Met. Mater. Int., Vol. 15, No. 3 (2009), pp. 409~419
doi: 10.1007/s12540-009-0409-9 Published 26 June 2009

Impact of Ammonia Producing *Bacillus* sp. on Corrosion of Cupronickel alloy 90:10

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(received date: 29 July 2008 / accepted date: 30 October 2008)

The objectives of the present investigation were to characterize ammonia-producing bacteria (*Bacillus* sp.) and study its impact on biodeterioration of cupronickel alloy 90:10. in a nitrogen free environment. It is well known that iron sulphate and molybdenum are good inhibitors when used in a cooling water system. The interactions between inhibitor and ammonia producing bacteria on the corrosion of cupronickel 90:10 were studied. The predominated ammonia producing bacteria *Bacillus* sp. (AG1-EU202683; AG2-EU202684; AG3-EU202685 and AG4-EU202686) were identified by 16S rRNA gene sequencing from the biofilm. These bacteria fixed with atmospheric nitrogen for their cell protein synthesis and converted into ammonia. Ammonia enhanced pH and ammonical solution were formed in the presence of *Bacillus* spp. which acted as an etchant. The presence of some anodic spots in the presence of bacteria was affected by ammonia and then underwent pitting corrosion. The present study reveals that *Bacillus* spp. encourage intergranular attack without any stress in the cooling water system.

Keywords: copper alloys, microbial corrosion, intergranular attack, *Bacillus* spp., biogenic ammonia

1. INTRODUCTION

Biofilms are important in a wide spectrum of industrially relevant situations and can lead to microfouling and microbially influenced corrosion [1,2]. Copper and its alloys are most commonly used in the fabrication of heat exchangers in cooling water systems. The alloys depend on their natural oxide for corrosion resistance. The corrosion of copper occurs with the outward movement of the cuprous ion rather than the inward movement of oxygen [3]. The slime layer forms a sticky surface, which allows silt and other suspended particles to adhere to the condenser tubes, thereby enhancing the aggregation of deposits on the material surface that increases fluid frictional resistance and heat transfer resistance [4] in cooling water systems. Pope *et al.* [5] have documented MIC of cupronickel (90:10), admirably brass and aluminum brass in the cooling systems of freshwater and backwater. It was also reported that copper alloy condenser tubes had under-deposit corrosion due to the formation of slime deposits and ammonia [6], which led to stress corrosion cracking [7-9].

Iron sulphate and molybdate [10] are added in many cooling water system as corrosion inhibitors [11]. It is also well known that iron and molybdate [12,13] act as co-factors for bacterial nitrogenase enzymes. However, no systematic study has been carried out on the interaction between microbial biofilms and corrosion inhibitors in a cooling water system. It was expected that the ammonia producing bacteria can proliferate as biofilm in a nitrogen free environment where the bacteria fixes the nitrogen from the atmosphere and converts it to ammonia. Hence, work was undertaken to discover the activities of ammonia producers on the corrosion of Cupronickel 90:10 in a nitrogen-free water system with the presence of corrosion inhibitors like iron sulphate and molybdate.

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2. EXPERIMENTAL PROCEDURE

2.1. Sample collection

The cupronickel coupons 6 cm × 4 cm were immersed for a period of 6 months in samples obtained from a Chavara-rare earth environment which extend over 22 km from Neendakara to Kayankulam in Kollam district, in Kerala, India. The six months old biofilm samples were washed with sterilized water in order to remove pelagic bacteria from the biofilm. The biofilm samples were also scrapped from the cupronickel (90:10) metal surface with the help of a sterile surgical knife. The biofilm sample was collected in a sterile conical flask, stored in an ice box, and transported for microbiological analysis at CECRI- microbiological lab. The water sample was collected from the site in a sterilized 10 liter polythene container and transported to CECRI in order to carry out corrosion study in the laboratory. The chemical characteristics of Chavara water were analysed by the standard method [14].

2.2. Bacterial Isolation and Identification

The samples were serially diluted using 9ml of sterile distilled water. Total viable bacterial counts were enumerated by the standard pour plate method using the nitrogen-free medium [15]. The composition of nitrogen free medium used was as follows (g/l): K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.050; $CaCl_2.H_2O$, 0.1; $Na_2MoO_4.2H_2O$, 0.0010 and Glucose, 10. The bacterial population was expressed as colony forming units per cm^2 (CFU/cm^2). Morphologically dissimilar colonies were selected and isolated from nitrogen-free agar plates. The isolated colonies were purified using the appropriate medium from the streak plate method. Biochemical characterization of the isolates was carried out by the API biochemical test kit (Bio-Merieux, SA, France) and by standard tests.

2.2.1. 16S rRNA gene sequencing and phylogenetic analysis

The genomic DNA was isolated from the four isolates according to the procedure described by Murmur *et al.* [16] and the small subunit rRNA gene was amplified using the two primers 16S1 (5'-GAGTTTGATCCTGGCTCA-3') & 16S2 (5'- CGGCTACCTTGTTACGACTT-3'). The purified PCR product, approximately 1.5 Kb in length, was sequenced using five forward and one reverse primer as described earlier [17]. The deducted sequence was subjected to blast search for the closest match in the database. The 16S rRNA gene sequence of ammonia producing *Bacillus* sp. AG1-AG4 was submitted to the Gene bank. The pair wise evolutionary distances were computed using the DNA DIST program with the Kimura 2 parameter model [18]. The phylogenetic trees were constructed by using four tree making algorithms (the UPGMA, KITSCH, FITCH and DNAPARS) of the PHYLIP package [19]. The stability among the clads of the phylogenetic tree was assessed by taking 1000 replicates of the dataset analyzing them using the programs SEQBOOT, DNADIST, UPGMA and CONSENSE of the PHYLIP package.

2.3. Ammonia spot test and estimation

Bacterial isolates were tested for the production of ammonia in a nitrogen-free broth. 10ml nitrogen free broth in tubes were inoculated with a freshly grown culture and incubated for 48-72 hours at 28 °C. Nessler's reagent (0.5 ml) was added in each tube. The development of a white or a yellow colour indicated ammonia production [15]. The culture samples were withdrawn at different times, centrifuged, and filtered (through cellulose acetate membranes; pore size, 0.45 μm). An appropriate amount of supernatant or filtrate was tested for the presence of ammonia by the indophenols method [20]. The mixture was incubated for 30 min at room temperature. The absorption value was measured at 625 nm by UV-spectrophotometers and the ammonia concentrations were estimated.

