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Soil Buried Mild Steel Corrosion by *Bacillus cereus*-SNB4 and its Inhibition by *Bacillus thuringiensis*-SN8

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Abstract.- Microbiologically influenced corrosion (MIC) of mild steel coupons (MSCs) under field simulated soil buried conditions was analysed up to exposure of 150 days. Water and nutrient broth impregnated soil buried MSCs were exposed to highly corrosive *Bacillus cereus*-SNB4, its non-corrosive antagonist *Bacillus thuringiensis*-SN8 and their co-culture. After 50 days, MIC rates of MSCs treated with *B. cereus*-SNB4 turned out to be 16.96 and 2.71 $mg.dm^{-2}.d^{-1}$ for wet and nutrient broth impregnated soils, respectively. After 100 days the corresponding values were 41.01 and 9.26 $mg.dm^{-2}.d^{-1}$, respectively. At the last observational point average percent weight losses due to MIC of the coupons exposed to the *B. cereus*-SNB4 in wet and nutrient broth impregnated soils, were 32.89% and 1.83%, respectively. The reductions in MSCs weights following exposure to *B. thuringiensis*-SN8 and the co-culture were found even lesser than those of the control coupons throughout the study period. MIC inhibitory rather protective role of the bacterial antagonist is suggestive of secretion and involvement of some exoproduct(s) which made the MSCs resistant to the bacterially accelerated corrosion.

Key words: Microbiologically influenced corrosion, biocorrosion and its control, MIC antagonist, soil buried corrosion and its biocontrol.

INTRODUCTION

It is known that around 50% of the underground pipes deteriorate due to microbial actions (Booth, 1964; Allsopp and Seal, 1987). Besides the external surface deterioration, 40% of all internal pipeline corrosion in the gas industry has been attributed to microbial corrosion (Graves and Sullivan, 1996; Pound, 1998). Microbiologically influenced corrosion (MIC) is of great economic consideration. For instance, cost of repair and replacement of piping material used in different types of services in USA had been estimated around 0.5-2 billion per annum (Vernon, 1957). MIC does not invoke any new electrochemical mechanism of corrosion rather it brings change(s) which promote establishment or maintenance of physicochemical reactions not normally favoured under otherwise similar conditions (Beech and Gaylarde, 1999). Main types of bacteria associated with corrosion failures of cast iron, mild and stainless steel structures include sulfate reducing (Hamilton, 1985), sulfur oxidizing (Cragnolino and Tuovinen, 1984), iron oxidizing/ reducing (Obuekwe *et al*., 1981), manganese-oxidizing (Dickinson *et al.*, 1997) and those which secrete organic acids, exopolymers and slime (Cragnolino and Tuovinen, 1984; White *et al*., 1986). Rozanova *et al*. (2003) have described likelihood of promoting corrosion of mild steel by sulfate reducing, iron oxidizing/reducing bacteria together with sulphur oxidizers. It has been established that most devasting MIC takes place in the presence of microbial consortium in which many physiological types of bacteria interact in complex ways (Pope *et al*., 1984; Little and Mansfeld, 1991). Various mechanisms of biocorrosion, which reflect the variety of physiological activities carried out by different types of microorganisms, have been identified. While under natural environments microbial processes are also influenced by and exert changes in prevailing chemical and electrochemical forces (Beech and Gaylarde, 1999). In such environments besides the biocorrosion initiating/ enhancing abiotic and biotic factors, presence of corrosion stopping/ reducing agents cannot be ruled out. For instance, Zuo (2007) described that in field, a consortium of aerobic and anaerobic bacteria is usually present. The author commented on necessity of protecting aerobic biofilm for reducing availability of oxygen hence decreasing cathodic reaction and to inhibit growth of corrosion- causing bacteria such as SRB, which depolarize cathode and

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stimulate localized corrosion. Likewise, 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 on mild steel and stainless steel. Presence of humidity, warm temperature and organic matter coming for openly drained domestic and industrial effluents create favourable conditions for MIC in this country. The present investigation reports association of bacteria with the process of MIC of soil buried mild steel pipes. They were isolated from soil sampled from vicinity of natural gas transporting corroded steel pipes.

