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Microbial corrosion inhibition of mild steel by *Bacillus thuringiensis*

ABSTRACT

The mechanism of microbial corrosion inhibition cannot be linked to a single biochemical reaction or particular species or group of microbes. Some microorganisms are able to both cause and inhibit corrosion. Studies on the effects of Bacillus thuringiensis on the corrosion behaviour of mild steel were carried out using gravimetric and atomic force microscopy (AFM) analysis. The mild steel coupons 2 x 2 x 2 cm in size were suspended with a cotton thread which passes through a hole in each coupon, inoculated with the bacteria culture and incubated aerobically. The coupons were retrieved at 10 days intervals progressively for 60 days and analyzed. The result revealed that B. thuringiensis inhibited the corrosion of mild steel. The corrosion rate showed clear decrease in rate from 0.45 mpy after 10 days to 0.03 mpy after 60 days of exposure to B. thuringiensis when compared to a significant increase in corrosion rate observed (from 0.67 mpy after 10 days to 3.98 mpy after 60 days) for mild steel not exposed to the bacterium respectively. The AFM analysis showed a wavy pattern of corrosion on the surfaces of the metals not exposed to bacteria due to heights differences, coupled with some peaks and valley formed as a result of uneven deposition of corrosion products. Bacillus thuringiensis is very effective in decreasing and inhibiting mild steel corrosion in an aerobic environment.

Keywords: *Microbial corrosion, inhibition, metals, corrosion rate, biofilm, Bacillus thuringiensis*

1. INTRODUCTION

Microbial corrosion of metal is due to biochemical reactions that release either electrons or ions from the metal in such a way that corrosive chemicals such as organic acids, microorganisms and their metabolites stimulate anodic reactions [1]. Most damaging effects of corrosion takes place in the presence of a biofilm especially when such biofilms are made up of different microorganisms. Microorganisms are capable of changing the electrochemical conditions by biofilm formation [1]. The interactions between various species in biofilm may increase the rate of corrosion by triggering a series of biochemical reactions in the biofilm's oxic and anoxic regions [1,2], while in some cases these changes can induce corrosion inhibition. Microorganisms can induce corrosion inhibition through different mechanisms or combination of some [1,3]. These include formation of protective films on the metal, alterations of the oxido-reduction potentials of the microenvironment, neutralizing the action of corrosive substances present in the environment or inhibition of the adhesion or growth of harmful microorganisms [3, 4].

The effect of microbial biofilm on metal surface can either be positive or negative depending on the concentration, absorption, charge, and three dimensional structure of each extracellular polymeric substances (EPS) component [5]. Majority of corrosion issues can be controlled by using conventional material protection techniques such as coatings, surface treatments, and corrosion inhibitors, however these methods will eventually produce environmental issues due to the accumulative release of hazardous compounds. A novel approach to corrosion inhibition is microbial influenced corrosion inhibition (MICI), an eco-friendly anticorrosion technique. Due to the complexity of the environment and the diversity of microorganisms, multiple MICI mechanisms can co-exist and synergistically inhibit corrosion [6].

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Recently, findings in the field of MICI have sparked interest due to a better understanding of the role of certain microorganisms in corrosion prevention. This research is therefore aimed at studying the inhibition effects of *B. thuringiensis* on the corrosion behaviour of mild steel.

B. thuringiensis are gram-positive, spore-forming, rod-shaped, motile bacteria. They are found mostly in soil and in different environmental conditions. Their survival in some harsh conditions can be attributed to their ability to produce endospores. *B. thuringiensis* have been found to provide protection to plants, and fish against microbial infection through mode of action including secretion of antibiotics, enzymes and volatile compounds [7;8]. Some strains of *Bacillus* sp. have been found to induce corrosion inhibition.

2. MATERIALS AND METHODS

Isolation and identification

The strain of *Bacillus* used in this study was isolated from biofilm covering an abounded metal storage container. The composition of the medium used for the enrichment and cultivation and identification of the *B. thuringiensis* was: glucose, 5 g/L; glycol, 1 g/L; sodium acetate, 0 g/L; $(NH_4)2SO_4$, 1 g/L; corn steep liquor, 2 mL/L; yeast extract, 1 g/L; hydrolyzed casein, 1 g/L; $CaCl_2$, 20 mg/L; $MgSO_4$, 50 mg/L; $MnSO_4$, 100 mg/L (Oxoid).