2.4. Corrosion studies

2.4.1. Weight loss measurement

Cupronickel coupons of 5 × 1 cm size with a hole on the top were used for weight loss experiments. The coupons were machine polished to mirror finish, degreased with trichloroethylene, and rinsed with deionized water. Four systems were designed by using Chavara water. System I consisted of water alone. System II consisted of chavara water with nitrogen-free medium without bacteria. System III had chavara water and nitrogen-free medium along with bacteria AG1, whereas System IV consisted of chavara water and nitrogen-free medium with bacteria AG3. The pre–weighed coupons were immersed in Chavara water and in a nitrogen-free broth with and without bacteria. Three coupons were exposed in a conical flask and duplicate cells were made for each system. In total, six coupons were used to measure the weight loss for each system. The average weight loss and standard deviations (SD) were calculated. The broth was continuously replaced once every 3 days in order to make sure the biofilm remained alive. Nitrogen-free medium without bacteria acted as the control system. The corrosion rate was calculated by using the method recommended by NACE [21].

2.5. Electrochemical studies

2.5.1. Potential & Polarization measurements

Cupronickel coupons of 1 × 1 cm dimension with an extended stem of 15 cm length were used for potential and polarization studies. Specimens were polished to mirror finish by using emery paper 1/0 down to 4/5. The specimens were also finally degreased with trichloroethylene, followed by deionized water. Three specimens were immersed in separate 250 ml conical flasks that contained nitrogen free broth and were then sterilized. AG1 and AG3 were inoculated in a nitrogen-free medium and used as the experimental system while the uninoculted specimen was used as the control system. OCP values were measured for time by using a digital

multimeter of high resistance.

Polarization measurements were carried out potentiodynamically employing a model PGP 201, potentiostat with voltamaster -1- software. Cupronickel 90:10 coupon of size 1 cm^2 as working electrode a Saturated Calomel Electrode (SCE) as a reference electrode and a large platinum electrode were employed for polarization study. The system was allowed to attain a steady potential value for 10 min. The study state polarization was carried out from OCP to -200 mV SCE and +200 mV SCE from the OCP separately by using separate electrodes at a scan rate of 1800 mV/h. The polarization study was done on the 20th day of the immersion period.

2.5.2. Impedance studies

The electrodes of the same specification that were employed for the polarization studies were also used for the impedance studies. Impedance studies were carried out using a computer control EG & G system: M6310 with software M398. After a steady state was attained, an AC signal of 10 mV amplitude was applied and impedance values were measured for frequencies ranging from 0.1 Hz to 100 KHz. The values of R_t were obtained from the Nyquist plot. Impedance measurements were also taken on the 20th day of the immersion period.

2.6. Surface studies

2.6.1. X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM)-Energy Dispersive X-ray spectroscopy (EDX) studies

Cupronickel coupons of 5 × 1 cm^2 size were machine polished to mirror finish, degreased with trichloroethylene, washed with deionized water and dried. The coupons were immersed in a nitrogen-free broth with and without AG1 and AG3 for 20 days and the coupons were removed. The nature of the oxides formed on the metal surface was estimated by XRD and SEM-EDX. The specimen that was prepared was analyzed by X'pert PRO PAN and analyzed by the X-ray diffractometer with Syn Master 793 software in order to identify the corrosion product. The XRD pattern was recorded using the computer controlled XRD-system, JEOL, and Model: JPX-8030 with a C α K radiation (Ni filtered = 13418 A°) at the range of 40 kV, 20 A. The 'peak search' and 'search match' program built in software (syn master 7935) was used to identify the peak table and ultimately the identification of the XRD peak. The same specimens were examined at different magnifications [500 X, 1000 X and 2000 X] by the scanning electron microscope [Model – Hitachi – S 3000 H]. The elements were identified by the Energy dispersive X-ray spectroscopy (EDX) model: Naron system SIX (Thermo electron corporation).

2.6.2. Atomic force microscope (AFM)

The specimens were immersed in a nitrogen-free medium in the presence and absence of bacteria at 30 °C for 5 days. The specimens were removed on the 6th day and pickled. The corrosion product was removed and dried at room temperature, and then characterized by an atomic force microscopy, with a model Pico scan 2100 (Molecular Imaging, USA) that used gold coated SiN_3 cantilevers (force constant 3 n/W) in a 30 nm tip area.

3. RESULTS AND DISCUSSION

3.1. Chemical characteristics of Chavara water

The water quality data showed that Chavara water contained chloride and oxygen in the range of 9000 ppm and 4.2 ppm to 5.0 ppm respectively. The pH of the water from the field was 7.2 to 7.8 and the total hardness was 3000 ppm.

3.2. 16S rRNA gene based identification

The bacterial density of the six months old cupronickel biofilm was 2.13 × 10^5 CFU/cm^2. Ammonia producing *Bacillus* sp. were identified by a biochemical test and confirmed by the 16S rRNA gene sequence analysis. The standard biochemical analysis revealed that the isolates AG1 and AG3 were almost similar, except in two tests. The isolate AG1 was catalase negative while the AG3 was catalase positive. Moreover, AG3 produced a pigment, but AG1 did not. The isolate AG2 and AG4 had close similarities, except for arabinose and lactose sugar fermentation. The blast results, as well as the phylogenic analysis (Fig. 1), revealed that the *Bacillus* spp. AG1 (EU202683), AG2 (EU202684), AG3 (EU202685) and AG4 (EU202686) had 99 % sequence similarity with *Bacillus anthracis* (AY138382), *Bacillus cereus* (AE016877) and *Bacillus thuringensis* (AF155955). Since *Bacillus anthracis* and *B. cereus we*re phenotypically similar, with the exception of the presence of the PA gene in the plasmid, the PA gene specific PCR was used to differentiate the four cultures. The results were found to be negative for the PA specific primers. This clearly indicates that none of the four isolates were *B. anthracis.* So far, there has been no report available on the effects of ammonia production by *Bacillus* sp. and its impact on the corrosion behavior of cupronickel alloy 90:10. Gaylarde and Johnshon [22] reported that *Desulfovibirio* bacteria were capable of attacking freshly cleaned 90/10 alloys. Similar trends were noticed in the present study with *Bacillus* sp. AG1 and AG3. These bacteria were resistant to copper toxicity and were capable of attacking mirror polished copper coupons in the medium when ammonia was released by the release of ammonia. *Bacillus* sp. was reported as manganese oxidizers and dominating genus on copper coupons in Tuticorin seawater (India) by Palanichamy *et al.* [23]. They suggested that *Bacillus* spp. were resistant to copper toxicity by the formation of spores.