MATERIALS AND METHODS

Source of bacteria

Mild steel corrosion influencing bacteria were isolated as described by Bano and Qazi (2008). Those designated as SNB-4 and SN-8 highly corrosive to mild steel and antagonistic to the earlier, respectively, were employed in these experiments.

Preparation of soil

For simulating field conditions soil was sampled at 2 m depth from the vicinity of Quaid-e-Azam Campus, University of the Punjab, Lahore. It was brought to the laboratory, air dried, thoroughly mixed several times and sieved through 20 mesh sieve. Then 300g of the prepared soil was added in screw capped glass cylinders. The containers (uncovered) were then placed in an oven for 2 days at 105 °C for removal of moisture contents. They were then closed with plastic caps and sterilized at 121°C for 30 minutes in an autoclave.

Preparation of mild steel coupons

Mild steel coupons (MSCs) measuring 2.5×2.5×0.1cm were degreased with analytical grade acetone (Merck) and gently polished with 240 grit polishing paper. They were then rinsed with distilled water and washed with ethanol. The MSCs were again rinsed with distilled water and wiped immediately with paper towel and dried in an oven at 80ºC for 10 minutes. Weight of each coupon (g) was measured with the help of an electric balance (Zuo and Wood, 2004). Then each coupon was placed in concavity of a piece of blotting paper folded in quadrant manner. The wrapped coupons were sterilized in a screw capped glass container at 121ºC for 15 minutes.

Inocula preparation

Overnight grown nutrient broth (Merck, 1996/97) culture of a given bacterium was centrifuged, aseptically at 5000rpm for 10 minutes. Pellets were washed twice with sterilized distilled water and then suspensions in water and nutrient broth prepared upto 0.80 O.D. of the cells at 600nm.

Experimental procedure

In control containers sterilized soil 300g was moistened with 80ml of sterilized distilled water or nutrient broth to provide about 25% moisture contents. For experimental containers, comparable amount of a given bacterially inoculated water or nutrient broth, as described above, was dispensed. In one set of the containers both the bacteria were co-cultured by suspending their equal amounts of cells. Then two mild steel coupons of known weights were inserted in the soils one cm apart from each other in middle of a glass container. The containers were capped, air tightened and sealed with thick plastic black tape. They were then placed in inverted position to minimize evaporation. Three containers from every category were used for observations at each of 50, 100 and 150 days.

Post-harvest preparation of MSCs

At termination of experiments, 1g of experimental soil was saved aseptically for viable counting of bacteria. Then the metal coupons were taken out from the containers and dried in oven at 80°C for half an hour. Soil from the surfaces was removed and finally the coupons were dipped in post harvest solution for 10-15 minutes as described by Angeles-Chavez *et al*. (2001) to remove corrosion product(s). The MSCs were then oven dried till consistent weight. Corrosion rate was determined according to Majumdar *et al.* (1999). The soil removed from the surroundings of coupons was oven dried, pulverized and passed through 20 mesh sieve. The material was then processed for estimating various soil characteristics such as pH

(Watson and Brown, 1998), chloride (Gelderman *et al*., 1998), sulphate (Combs *et al*., 1998), phosphorous (Frank *et al.,* 1998) and organic contents (Combs and Nathan, 1998). For estimating viable bacterial counts, 1g of soil was mixed in 10ml of sterilized distilled water. Further dilutions of the sample were made in distilled water and 0.1ml of a given dilution was spreaded on nutrient agar plate. Bacterial colonies were counted after incubation (37°C) for 24 h to enumerate colony forming units (CFU)/g of soil.

Identification of the bacterial isolates by 16s rRNA gene sequencing

The bacterial isolates designated as SNB-4 and SN-8 were revived in nutrient broth. Cells were harvested at exponential phase and lysed. The 16S rRNA genes of the bacteria were amplified using the eubacterial specific primers set 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) (Lane, 1991), and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) (Marchesi *et al*., 1998) by polymerase chain reaction (PCR) as follows: denaturation for 30 s at 95 °C; annealing for 30 s at 55 °C, elongation at 72 °C for 90 s; and a final 10 min incubation period at 72 °C. An aliquot of each PCR, product was analyzed on a 0.7% (w/v) agarose gel to confirm that a product of the correct size (~ 1.5 kb) was amplified. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN). The sequences of the purified PCR products were analyzed using CEQ 2000 Dye Terminator Cycle Sequencing (Beckman Coulter, U.S.A.). The sequence data were compared with 16S rRNA sequences deposited in public databases by using the BLAST search program (Altschul *et al*., 1997). On the basis of highest % similarity the test organisms were identified.