A 10-fold serial dilution of the corroded metal sample suspension was prepared by weighing out 1 g of corroded metal samples into 9 mL of sterile distilled water in sterile 20 mL test tube. This constituted 10^{-1} dilution. The corroded metal suspension was vigorously shaken for 3 minutes by hand. After shaking, the 10^{-1} dilution was allowed to stand for 30 seconds. Then using a sterile pipette, 1mL was removed from the middle of the suspension and transferred into 9 mL sterile distilled water to achieve 10^{-2} dilution. The content of the 10^{-2} dilution test tube was shaken and dilution continued until the 10^{-7} dilution was obtained. After the serial dilutions, aliquots (0.1 mL) of dilutions 10^{-5} to 10^{-7} were streaked on a nutrient agar (NA) plates amended with cycloheximide (100 µmg) to prevent fungal growth and incubated at 37ºC for 24 hours.

Biochemical identification

B. thuringiensis strain was identified on the basis of morphology, Gram staining, biochemical characterization and confirmatory tests. Subsequent identification tests include hemolysis, starch hydrolysis, gelatin hydrolysis, citrate test, urease, motility, lactose test and production of endotoxin proteins, which are produced during sporulation [7]. Presence of crystal protein was confirmed using Lowry's method [9].

Corrosion test

The mild steel metal used for this experiment was first cut into coupons of specific dimension 2cm x 2cm x 0.14 cm for mild steel. Thereafter the coupons were polished with silicon carbide abrasive paper (from grade no. 400 to 1000), then cleaned with distilled water, dried in acetone and weighed with electronic weighing balance (Nicolet Model 37500). Weighed coupons were stored in moisture-free desiccators prior to use.

The test coupons were placed suspended with a cotton thread which passes through the hole in each coupon. Thereafter, 20 mL of 0.01 dilution of sterile nutrient broth was added and the medium was inoculated with 0.1 mL Mac Farland Standard inoculum of *B. thuringiensis*. The entire sets of experiments were uniformly prepared in duplicate and incubated in aerobically on the same day at 37ºC in a stagnant condition. The coupons were retrieved at 10 days intervals progressively for 60 days and the pH values recorded. Thereafter the metal coupons were scraped with spatula, washed with distilled water, dried on the lab bench and weighed. The weight loss was taken to be the difference between the weight of the coupons at a given time and its initial weight.

Average values for each experiment were obtained and used in subsequent calculations. Data collected were subjected to statistical analysis using one way analysis of variance (ANOVA) procedure by IBM SPSS (2012 Version).

Corroded coupon surface analysis

An atomic force microscopy (AFM) analysis was carried out to visualize the metal surface after the corrosion experiment. The coupon was removed from the media, lightly rinsed in sterile distilled water, and air-dried. The coupon was examined under an AFM (Bruker Dimension Icon Nanoscope V) in the tapping mode to capture the images of biofilms on the coupon surface. Silicon N-type cantilever nanoprobes with a spring constant of k = 25–75 N/m (App NANO) were used. The coupon samples were taken to AFM for micro scanning of the surface features. The heights of the AFM results were used to predict the surface topography of the samples due to corrosion.

3. RESULTS

The result of the identification of the bacteria by morphological, biochemical and confirmatory test indicated that the isolate was *B. thuringiensis*. Table 1 shows the result of the morphology and biochemical test. The isolate showed typical colony morphology, spore forming, predominantly off white to creamy with irregular edges with presence of ovoid para-sporal crystals. Lowry's test confirmed the presence of crystal protein.

The gravimetric results of microbial corrosion inhibition of mild steel after 60 days by *B. thuringiensis* is shown in Table 2, and also in Figure 1 and 2. The results shows a steady and significant decrease in weight loss and corrosion rate for mild steel exposed to *B. thuringiensis.* The weight loss decreased from 0.002 g to 0.001 g while the corrosion rate decreased from 0.45 mpy to 0.03 mpy respectively. In the case of the mild steel not exposed to the bacteria, there were significant increase in weight loss from 0.003 g to 0.096 g while the corrosion rate also increased significantly from 0.67 mpy to 3.98 mpy respectively over the exposure period.