3.3. Ammonia spot test and estimation

The appearance of a yellow colour in the nitrogen-free medium inoculated with *Bacillus* sp. within 72 hours of

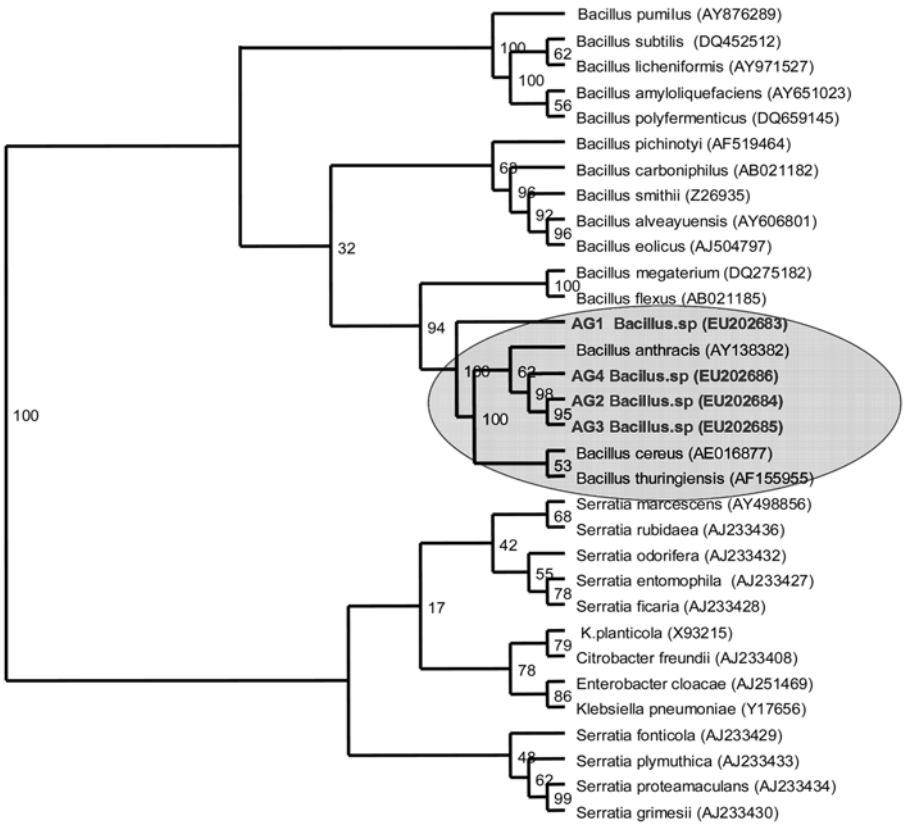

Fig. 1. UPGMA phenogram showing the phylogenetic position of Ammonia producing *Bacillus* strain AG1-AG4 based on 16s rRNA gene sequence analysis. Bootstrap values are given at the nods.

Table 1. Estimation of ammonia and pH in nitrogen free medium with and without bacteria

No	Bacterial strain	Concentration of ammonia (ppm) level	pH
1	CONTROL	0.00	7.2
2	AG1	4.6	8.4
3	AG2	2.5	8.2
4	AG3	8.0	8.5
5	AG4	5.0	8.4

incubation indicated the production of ammonia by *Bacillus* sp. Ammonia production by the bacteria would have resulted in a sudden increase of the medium's pH. The pH of the medium in the presence and absence of the bacteria were measured and presented in Table 1. The pH of the nitrogen-free medium in the absence of *Bacillus* sp. was 7.2.When bacteria were present, the quantity of ammonia produced was in the range of 2.5 ppm and 8.0 ppm and the pH was in the range of 8.2 and 8.5. This showed that ammonia production has caused an increase of the medium's pH. Ammonia concentration was estimated by Rao and Nair [3] in natural biofilms and they noticed that ammonia generation was caused by Nitrate Reducing Bacteria (NRB). The denitrifying bacteria observed during the course of this study were *Alcaligenes* sp., *Bacillus* sp., *Micrococcus* sp., *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. The estimated amount of ammonia in the natural biofilms was in the range of 0.5 and 5.5 ppm. The present result supports the observation made by Kanamori *et al.* [24], who suggested that *Bacillus* produced ammonia. Ahmad *et al.* [25] also identified *Bacillus* sp. from different rhizospheric soils and plant root nodules in the vicinity of Aligarh and found that 80 % of the isolates were ammonia producers.

3.4. Weight loss

The corrosion rate of cupronickel 90/10 in Chavara water (India) in the presence or absence of *Bacillus* sp. and nitrogen-free medium are presented in Table 2. The corrosion rate of cupronickel in Chavara water (Control I) was in the range of 0.046 mm/year and 0.052 mm/year. In Control System II (Chavara water with nitrogen free medium), the corrosion rate was 0.008 mm/year whereas in the presence of (AG1 and AG3) *Bacillus* sp., the corrosion rate was in the range of 0.023 mm/year and 0.030 mm/year. Harrison and Kennedy [26] reported that corrosion rates exceeding 1.0 mm/year were considered very high for copper alloys. It can therefore be concluded that the corrosion rate in this study was lower in the presence of *Bacillus* spp.

Table 2. Corrosion rate of cupronickel (90/10) in different systems

No	System	Immersion periods (day)	Average weight loss (mg)	Corrosion rate (mm/year)	Form of corrosion
	Control (System-I)				
1	500 ml of Chavara water immersion on cupronickel(90/10) coupon (5 cm × 1 cm)	10	11.18 ±.2	0.046	Uniform corrosion
2	500 ml of Chavara water immersion on cupronickel(90/10) coupon (5 cm × 1 cm)	18	23.13 ±.3	0.052	Uniform corrosion
	Control (System-II)				
3	500 ml of nitrogen free medium broth and cupronickel immersion without (bacteria)	20	4.1 ±.3	0.008	Slight uniform corrosion
	(System III)				
4	500 ml of nitrogen free medium broth and cupronickel immersion and inoculated with bacteria (AG1)	20	11.4 ±.3	0.023	Pitting corrosion
	(System IV)				
5	500 ml of nitrogen free medium broth and cupronickel immersion and inoculated with bacteria (AG3)	20	14.7 ±.2	0.030	Pitting corrosion

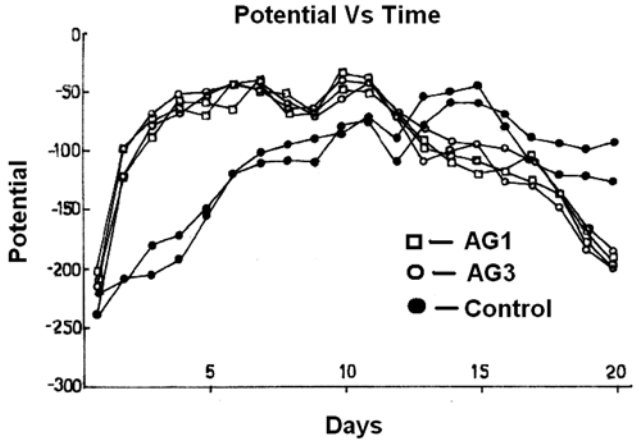

Fig. 2. Potential measurement for cupronickel 90:10 in the presence of *Bacillus sp* and control system.

