iron oxides such as goethite (α-FeOOH) and Hematite (Fe_2O_3) (Hamilton, 1985; Lee and Newman, 2003). Apart from different electrochemical characteristics of the environment the processes is also influenced by the activities of microorganisms. Microbiologically influenced corrosion (MIC) is extremely harmful to both industry and the environment (Javaherdashti, 1999). Traditionally many microorganisms have been identified a culprits for MIC. While, some recent investigations have begun to show protective effects of some bacterial biofilms for metals against corrosion (Zuo, 2007). Some biofilms have been shown to be beneficial for reducing corrosion damage (Potekhina *et al*., 1999; Little and Ray, 2002). Jayaraman *et al*. (1999a) identified novel biofilms that inhibit the development of sulphate reducing bacteria (SRB) on metal surfaces such as mild steel and stainless steel. An aerobic biofilm-forming *Bacillus* bacterium producing antimicrobials, such as indolicidin and bactenecin, effective in preventing the SRB (*D. vulgaris*) from colonizing the metal surfaces has been genetically engineered by Jayaraman *et al*. (1999b). Zuo *et al*. (2004) have demonstrated that antimicrobial-producing bacterial biofilm on mild steel, prevented growth of SRB present in Three- Mile- Island process water as well as that of added cells of *D. vulgaris* and *D. orientis*. They found secretion of antimicrobials; gramicidin, gatavalin, polymxin, subtilin, and tyrocidine by biofilms in continuous reactors. Jayaraman *et al*. (1999c) demonstrated natural secretion of antimicrobial peptide gramicidin S by biofilm-forming *B. brevis* that inhibited SRB colonization and resulted in corrosion reduction of mild steel and stainless steel. Azuma *et al*. (1992) had isolated two gramicidin-S hyperproducing strains of *Bacillus brevis*, which secreted the product up to 350 µg/ml, while the mutant *B. bervis* 18-3 yielded up to 590 µg/ml of the gramicidin.

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These gramicidin-S producing bacterial strains inhibit growth of corrosive microorganisms such as Gram-positive SRB (*D. orientis*) and Gram-negative SRB (*D. vulgaris*). Zuo *et al*. (2004) reported that 93.5% of *D. orientis* were killed at 1 µg/ml and 93.5% of *D. vulgaris* killed at 100 µg/ml of gramicidin-S. This antimicrobial agent had also been used to inhibit another corrosion causative bacterium *L. discophora* SP-6 and found to kill 97% of the cells at a concentration of 20 µg/ml. Zuo and Wood (2004) have carried out experimental inhibition of mild steel corrosion from SRB by using gramicidin-S in continuous culture and reported that in absence of the gramicidin-S, H_2S was generated after 1 day of continuous operation while at the end of experiments a thin black film of ferrous sulfide was seen on metal surface indicative of corrosion of iron by SRB. The present study reports independent bacterial secretion of substances expressing protective role against MIC. Interestingly such microbes were isolated from the same soil sample wherein the corrosion causing/promoting bacteria were screened.

MATERIALS AND METHODS

Microorganisms

Based upon their highest corrosion causing abilities, the bacterial isolates *Bacillus cereus*-SNB-4 and *Bacillus coagulans*-SS-5, and their non-corrosive antagonistic *Bacillus* sp., SN-8 and SS-14, respectively reported by Bano (2008) were employed in this study. The bacterial cultures were revived and grown at their pre-optimized conditions as described elsewhere (Bano, 2008). Antibiosis was visualized by paper disc diffusion and deep well methods. For these purposes 24 hours incubated bacterial cultures raised in nutrient broths of a given bacterium and its respective antagonist were used. For disc diffusion method discs of Whatman filter paper No.1 having a diameter of 8mm were prepared and autoclaved. Non-corrosive cultures of SN-8 and SS-14 having O.D. of 0.52 and 0.92, respectively at 600nm were centrifuged at 5000rpm for 10 minutes. The supernatants were then filter sterilized employing Sartorius 0.2µm Millipore filters. The cell free cultural fluids (CFCF) were then loaded on the pre-sterilized filter paper discs in an amount of 50µl/disc. The test organisms were inoculated by spreading 25µl of overnight incubated culture on nutrient agar plate. The CFCF loaded filter papers discs were then placed on to surface of the inoculated agar plates which were subsequently incubated at 37ºC. For deep well method the test organisms were inoculated as described above. Then wells were digged in the inoculated medium with the help of flame sterilized pipe having 0.4 cm diameter. Antagonist bacterial cultures were then dispensed in amounts of 25, 50 and 100 µl/well. In another series of experiments cell free fluid was introduced in an amount of 50µl/well. The petri plates were then incubated as described above and growth inhibition zones in all the experiments were recorded after 24 hours of incubations.