Table 1: Characterization of B. thuringiensis isolated from metal storage tank

Characteristics	Result
Cell morphology	
Colony	Filamentous, off-white, elevated, irregular
Gram Stain	+
Shape	Rod shaped
Motility	+
Sporulation	+
Biochemical reaction	
Indole production test	+
Citrate	+
Oxidase	+
Urease	+
Catalase	+
Glucose	+
Galactose	-
Starch hydrolysis	+
Gelatin hydrolysis	+
Lactose	-
Endotoxin protein	+

+Positive; -Negative

Table 2. Corrosion data for mild steel in the presence and absence of B. thuringiensis at different exposure period

Duration	Mean weight loss (g)		Corrosion rate (mpy)	
	Control	With *B.thuringiensis*	Control	With B.thuringiensis
Day 10	0.003	0.002	0.67	0.45
Day 20	0.032	0.010	3.40	1.1
Day 30	0.061	0.014	4.52	1.05
Day 40	0.086	0.008	4.87	0.45
Day 50	0.008	0.003	3.92	0.13
Day 60	0.096	0.001	3.98	0.03

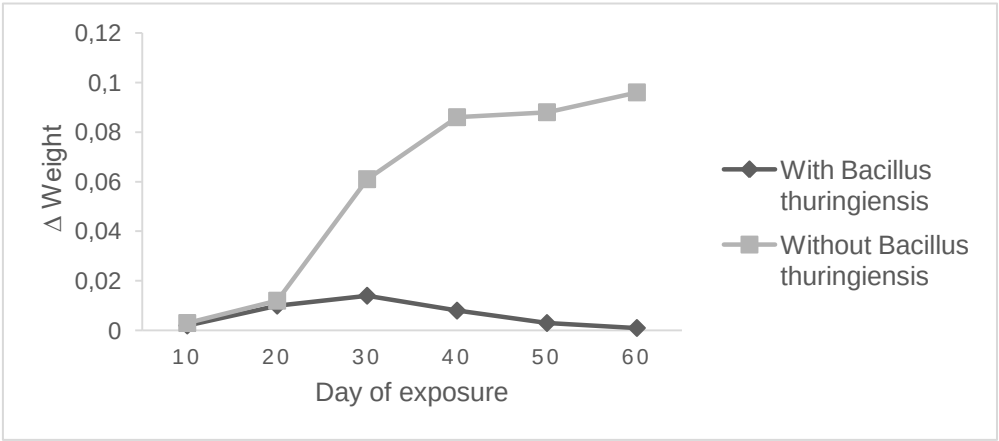


Figure 1. Gravimetric date of mild steel in the presence of B. thuringiensis

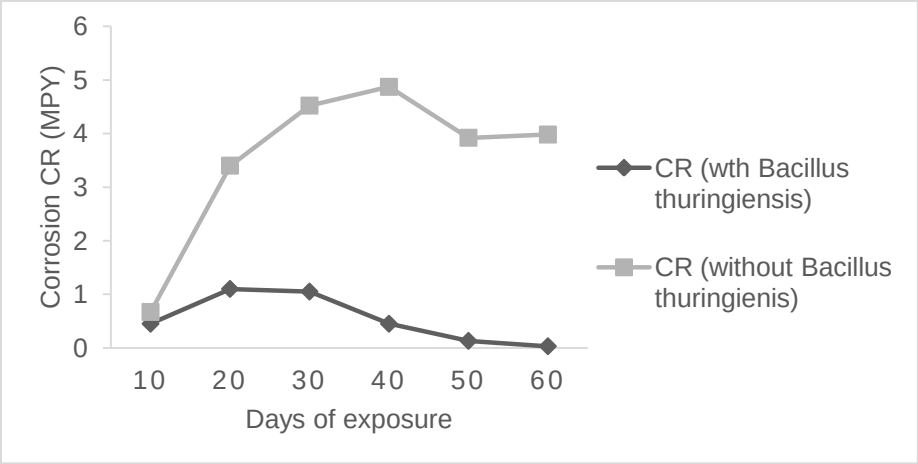


Figure 2. Corrosion rate of mild steel in the presence of B. thuringiensis

Effect of pH on the corrosion behaviour of mild steel in the presence of B. thuringiensis

The variation in pH with exposure time (days) for mild steel in the presence of *B. thuringiensis* is shown in Table 3 and Figure 3. The readings showed that there was relatively increase in the pH throughout the 60 days for samples exposed to bacteria when compared to the control without bacteria.