RESULTS

Based upon % identities of 16S rRNA genes sequences of the corrosion promoting and inhibiting bacterial isolates employed in this study were identified as *B. cereus* and *B. thuringiensis,* respectively. The strains are designated as *B. cereus*-SNB4 and *B. thuringiensis*-SN8. Corrosion rate of control MSCs at day 50 turned out to be 68.2 and 8.5 $mg.dm^{-2}.d^{-1}$ for the wet and nutrient broth impregnated soils, respectively (Table I). On next observational points ***i.e.,*** 100 and 150 days' exposures the corrosion rates for the experiments provided with nutrient broth became several folds higher than the values obtained for the first study period. However, corrosion rates of the coupons exposed to nutrient broth impregnated soils remained significantly lesser than those treated with wet soils throughout the study period. This indicated corrosion protective role of organic components of the bacteriological medium. Average percent weight loss (APWL), ranged from 4.09-6.69% for the wet controls and 0.81 to 6.66 % for controls supplied with nutrient broth during first through last observational points (Table I, Figs. Ia, b, c). This trend also appeared for the MSCs exposed to the cells of *B. cereus*-SNB4 in otherwise similar conditions and the differences were several folds (Table I, Figs. Ia, b, c). Accordingly, the coupons exposed to the corrosion enhancing bacterium in wet soil showed MIC rates as to be 16.96, 41.01 and 59.15 $mg.dm^{-2}.d^{-1}$ at 50, 100 and 150 days, respectively. While the MSCs treated with *B.cereus*-SNB4 in nutrient broth impregnated soils expressed MIC rates as 2.71, 9.26 and 9.86 $mg.dm^{-2}.d^{-1}$ at first through last study period, respectively (Table 1, Figs. Ia, b, c).

Maximum APWL (39.58%) was obtained for the coupons exposed to *B. cereus*-SNB4 at the last observational point. The corresponding figure for the experiment in which nutrient broth was provided to enhance the microbial growth was found to be 8.49 %. Albeit the provision of nutrient broth enhanced the bacterial CFU/g of the soil up to 3.4×10^{13} (Figs. Ia, b, c). The figure turned out to be two log values higher than the corresponding CFU/g for the bacterially inoculated wet soil. Thus provision of nutrient broth enhanced bacterial growth but it decreased the corrosion process geared by the *B. cereus*-SNB4. The soil inoculated with *B. thuringiensis*-SN8 showed APWL values even lesser than those found for both categories of the controls. Co-culturing of the corrosion causative and protective bacteria showed promising results in terms of significantly inhibiting corrosion rates throughout the study periods. APWL also followed the trend of corrosion rates for the different

Table I.- Corrosion rate in mg $dm^{-2}d^{-1}$ and average percent weight loss (APWL) of mild steel coupons buried in wet and nutrient broth impregnated soils treated with *Bacillus cereus*-SNB-4, *Bacillus thuringiensis*-SN8, and their co-culture for different periods of time.

Experiments	Corrosion rate (mg/dm^2d)					
	50 days		100 days		150 days	
	Wet soil	Nutrient broth impregnated soil	Wet soil	Nutrient broth impregnated soil	Wet soil	Nutrient broth impregnated soil
Control (1)	68.2±0.13[a] (4.09±0.22)[b]	8.5±0.8 (0.81±0.5)	52.6±12.0 6.33±1.47	40.74±2.55 (4.86±0.3)	38.45±4.53 (6.69±0.65)	37.08±3.25 (6.66±0.6)
B. cereus-SNB4 (2)	85.16±9.31[3,4] (5.03±0.6)	11.21±6.5[1,3,4] (2.0±0.37)	93.61±10.87[3,4] (9.87±2.52)	50±9.03 (5.96±1.09)	94.6±8.82[1,3,4] (39.58±21.33)	46.97±5.19 (8.49±0.92)
B. thuringiensis-SN8 (3)	56.53±1.6 (3.37±0.1)	7.25±1.08 (0.3±0.06)	39.73±5.32 (4.81±0.64)	28.45±4.16 (3.4±0.50)	31.79±8.82 (5.79±1.40)	30.77±3.6 (5.7±0.54)
B. cereus-SNB4 + *B. thuringiensis*-SN8 (4)	51.07±7.77 (3.06±0.47)	7.64±0.86 (0.52±0.03)	29.28±10.18 (3.52±1.23)	26.57±4.36 (3.12±0.51)	24±9.5 (6.03±0.02)	27.56±11.75 (6.59±0.85)