Experimental procedure

Mild steel coupon (MSC) used in this study comprised of Fe (95.68%), Si (1.916%), Al (0.516%), Mo (0.30%), Co (0.216%), Mn (0.205%), P (0.203%), S (0.20%) and trace elements (Bano, 2008). The MSC were prepared as described by Zuo and Wood (2004). The coupons were accordingly degreased before experimental exposure with acetone and then gently polished with 240 grit polishing paper followed by rinsing with distilled water. They were again washed with ethanol followed by rinsing with distilled water and wiped immediately with paper towel and dried in an oven at 80ºC for 10 minutes and then cooled to room temperature. Weight of each coupon (g) was recorded with the help of an electric balance. Then each coupon was placed in concavity of a piece of blotting paper folded in quadrant manner and sterilized while keeping in screw capped glass container in autoclave at 121ºC for 15 minutes. In one series of the experiments MSC were exposed to 30 ml nutrient broth inoculated with a given corrosion promoting bacterium and incubated at its respective growth optima for three months. Uninoculated but otherwise treated similarly MSC coupons served as controls.

One hundred ml of supernatant of a given overnight incubated culture was filter sterilized as described above. Then 10ml of the CFCF was mixed with 20 ml of sterilized nutrient broth. Prepared MSC (20×20×1mm) was introduced into

the preparation. The contents were then inoculated with overnight grown culture of a given corrosion promoting bacterium. For this purpose, 100 ml of a bacterial culture was centrifuged at 5000 rpm for 10 minutes and the supernatant discarded. The pellet was suspended in normal saline to achieve atleast 0.52 OD at 600nm. Then 0.1 ml of saline suspended bacteria were used as inoculum for aforementioned experiment. Uninoculated experiments served as control. The experimental as well as control flasks were incubated at 37ºC for three months.

At the end of experiment, the corrosion product was removed from coupons surface by the method of Angeles-Chavez *et al*. (2001). Accordingly, the coupons were dipped in a solution of Sb_2O_3 and $SnCl_2$ dissolved in HCl for 5-15 minutes to remove corrosion product. Then the coupons were washed with distilled water, dried at 80ºC for 20 minutes and weighed to determine the weight loss. Weight loss of each coupon was calibrated as percent loss from its initial mass. The weight loss of control coupon was subtracted from the experimental coupons to calculate their percent corrected weight loss *i.e.* the loss that occurred to MIC. Corrosion rate (mg $dm^{-2}d^{-1}$) was then calculated according to Majumdar *et al*. (1999) by the following formula:

$$C=W1-W2/AT$$

where C is the corrosion rate (mg $dm^{-2}d^{-1}$), W1 and W2 are weights of MSC before and at the completion of experiment, respectively, A is the surface area of coupon and T duration of immersion in days.

The cleaned MSCs were observed microscopically for their surface deterioration.

RESULTS AND DISCUSSION

When the corrosion promoting bacteria were lawned on nutrient agar plates and exposed to respective antagonists cultures and their cell free culture fluids (CFCF), it was found that, in general, cultures caused formation of larger growth inhibition zones (GIZ) than their CFCF. Values of diameters of GIZ ranged from 8-15 mm (Table I). In case of the well method, amount of loaded CFCF of an antagonist had a dose-dependent growth inhibition effect on the test organism (Table I). Corrosion studied by exposing the MSCs to the corrosion causative microbes in the absence and presence of CFCF of a respective antagonist demonstrated the shielding effect of the latter microbes on MIC. After 90 days, the corrosion rates for the isolates *B. cereus*- SNB-4 and *B. coagulans*-SS-5 cultured in the presence of CFCF of their respective antagonists were found even less than the control (uninoculated) samples. In case of MSC exposed to *B. cereus*-SNB-4 in presence of respective CFCF, the corrosion rate was found to be 9.53 $mg.dm^{-2}d^{-1}$ and average percent weight loss was recorded as 0.73%, which are less than the corresponding control values of 16.67 $mg.dm^{-2}.d^{-1}$ and 1.32% (Fig. 1). For MSC exposed to *B. coagulans* in presence of CFCF of respective antagonists, the corrosion rate and average percent weight loss were found to be 9.44 m $g.dm^{-2}.d^{-1}$ and 0.117 %, respectively (Fig. 2). These values again appeared less than the corresponding control values of 14.8 $mg.dm^{-2}.d^{-1}$ and 1.54%. Corrosion rates for MSC exposed to corrosion causative *B. cereus*-SNB-4 and *B. coagulans*-SS-5 in nutrient broth were found to be as 39.5 and 37 $mg.dm^{-2}d^{-1}$, respectively, as compared to the corresponding control values of 24.99 an 22.2 $mg.dm^{-2}d^{-1}$ (Figs. 1,

Table I.- Growth inhibition zone (GIZ) of *B. cereus*-SNB-4 and *B. coagulans*- SS-5 bacterial isolates against their respective antagonistic microbes.

Bacteria	Application	Amount (µl)	GIZ (diameter; mm)	
			SN-8	SS-14
B. cereus - SNB-4	Disc diffusion	50a	12	-[c]
		50b	11	-
	Deep well	50a	9	-
		25b	10	-
		50b	15	-
		100b	10	-
B. coagulans-SS-5	Disc diffusion	50a	-	10
		50b	-	12
	Deep well	50a	-	10
		25b	-	8
		50b	-	10
		100b	-	10

a, Bacterial cell free culture; the prefix indicates the amount in µl loaded.
b, Bacterial culture.
c, Test was not performed.