Table 3. Average pH readings for mild steel in the presence of B. thuringiensis

Days	Day 10	Day 20	Day 30	Day 40	Day 50	Day 60
pH	6.9	7.0	7.2	7.4	7.7	7.9
pH (control)	6.9	5.9	5.7	5.2	4.7	4.2

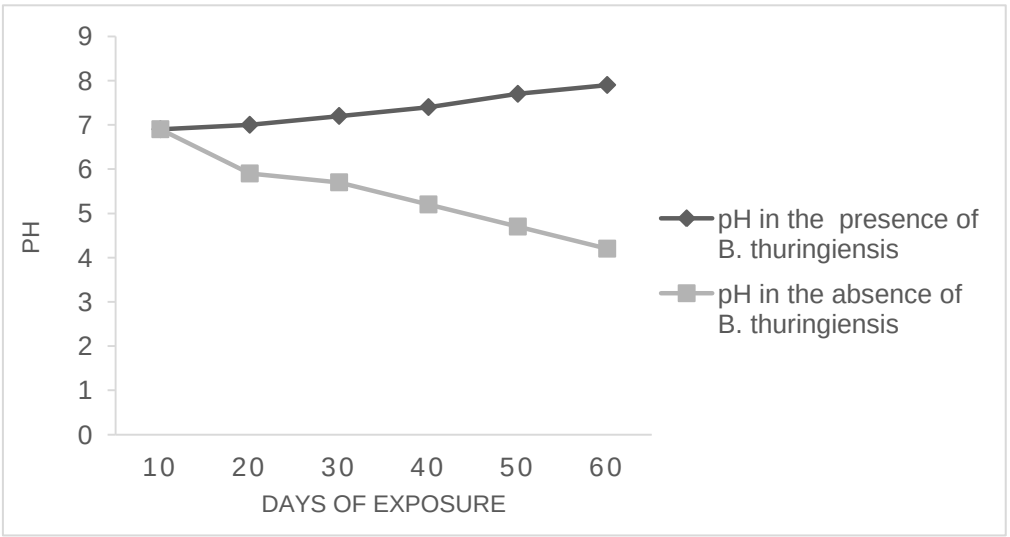
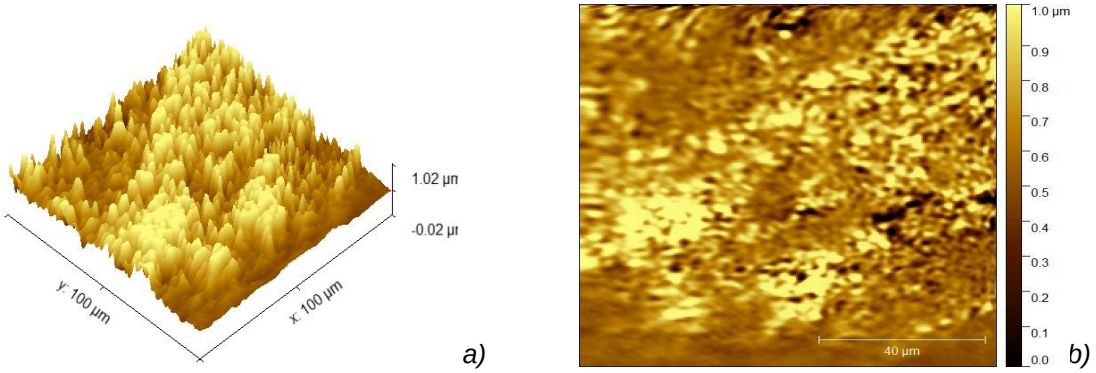


Figure 3. Change in pH in aerobic corrosion of mild steel in the presence of B. thuringiensis