a=Corrosion rate; b=APWL; Means of three replicates ± S.E.M.
Values of a given observational period within a column were compared employing single factor analysis of variance, superscripts refer to the experiment number given in parentheses in first column from whom the given value is significantly different at $p \leq 0.05$.

experiments. When APWL of respective control coupons were subtracted from the experimental MSCs, to depict effects of MIC, it appeared that exposures to the *B. thuringiensis*-SN8 and the co-culture gave negative values for all the experiments throughout the study period. This clearly indicated MIC controlling as well as abiotic corrosion protective potential of the bacterium *B. thuringiensis*-SN8

APWL due to MIC caused by *B. cereus*-SNB4, turned out as to be 0.94, 3.54 and 32.89% at 50, 100 and 150 days following the exposure of MSCs in wet soils. The corresponding values for coupons exposed to bacterium in nutrient broth impregnated soils appeared as 1.19, 1.1 and 1.83%, respectively. All the MSCs treated with *B. thuringiensis*-SN8 and the co-culture in wet as well as nutrient broth impregnated soils yielded negative values of APWL due to MIC.

pH of experimental soils, remained, in general, unaffected except a drop of about one unit for the soil exposed to *B. cereus*-SNB4 in the presence nutrient broth. Chloride contents of the control soils showed great variation (Fig Id). While the bacterially inoculated soils showed several folds increase in the chloride contents at second and third observational periods as compared to the value of first sampling point. Chloride contents of wet and nutrient broth supplemented soils inoculated with *B. cereus* -SNB4 bacteria were found as to be116 ppm and 684.95 ppm, respectively. Chloride contents for the wet and nutrient broth impregnated control soils turned out to be 307.95 and 2.11 ppm, respectively. Concerning the organic contents, all the experiments invariably showed higher values for wet soils than those supplied with nutrient broths at first sampling point. Organic content values of wet soils for the different experiments ranged from 0.456 to 0.74%. While for soils impregnated with nutrient broth it ranged from 0.045 to 0.747% (Figs. 1d, e, f). The wet soil showed about 55% higher sulphate contents than nutrient broth impregnated soil at first sampling point. For the experiment involving *B. cereus*-SNB4 no difference was noticed in sulphate contents, while for *B. thuringiensis*-SN8 inoculated experiments, the wet soil had 126% higher sulphate contents than the corresponding experiment impregnated with nutrient broth. Following co-culturing the situation became inverse otherwise and the soil receiving nutrient broth had 37% higher sulphate contents than the wet soil. No such vivid difference was noticed incase of phosphorous contents for mono and co-cultured experiments, however, the nutrient broth controls had 58% higher phosphorous than the corresponding wet soil controls.