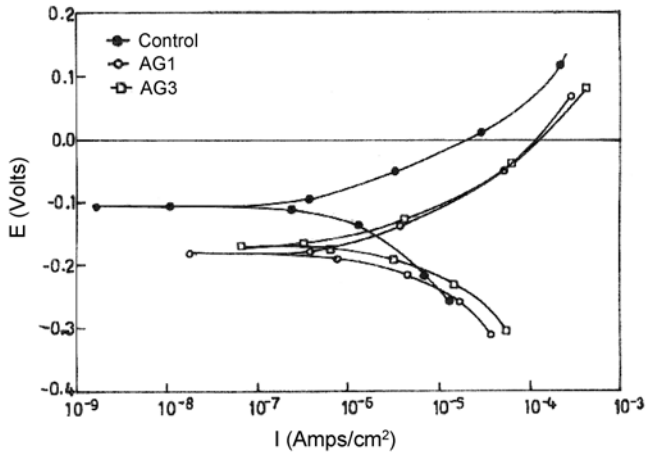

Fig. 3. Polarization studies for cupronickel in different systems of control and bacterial systems with nitrogen free medium.

3.5. Potential measurement

The potential measurement versus time in the presence and absence of *Bacillus* sp. are presented in Fig. 2. The initial potential was about −225 mv SCE in the presence of *Bacillus* sp. and it increased rapidly to –50 mv within 5 days. After the 11th day, the potential slowly shifted to the negative side of the range (–190 mV). But in the absence of *Bacillus* sp., the potential slowly shifted towards the positive side and reached about 50 mV on the 11th day. The potential maintained its position at the range of –100 mV on the 20th day. The shifting of potential to the negative side in the presence of *Bacillus* indicates that bacteria enhance corrosion when compared to the control.

3.6. Polarization

The polarization data for cupronickel (90/10) in the presence and absence of *Bacillus* spp. are presented in Fig. 3 and Table 3. In the control system (which had the presence of nitrogen-free medium), the icorr (Corrosion current) was 7.69×10^{-7} A/cm^2. When there was *Bacillus* spp. present in the nitrogen-free medium, the icorr value was in the range of 1.21×10^{-6} and 1.65×10^{-6} A/cm^2. The nature of the curve also indicates that *Bacillus* spp. enhance corrosion by enhancing the anodic reaction through ammonia production.

3.7. Impedance

Impedance spectroscopy data are presented in Table 4 and

Table 3. Polarization studies for cupronickel in different systems with and without bacteria

S.no	System	Ecorr (mv)	Ba (mv/decade)	Bc (mv/decade)	Icorr(Amp/cm^2)
1	Control	−100	70	−123	7.69×10^{-7}
2	AG1	−180	87	−72	1.21×10^{-6}
3	AG3	−106	80	−80	1.65×10^{-6}

Table 4. Impedance spectroscopy studies for cupronickel in different systems with and without bacteria

S.no	System	Rs ohms.cm^2	Rct k.ohms.cm^2	Rt k.ohms.cm^2	Cdl F/cm^2
	Control				
1	nitrogen free medium	133	33.06	32.93	4.98×10^{-4}
2	nitrogen free medium + AG1 strain	114	16.48	16.37	7.07×10^{-4}
3	nitrogen free medium + AG3 strain	102	18.01	19.02	6.08×10^{-4}

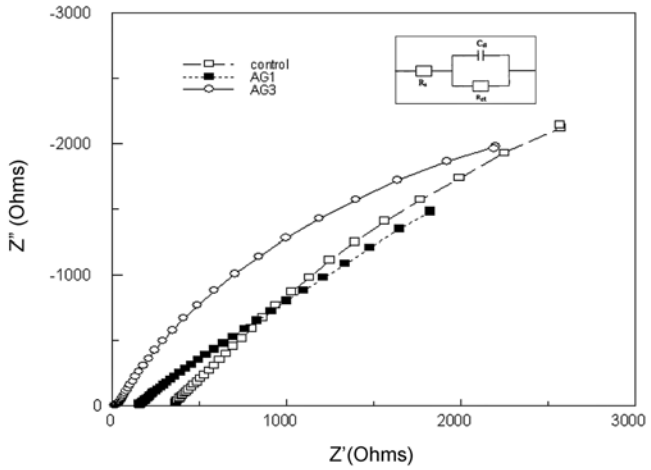

Fig. 4. Impedance spectroscopy studies for cupronickel in presence of *Bacillus* sp. and control system with nitrogen free medium. (R_s, solution resistance; R_{ct}, charge transfer resistance; C_{dl}, double layer capacitance).

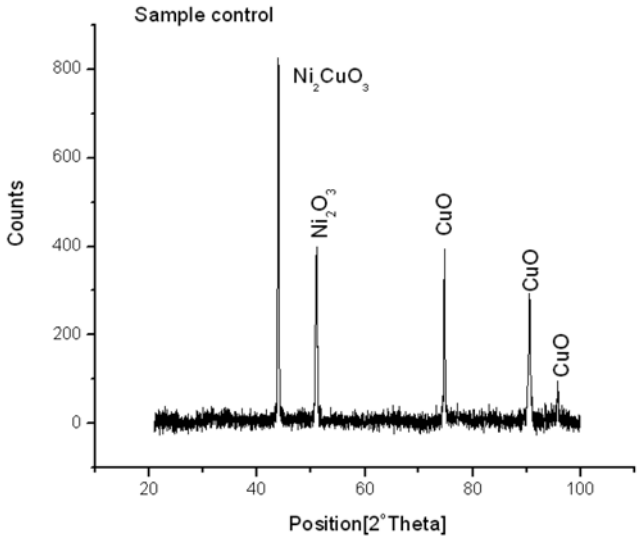

Fig. 5. XRD spectrum for cupronickel (90:10) in control system (nitrogen free medium).