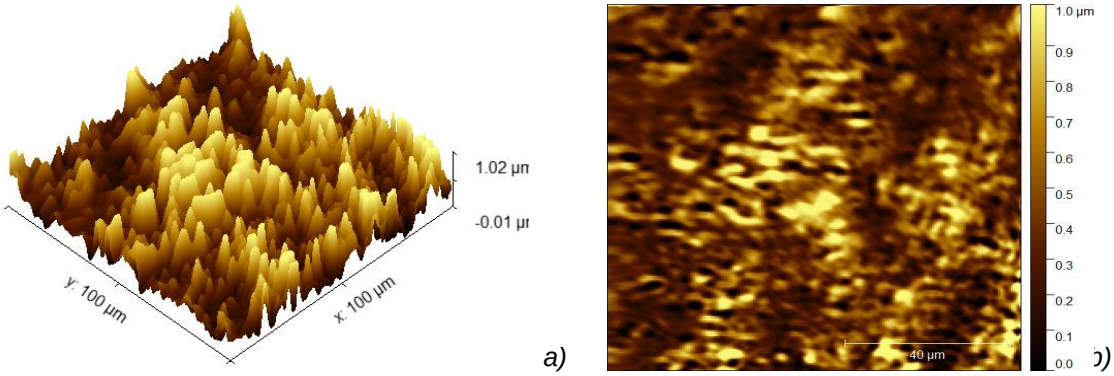
Surface analysis

The AFM of the metal coupons exposed to bacteria (after 60 days) and the coupon in the absence of bacteria is shown in Figure 4 and 5. The Figures shows the topography of the coupons after 60 days of exposure. In Figure 5, some pits and corroded regions were observed mainly on the surface of the control metal sample. The pits observed on the coupon without bacteria are covered with black regions which indicate that the pits does have accumulation of corroded products. These deposition of corrosion products on the surface were more evident on the coupon not exposed to bacteria (Figure 5) when compared to coupon exposed to bacteria (Figure 4).



a) *b)*

Figure 4. Three dimensional (3D) AFM image (a) and surface topography (b) of mild steel after exposed to B. thuringiensis for 60 days



a) *b)*

Figure 5. Three dimensional (3D) AFM image (a) and surface topography (b) of mild steel without B. thuringiensis for 60 days

4. DISCUSSION

The identification of the isolated bacteria species showed the presence of *B. thuringiensis*. In a similar study, [10] isolated *B. thuringiensis* from biofilm samples on metal surface. Moreover, many other studies have shown that microorganism can be isolated from metals. *Desulfotomaculum* sp. from corroded metals by [11]. Studies conducted by [12] also reported the isolation of *Aspergillius fumigatus* from corroded pipeline. In most of these studies, it was reported that the microorganisms isolated promoted the microbial influenced corrosion of metal under laboratory conditions. However, [1] observed that, some microorganisms are able to both cause and inhibit corrosion. According to their findings, microorganisms do not only influence corrosion, but they can also inhibit or protect against corrosion.

The corrosion observed on metal not exposed to the bacterium could be mainly due to electrochemical corrosion [2]. It was observed that the corrosion rate decreased with time when compared with the control. This signifies a decrease in the rate of the anodic dissolution of iron in the presence of *B. thuringiensis*. In a study conducted by [1], they observed, that aerobic biofilms of *Bacillus* sp. decreased corrosion to a great extent when compared to anaerobic biofilms. According to [1], this shows that oxygen removal has a significant role in MICI [13]. The authors [5] reported that *Shewanella oneidensis* species, a facultative anaerobic iron-reducing bacterium (IRB), could inhibit corrosion of X52 carbon steel through aerobic respiration and iron respiration. According to [14] the microbial influenced inhibition of carbon steel ST37 was mainly due to biosurfactant produced by *Bacillus* sp. The authors in [4] observed that *B. thuringiensis* caused reductions in the weight and subsequently the corrosion rates of mild steel. They attributed the MICI to the secretion exo-products by the bacteria. Studies have also shown that genetically modified *B. subtillus* secreting the antimicrobials indolicidin and bactenecin also reduced SRB-induced corrosion significantly [1;15].