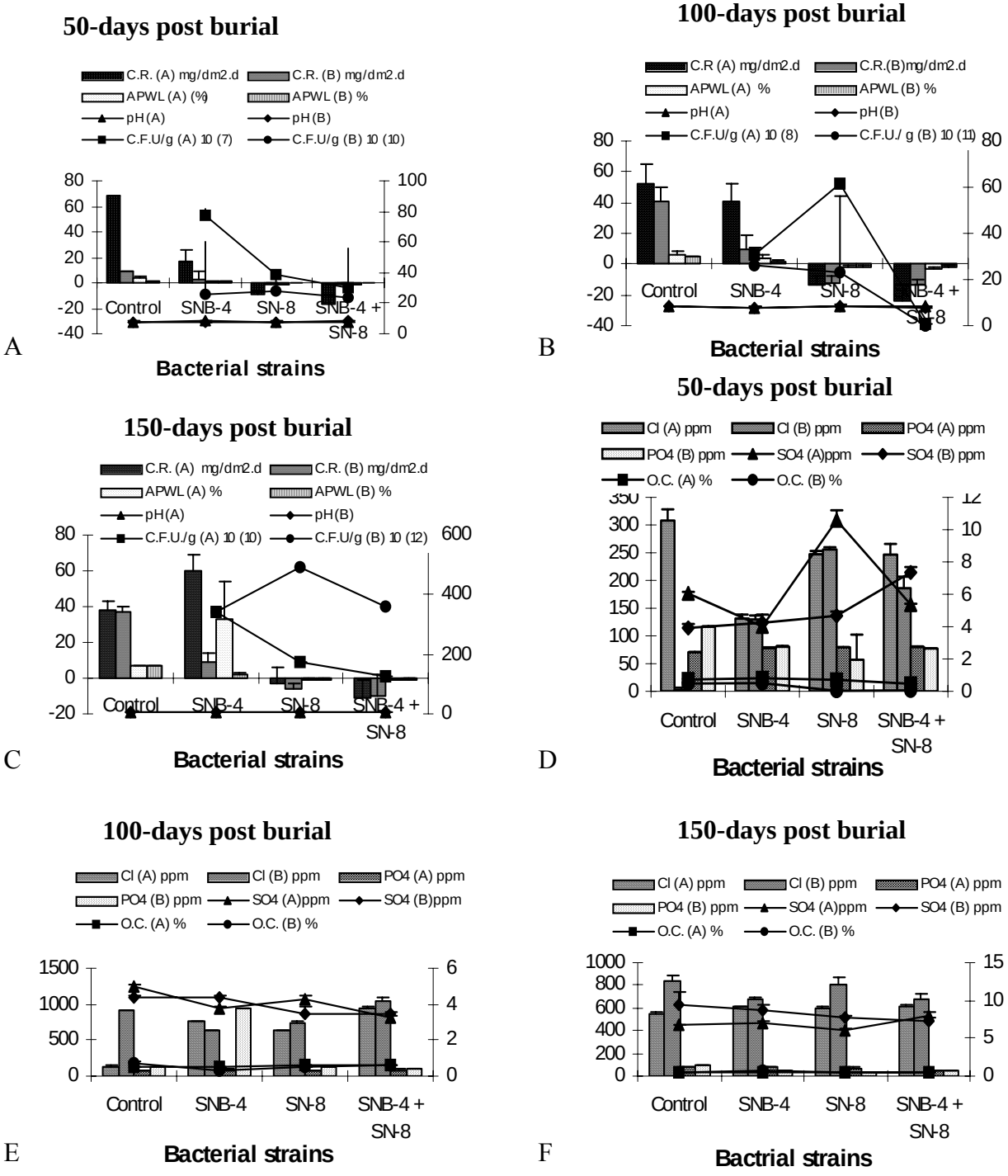

Fig. 1. Microbiologically influenced corrosion of buried mild steel coupons and accompanying levels of pH, different chemical contents and bacterial C.F.U./g following inoculations with *Bacillus cereus*-SNB4, *Bacillus thuringiensis* -SN8 and their co-culture in wet (A) and nutrient broth impregnated soils (B) at different post-burial periods. C.R., corrosion rate; APWL, average percent weight loss; O.C., organic contents. Values in parentheses with C.F.U/g is dilution factor.