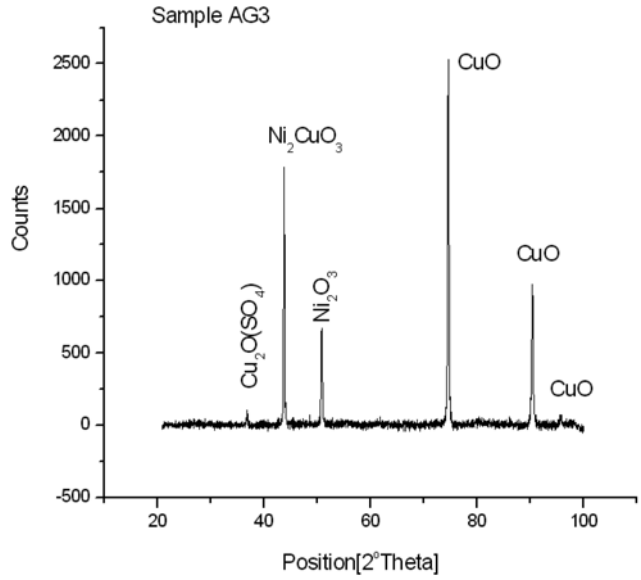

Fig. 6. XRD spectrum for cupronickel (90:10) in presence of *Bacillus* sp. (AG3) with nitrogen free medium.

Fig. 4. In the control system, the Rt value was 32.93 kΩ/cm^2, whereas in the bacterial system, the Rt value was in the range of 16.37 and 19.02 kΩ/cm^2. This indicates that corrosion is higher in low resistance systems. The nature of the curve in the *Bacillus* spp. system also indicates that the corrosion is due to activation control. Rs' value was lower in the presence of bacteria when compared to the absence of bacteria. This may be due to the dissolution of metal, which may then reduce the Rs of the electrolyte. The electrochemical impedance spectroscopy was used by Hashem [27] to study the effect of ammonia residuals on the corrosion of copper in seawater polluted with ammonia and it was suggested that ammonia enhances the attack by dissolving the complex with copper ion.

3.8. Surface analysis by X-ray diffractometer

The X-ray diffraction peak for corrosion products from the control and *Bacillus* sp. (sample) system are presented in Figs. 5, 6, and 7. Ni_2CuO_3, Ni_2O_3 and CuO were noticed in all the systems, whereas the maximum scale of intensity was in the range of 800 counts. Besides noticing the high intensity peaks in the presence of bacteria (maximum 2500 counts) in the *Bacillus* sp. system when compared to the uninoculated system, it was also interesting to note that the adsorption of the SO_4 complex also occurred in the presence of bacteria in the nitrogen free medium. Xiao *et al.* [28] noticed Cu_2O (Cuprite) as a major corrosion product when they exposed copper to drinking water environments for a period of six months. They suggested that Cu_2O oxidation to CuO increased with alkalinity which depended on time and pH.

It was also found in the present study that CuO is the major corrosion product at high pH electrolyte. Cassagne *et*

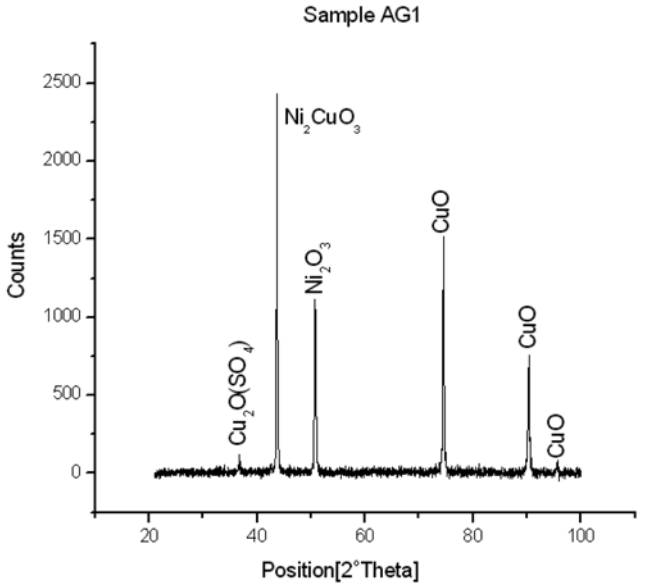

Fig. 7. XRD spectrum for cupronickel (90:10) in presence of *Bacillus* sp. AG1 with nitrogen free medium.

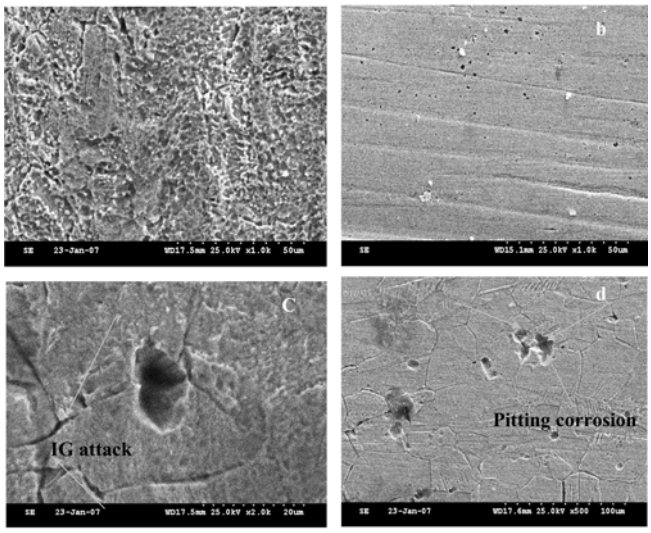

Fig. 8. Cupronickel metal surface analysis by Scanning Electron Microscope (SEM) for various systems: (a) Chavara water system, (b) Control (NFM), (c) Bacterial system (AG1), (d) Bacterial system (AG3).

al. [29] concluded from their detailed studies that cuprous oxide broke down when micropitting yielded to the initiation of transgranular SCC. Rao and Nair [3] noticed the cuproammonium complex and copper oxide as minor peaks on the admiralty brass. But in the present study, cupro ammonium complex was not noticed while $Cu_2O(SO_4)$ was noticed on the surface of the cupronickel 90:10 in the presence of bacteria. Since cuproammoniun complex is an unstable compound, it could not be detected in XRD.