2). Average percent weight loss (APWL) of the three months exposed MSC was found to be 3.13 and 2.93% for *B. cereus*-SNB-4 and *B. coagulans*-SS-5, respectively. Microscopic observation of corroded MSCs revealed that their surfaces had pits of varying dimensions.

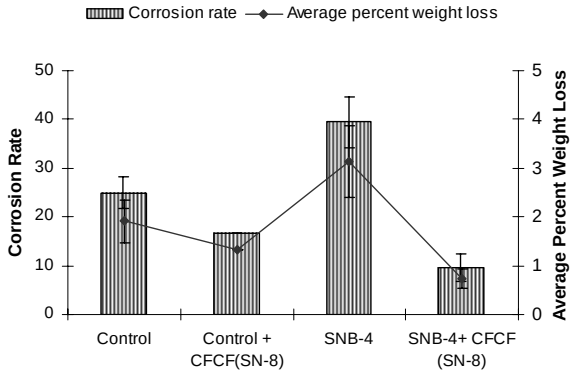

Fig. 1. Corrosion rate in $mg.dm^{-2}.d^{-1}$ and average percent weight loss in % of MSC exposed to *B. cereus*-SNB-4 cultured in nutrient broth and in the presence of CFCF of *Bacillus* sp. SN-8 upto a period of 90 days.

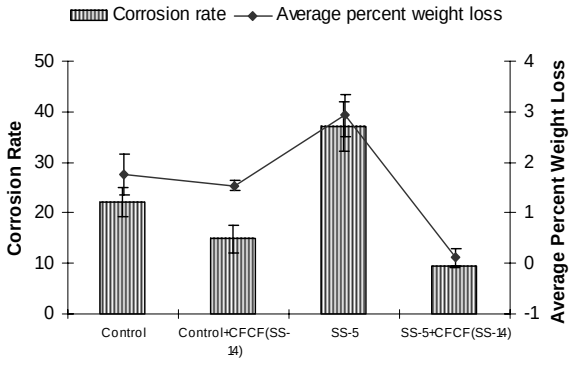

Fig. 2. Corrosion rate in $mg.dm^{-2}.d^{-1}$ and average percent weight loss in % of MSC exposed to *B. coagulans*-SS-5 cultured in nutrient broth and in the presence of CFCF of *Bacillus* sp. SS-14 upto a period of 90 days.

The MSC exposed to *B. cereus*-SNB-4 and *B. coagulans*-SS-5 cultured in CFCF of their respective antagonists showed 74.92% and 56.78% decreases in corrosion rate as compared to the ones exposed to *B. cereus*-SNB-4 and *B. coagulans*-SS-5 cultured in nutrient broth. When corrosion rates of MSCs exposed to un-inoculated nutrient broths were compared with those treated similarly but in the presence of CFCF of antagonistic bacteria, it appeared that the latter coupons had 33% less corrosion rates. The APWL of these experimental coupons also showed a comparable trend. These decreases in corrosion rates and APWL as compared to control MSCs as well as those exposed to corrosive bacteria demonstrate that the presence of antagonists' CFCF not only stopped the deteriorative (corrosion) activities of the micro-organisms but they some how protected the MSCs from abiotic corrosion also.

Metals get corroded naturally and the deteriorative process is influenced by a number of known factors. Iron/ mild steel corrosion in the presence of moisture/ water contents is a common phenomenon (Lee and Newman, 2003). The process of corrosion is also influenced positively/ negatively by certain biotic factors including bacteria. The latter aspect has been variously termed as microbiologically influenced corrosion (MIC). In the present study the bacteria *B. cereus*-SNB-4 and *B. coagulans*-SS-5 were found to be corrosive for mild steel coupons (MSCs). Thus the bacterium SNB-4 accelerated the process of corrosion by 36%.

Some polysaccharides are reported to exhibit stronger stability constant for Fe^{+3} ions (Ford *et al*., 1988). Such complexes may serve as a corrosion inhibitor. Majumdar *et al*. (1999) described that inverse relationship between exopolysaccharides (EPS) and the corrosion rate of mild steel was probably involved in developing a protective film on metal surface in sea water. Hefter *et al.* (1997) reported that straight chain aliphatic mono-carboxylates and dicarboxylates decreased corrosion rate of mild steel as much as upto 99.9%. McLean *et al.* (1990) investigated metal binding affinity of γ-polyglutamate produced by *B. licheniformis* and found that Cu^{+3}, Al^{+3} and Fe^{+3} had affinity for the *B. licheniformis* capsule. Although such details of corrosion inhibition mechanisms for the antagonistic bacteria have not been worked in this study. However, significant decreases in APWL of MSC exposed to corrosive bacteria together with antagonists' products as compared to MSCs exposed to un-inoculated control and corrosive bacteria are suggestive to speculate involvement of comparable

protective shielding effects of antagonist bacteria for the MIC. Further work is likely to describe details of such phenomenon.

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