DISCUSSION

In the present experiments provision of nutrient broth, besides enhancing the bacterial growth, invariably resulted into lesser corrosion rates as well as % APWL for the soil buried MSCs. For instance, MIC rates of MSCs treated with *B. cereus*-SNB4 in wet soils appeared as 16.25, 4.43 and 6.80 folds higher than the values obtained for the exposure in the presence of nutrient broth at first through last study periods, respectively. Likewise *B. cereus*-SNB4 caused 1.83% and 32.89% APWL in soils supplied with nutrient broth and water, respectively at end of the experimental period. A possible explanation is supported by earlier workers. For example, Borenstein (1994) described that a thick biofilm (layer of live bacteria) develops on the susceptible metal surface. The microorganisms develop colonies and form nodules (biomass containing microbiological/corrosion by-products and deposits). These formations then trap ions and occlude the metal surfaces directly beneath them from oxygen dissolved in water. Similarly, Fang *et al*. (2002) while studying corrosion of mild steel described that microorganisms tend to colonize on solid surface in natural environment and that the biofilm forms a protective layer, reducing exposure of the solid surface to external environment. These workers further mentioned that corrosion appeared to occur mostly in the regions between microbial clusters. Accordingly, aggregation of microbial cells leads to the development of gradient in electrochemical activity. The microbial clusters become barriers to diffusion and the area under them act as cathode, whereas the region between microbial clusters allows the surface to have greater access to chloride and sulfate in the sea water medium and to act as anode resulting in accelerating the electrochemical corrosion reactions. Furthermore, the regions between microbial clusters were likely to have higher expolymeric complex (EPS) concentration (Fang *et al*., 2002). This situation may enhance increased surface corrosion due to acidic (Lewandowski, 1994) and iron binding nature of the EPS (Beech *et al*., 1998). Thus the MIC is a complex phenomenon and bacterial clustering, regions between the cells aggregations and presence of a more or less uniform biofilm of bacteria are likely to affect the corrosion process differently. This notion must be taken into account while discussing MIC. For the present results it may be speculated that provision of nutrient broth might had created a more or less continuous biofilm across MSCs' surfaces, and played a role for lessening the corrosion process. Whereas in case of MSCs from wet soils viscous mats of bacterial growth might had not developed. And sparsely distributed microbial cells/ clusters, might have caused generation of gradient in electrochemical activity and then accelerated the corrosion processes as became evident by 18 folds higher % APWL in such cases as compared to nutrient broth supplemented soil experiments. Thus it is imperative to take into account the complex nature of biocorrosion that may not be explained by the familiar laboratory protocols of the microbiologists, which heavily depend and explain microbial activities on the basis of growth profiles. Albeit in the present study bacterial CFU/g at the end of experiments turned out higher following provision of nutrients but corrosion process did not follow the microbial growth pattern. In fact corrosion depends on various abiotic and biologically generated physicochemical conditions. Corrosion enhancing chemical factors, such as SO_4 and Cl_2 might had been masked by the presence of nutrient broth in the present experiments. Buffering capacity of amino acids present and molecular adsorption by small peptide of nutrient broth for some corrosion enhancing/causing metabolites can also be considered for explaining these results.

Regarding phosphate, sulfate, chloride and organic contents of the soil used in this study, no definitive trend related to MIC was noticed. Soil heterogenity in different containers in the present experiments had not allowed to predict the underlying processes of corrosion that might had influenced by the inorganic contents. It is suggested that some "artificial soil" can be tried in future for such studies that may bring more informative data. However, the corrosion protective role of the microbe has a potential for employing under field conditions, comparable to the experimental set up. Bacteria forming biofilms are well known to protect

different kinds of metals by consuming dissolved oxygen, and making it inaccessible to the corrosion reaction (Jayaraman *et al*., 1999a; Ornek *et al*., 2002).

MIC reducing as well as inhibiting potentials of the bacterium *B. thuringiensis*-SN8 were evidenced both by the corrosion rates and APWL, of MSCs exposed to the mono and co-cultures of the bacteria reported in this study. Secretion of some substance by the *B. thuringiensis*-SN8 might had been responsible for the observed corrosion protection effects. Infact secretion of antimicrobial substances (AMS) against SRB and other harmful bacteria which constitute the biofilm is well documented (Jayaraman *et al*., 1997, 1999b). AMS produced by *Bacillus* strains can be small molecules, which are structurally rather diverse, including bacteriocin and exoenzymes, like proteases, RNA-degrading enzymes, cell wall lytic enzymes and amylases (Hyronimus *et al*., 1998; Cherif *et al*., 2001; Van t'Hof *et al*., 2001). Some of these substances are active only against the same and closely related species (Tagg *et al*., 1976), while others have a broad spectrum activity (Azuma *et al*., 1992; Fujitita-Ichikawa and Tuchikubo, 1993). Further work must evaluate MIC protective role of cell-free culture fluids of antagonist bacteria as well as effects of growth of corrosion causative microorganisms at a bit distance from metal coupons.

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