3.9. Scanning Electron Microscopy

SEM results are presented in Figs. 8(a), (b), (c), and (d). Uniform corrosion was noticed in the Chavara water (Fig. 8(a)). In the absence of bacteria the in nitrogen-free medium, an uniform film was noticed on the cupronickel (Fig. 8(b)) while some holes on the figure indicated the heterogeneous adsorption of chemicals present in the nitrogen free medium. Figures 8(c) and (d) show the pitting of cupronickel, which was exposed to the nitrogen-free medium along with *Bacillus* spp. Moreover, etchings of grain boundaries were noticed, as shown in Fig. 8(d). It indicates that *Bacillus* spp. encourages pitting corrosion as well as intergranular attack. The present study also indicates that the adsorption of nutrients on cupronickel present in the medium results in the formation of film and is broken down by the bacteria.

3.10. EDX

Figures 9 and 10 show the spectrum received from EDX for cupronickel in the presence and absence of *Bacillus* sp. in nitrogen free medium. The percentage weight of copper, nickel, iron and oxygen were analyzed on the cupronickel and presented in Table 5. In the control system, 87.68 % of

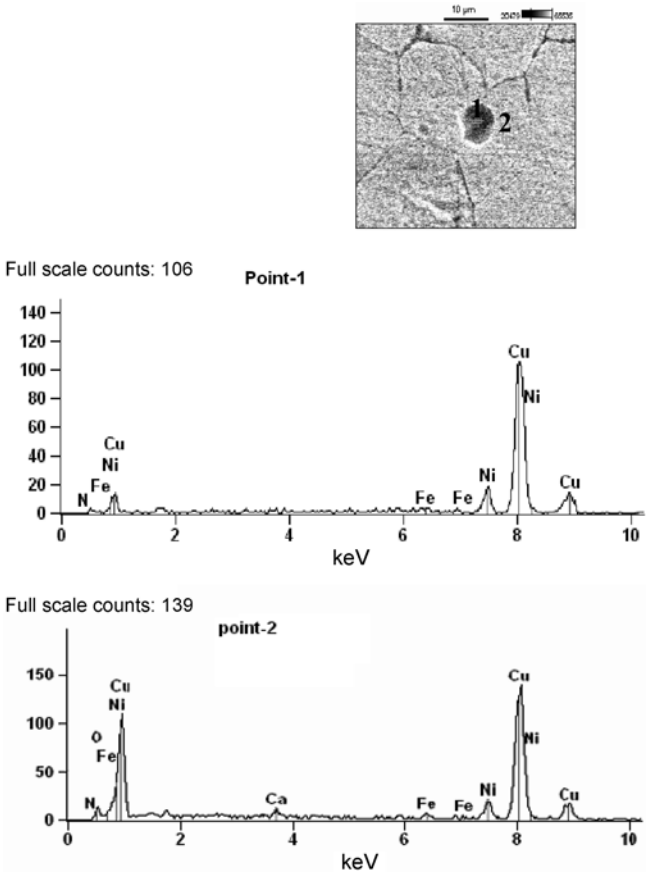

Fig. 9. Energy Dispersive X-ray Spectroscopy analysis for cupronickel in presence of *Bacillus* sp.

copper and 10.40 % of nickel were noticed, along with 2 % iron, where iron acts as a passivator. In the experimental system, the center of the pitted surface had 90.53 % of copper,

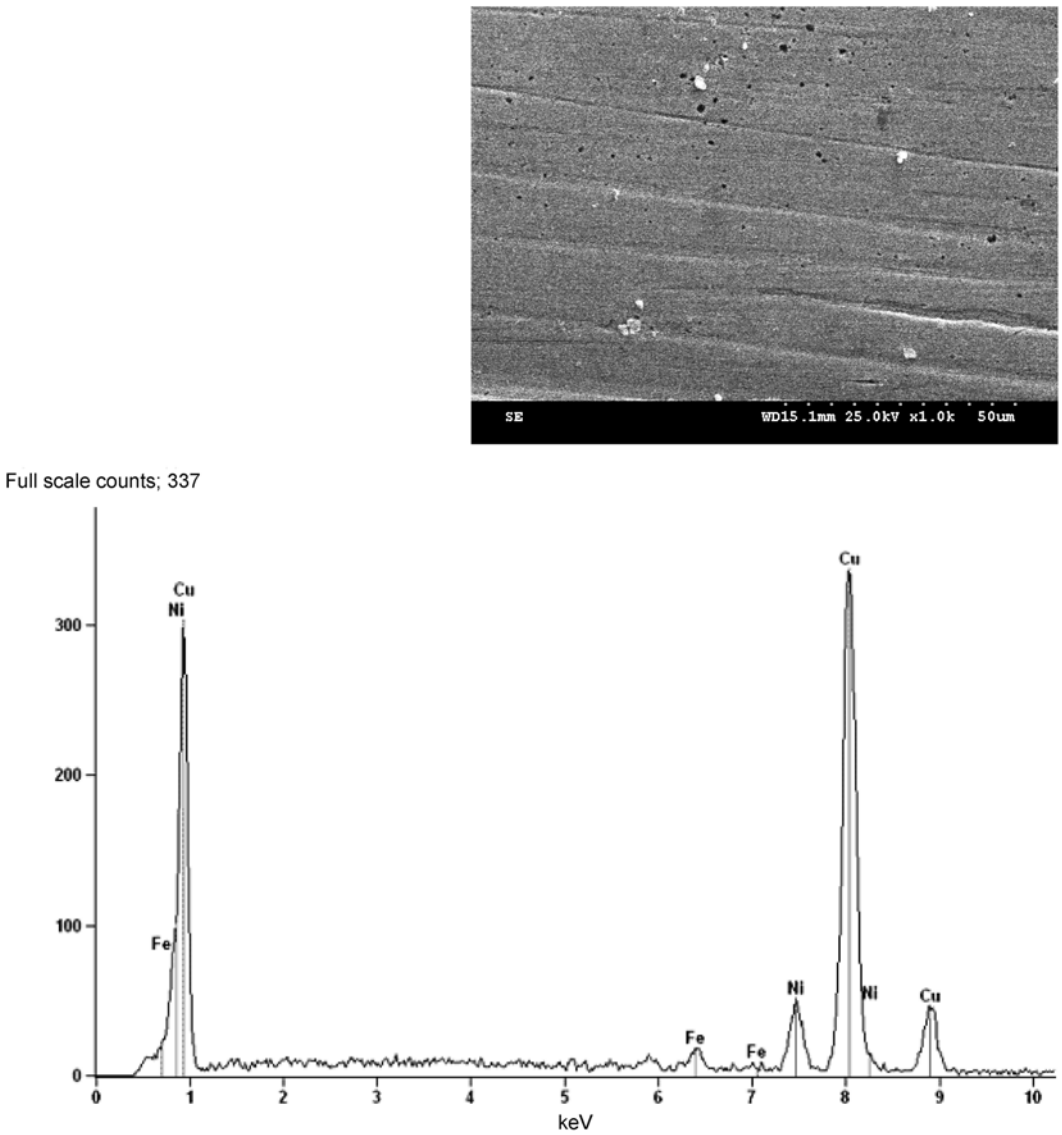

Fig. 10. Energy Dispersive X-ray Spectroscopy analysis for cupronickel (90:10) in control system (nitrogen free medium).