The authors in [4] also reported the inhibition of mild steel coupons corrosion by *B. thuringiensis* under field soil buried conditions. The authors stated that some species of *Bacillus* sp. such as *B. thuringiensis* have been implicated with inhibition of corrosion. According to them, secretion of some substances by *B. thuringiensis* were responsible for the observed corrosion protection effects. The secretion of antimicrobial substances against sulfate reducing bacteria (SRB) and other harmful bacteria which constitutes biofilm is well

documented [4]. Antimicrobial substances produced by *B. thuringiensis* are small molecules, which are structurally diverse, including bacteriocin and exoenzymes like proteases. The authors in [16] also reported the inhibition of biofilm formation and subsequent corrosion of mild steel by *Bacillus* sp. According to [17], microbial corrosion inhibition is frequently accomplished through a decrease in the corrosion rate as was observed in this present study, decreasing of the medium aggressiveness in restricted areas of the metal solution interface (e.g., by neutralizing acidity) and providing or stabilizing protective films on metal [18-20]. These earlier findings supports the results obtained in the present study.

Another possible explanation of MICI by *B. thuringiensis* is supported by the result of the pH reading obtained throughout the period of the study. The result showed that there was relatively increase in the pH throughout the 60 days for samples exposed to bacteria when compared to the control without bacteria. Similarly, [11] reported that corrosion rate increased with decrease in pH. It therefore follows that decrease pH is proportional to increase in corrosion rate. In this study, the increase in pH followed a decrease in corrosion rate of the metal.

Figures 4 and 5 shows the three dimensional (3D) AFM images of metal coupon exposed to bacteria and coupon not exposed to bacteria after 60 days respectively. According to [21], AFM is used for the study of corrosion susceptibility and inhibition of metals and alloys at sub-micrometer resolution. In his investigation of corrosion damage through microscopy and stress analysis, [22] noted that the accumulation of corrosion products causes the area that was corroded on the surface of a metal to rise in heights. In line with these finding, we observed that, the regions which are not corroded were at lower heights than of the corroded regions. Additionally, the coupons that were not exposed to bacteria displayed more wavy patterns. This is because the height disparities between the corroded parts appear to form patterns resulting in peaks and valleys as a result of uneven deposition of corrosion products.

5. CONCLUSION

Microbial influenced inhibiting potential of the bacterium *B. thuringiensis* was studied by gravimetric techniques and AFM analysis of the corroded metal surfaces. The result revealed that *B. thuringiensis* inhibited the corrosion of mild steel. The AFM analysis showed a wavy pattern of corrosion on the surfaces of the metals not exposed to bacteria due to heights differences, coupled with some peaks and valley formed as a result of uneven deposition of corrosion products. The increase in pH observed throughout the period of the study followed the trend in the gravimetric results. In conclusion, it was observed that *B. thuringiensis* was very effective in decreasing and inhibiting mild steel corrosion in an aerobic environment.

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IZVOD

INHIBICIJA MIKROBNE KOROZIJE MEKOG ČELIKA POMOĆU *BACILLUS THURINGIENSIS*

Mehanizam inhibicije mikrobne korozije ne može se povezati sa jednom biohemijskom reakcijom ili određenom vrstom ili grupom mikroba. Neki mikroorganizmi su u stanju da izazovu i inhibiraju koroziju. Studije o uticaju Bacillus thuringiensis na koroziono ponašanje mekog čelika sprovedene su primenom gravimetrijske i mikroskopske analize atomske sile (AFM). Taloni od mekog čelika veličine 2 x 2 x 2 cm su okačeni pamučnim koncem koji prolazi kroz rupu u svakom talonu, inokulisani kulturom bakterija i inkubirani aerobno. Taloni su preuzimani u intervalima od 10 dana progresivno tokom 60 dana i analizirani. Rezultat je otkrio da B. thuringiensis inhibira koroziju mekog čelika. Stopa korozije je pokazala jasno smanjenje brzine sa 0,45 mpi nakon 10 dana na 0,03 mpi nakon 60 dana izlaganja B. thuringiensis u poređenju sa značajnim povećanjem uočenog stepena korozije (sa 0,67 mpi nakon 10 dana na 3,98 mpi nakon 60 dana) za blagi čelik koji nije izložen bakterijama, odnosno. AFM analiza je pokazala talasast obrazac korozije na površinama metala koji nisu bili izloženi bakterijama zbog visinskih razlika, zajedno sa nekim vrhovima i dolinama nastalim kao rezultat neravnomernog taloženja produkata korozije. Bacillus thuringiensis je veoma efikasan u smanjenju i inhibiciji blage korozije čelika u aerobnom okruženju.

***Ključne reči**: mikrobna korozija, inhibicija, metali, brzina korozije, biofilm, Bacillus thuringiensis*

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