Table 5. Energy Dispersive X-ray Spectroscopy for cupro-nickel in presence/absence of Bacillus sp. system

No	Position	Copper%	Nickel%	Iron%	Oxygen%
	Control				
1	Whole area	87.68	10.40	2.00	0.00
2	Point-1	90.53	8.85	0.62	0.00
3	Point-2	76.96	8.60	0.19	8.96

8.85 % of nickel, and 0.62 % of iron. The iron content was lower on the pitted surface, indicating the initiation of pitting at the lower iron adsorbed area. At Point 2 (the edge of the pit), the iron and copper contents were 0.19 % and 79.96 % respectively, whereas oxygen was 8.96 %. This indicates that the edge consists of cuprous oxide on the metal surface. The reduction of iron on the cupronickel in the presence of *Bacillus* sp. may be due to the use of iron as a nutrient by *Bacillus* sp., which is a cofactor for nitrogenase enzyme [12]. It can also be inferred that the oxide film was broken down by biogenic ammonia which resulted in pitting.

3.11. Atomic force microscope

Atomic force microscopic observations were done for cupronickel 90:10 in the presence and absence of bacteria and are presented in Figs. 11(a) and (d). The adsorption of nutrients present in the nitrogen-free medium can be seen in the control system. The film was heterogonous in nature (Figs. 11(a) and (b)). This indicates the presence of inhibitors, like iron sulphate and molybdenum, adsorbed on the metal surface. In the bacterial system, pitting and inter granular crack could be noticed. The depth of the largest pit was 1000 nm (Fig. 11(d)).

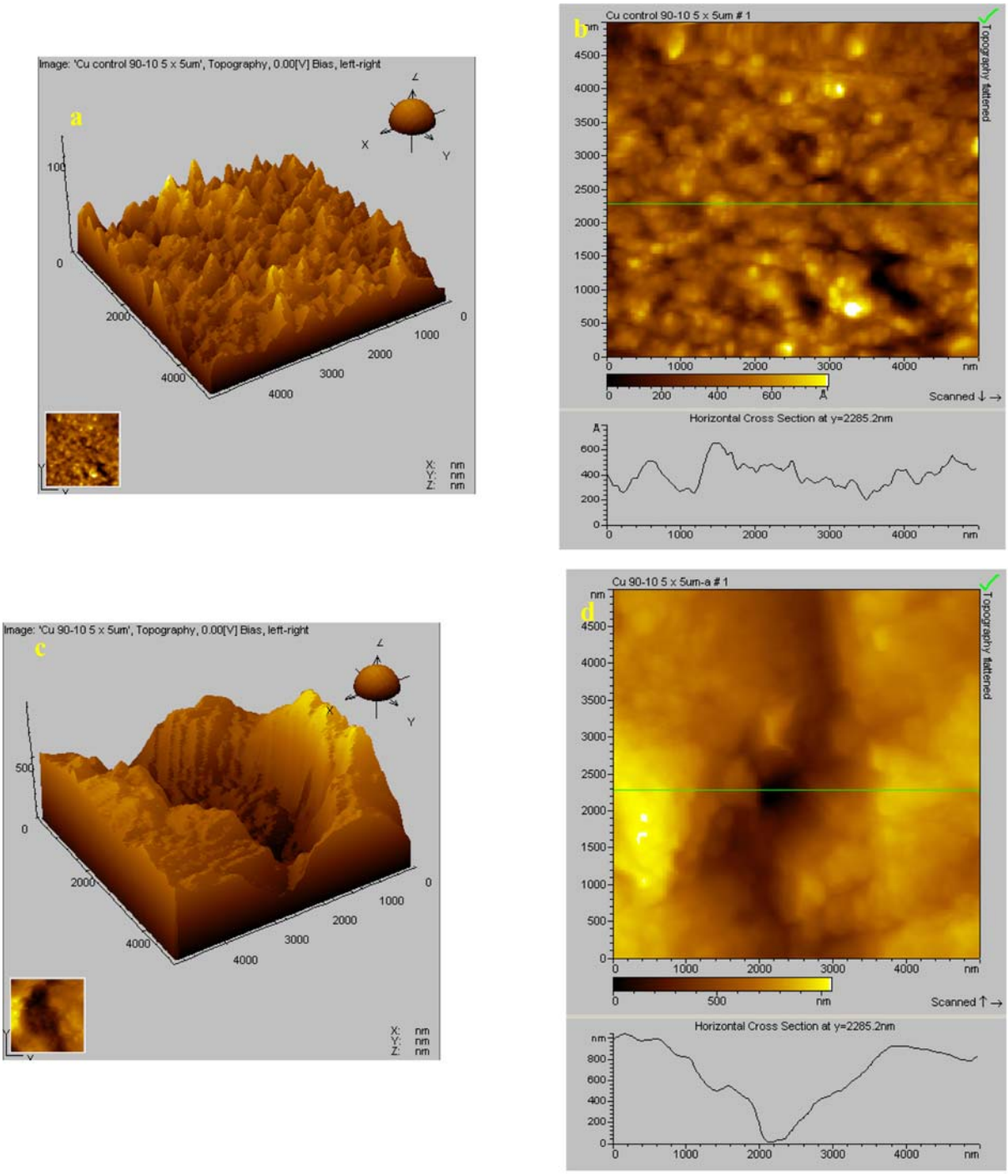

Fig. 11. AFM (Atomic Force Microscope) analysis for cupronickel in different systems: (a) Control (nitrogen free medium)-Topography, (b) Control-Horizontal cross section, (c) Sample system (Nitrogen free medium with bacteria)-Topography, and (d) Sample system-Horizontal cross section.

In the present study, it is assumed that nitrogenase enzyme is the most important factor in nitrogen fixation [30] from atmospheric di-nitrogen with available iron and molybdenum that act as cofactors [12,31]. When *Bacillus* spp. produces ammonia, it is volatilized within a short time with an increase in pH. The ammonical solution formed in the presence of *Bacillus* sp. acts as an etchant. Since nitrogen-free medium contains sulphate, it can be assumed that the ammonical solution may enhance the production of ammonium persulphate, which is a good etchant for copper alloys [21]. The analysis of ammonia and pH from the 20 days confirms the continuous formation of etchant solution in the presence of

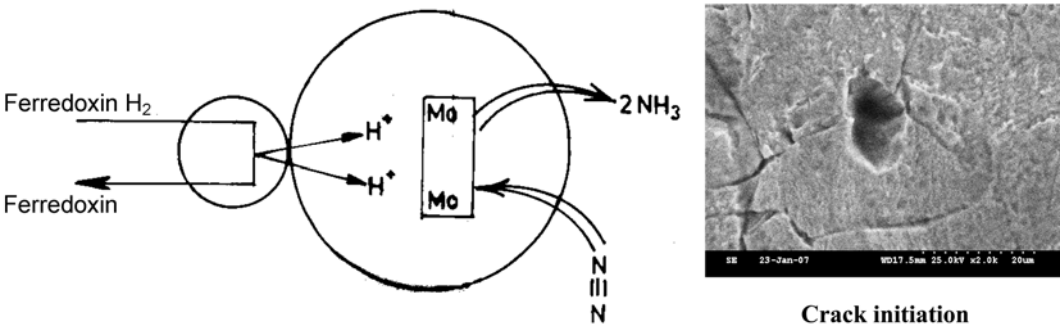

Fig. 12. Mechanism of MIC on Cupronickel (90:10) in the presence of *Bacillus* sp.

Bacillus sp. EDX analysis indicates the presence of oxide films on the edges of the pit and on the fracture surface. This might be due to the presence of CuO that was noticed as major peaks in XRD. Venugopal and Rawat [9] reported the profile of cracks observed on the inner side of the admiralty brass condenser tube in a nuclear cooling water system. Both circumferential and longitudinal cracks were observed on the external surface of the failed tubes and pits were observed close to the cracked regions. The nature of the crack indicated that admiralty brass tubes had failed due to SCC. But in the present study, an inter-granular crack was noticed. This was due to the biogenic ammonical action [32] of *Bacillus* sp. After ammonia formation, the following possible reaction took place [33].

$$NH_3 + H_2O \rightarrow NH_4^+ + OH^-$$
$$Cu + 2OH^- \rightarrow Cu(OH)_2 + 2e\text{-} \text{ (oxidation process)}$$
$$H_2O + 1/2O_2 + 2e\text{-} \rightarrow 2OH^-$$
$$Cu(OH)_2 + 2NH_3 + 2NH_4^+ \rightarrow [Cu(NH_3)_4]^{2+} + 2H_2O$$

The enhancement of anodic current in the polarization study can be explained by the fact that the corrosion on cupronickel was due to the oxidation of $Cu(OH)_2$. Besides, The inter granular attack was also formed due to the over saturation of the $[Cu(NH_3)_4]^{2+}$ ions [33,34].

$$Cu[(NH_3)_4]^2 + H_2O \rightarrow CuO + 2NH_3 + 2NH_4^+$$

The formation of CuO at the metal surface is a critical step needed for the occurrence of Stress Corrosion Cracking (SCC) which was noticed on XRD. This supports the observation made by Mori et al. [33].

Nitrogenase enzyme is the major causative factor for the production of ammonia [12]. Nitrogenase works at room temperature where nitrogen gas requires reduced ferredoxin or flavodoxin and ATP as substrates. Reduced ferredoxin or flavodoxin transfers electrons to the azoferredoxin. At the expense of energy being lost from ATP hydrolysis, the potential of redox groups of the enzyme is lowered further. Finally, a super-reduced molybdoferredoxin is formed, which binds N_2 and reduces it stepwise to ammonia. The binding of the dinitrogen molecule occurs when it is inserted into a metal-hydride bond that has molybdenum [35]. A MO=N-NH_2 group possibly functions as an intermediate. Only the azoferredoxin component of nitrogenase has ATP-binding sites and ATP hydrolysis is primarily associated with the formation of a super-reductant. Using cell-free nitrogenase preparation, the reduction of N_2 has been found to be coupled to the hydrolysis of an enormous amount of ATP, in the order of 16 ATP per N_2.

$$N_2 + 6H + 16\ ATP \rightarrow 2NH_3 + 16\ ADP + 16\ Pi$$

It is also well known that iron sulphate is a good passivator for cupronickel, which adsorbs on the metal surface and improves passivity. Some anodic spots on the metal surface will be affected by ammonia and undergo pitting corrosion. Subsequently the ammonical solution enhances the attack at the grain boundaries and accelerates intergranular corrosion without any stress. The mechanism has been explained in Fig. 12. It concludes that *Bacillus* sp. may encourage intergranular attack without any stress on the cooling water system. When the alloys stressed in tension are also exposed to a corrosive environment, the ensuring localized electrochemical dissolution of metal, combined with localized plastic deformation, opens up a crack [36]. Protective films that form at the tip of the crack rupture, causing fresh anodic material to be exposed to the corrosive medium and SCC, is then propagated [34].

4. CONCLUSIONS

Cupronickel is a very good engineering alloy that can be used as heat exchanger tubes in seawater and freshwater cooling systems. If the environment does not contain nitrogen, *Bacillus* sp. will fix nitrogen from the atmosphere and enhances the production of ammonia in a cooling water system. The ammonia produdced is prone to Inter Granular Corrosion (IGC) by *Bacillus* sp. in a nitrogen-free environment. Hence, the addition of iron sulphates and molybdates

as corrosion inhibitors in cooling water systems is questionable. The present study has created an awareness on the impact of corrosion inhibitors on microbiologically influenced corrosion of copper alloys in cooling water systems.

ACKNOWLEDGMENTS

The authors wish to thank Mr. R. Ravishanker, Mr. A. Rathiskumar and Miss S. Krithika for their assistance in the utilization of the facilities SEM, AFM and XRD in the Instrumentation division, CECRI, in India respectively. We also thank Dr.Thahira Rahman, Newcastle University, UK for her suggestions and advice. Authors thank BRNS (DAE) for sponsoring a project (GAP26/05) entitled "Evaluation of Engineering materials in rare earth environment with special reference to biofouling research". We also thank Dr. V. P. Venugopalan and Dr. T. S. Rao, Biofouling and Biofilm Processes Section, Baba Atomic Research Centre, Kalpakkam, India for their valuable discussions on this work.

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