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Bacterial Attachment on Magnesium Alloy AZ31B with a Note on its Antibacterial Property

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Abstract

Magnesium (Mg) alloys attract the focus of leading industries such as telecom, computer etc, and medical and household appliances due to their strength and weightlessness. However, magnesium is one of the major cell components of bacteria, which predisposes it to microbial attachment and growth. To understand its vulnerability to bacterial attachment that leads to microbiologically influenced corrosion (MIC), laboratory exposure studies using a magnesium alloy AZ31 B were carried out with a biofilm forming bacteria Pseudomonas sp. A high rate of bacterial attachment (calculated using image processing software) viz. 14.33±6.72% and 33.69±10.25%, was observed for one and two days of exposure, respectively. However, the area of bacterial adhesion reduced to 2.71±1.36% by the sixth day. The total viable count (TVC) on the surface of the experimental coupons also showed the maximum value on the first day, and reduced to a very low number by the sixth day. Mg ion concentration in the experimental medium showed a gradual increase over the time period. This was true with the control as well. The maximum concentrations were 25.5 and 23.5 mg/ml for control and experimental samples respectively by the sixth day. Consequently, the medium pH also increased to 9.7 and 9.8 in the control and experimental media respectively. The coupon surface pH also rose to more than 11 by the third day. Magnesium reacts with water producing magnesium hydroxide, which forms a protective film over the coupon surface in addition to the dissolution into the medium. The formation of such a film and the dissolution of Mg elevated the pH of the coupon surface and the medium to the alkaline range. The combined effect of high pH and high concentration of magnesium ion adversely affected the growth and survival of Pseudomonas sp. both in the medium and on the coupon surface resulting in a modest attachment and very low TVC by the sixth day. Thus, Magnesium alloys exhibited antibacterial property that prevented bacterial attachment and formation of biofilm.

KEY WORDS: (Bacterial adhesion) (Magnesium alloy) (pH) (Magnesium ions) (Antibacterial effect) (*Pseudomonas* sp.)

1. Introduction

Magnesium (Mg) and its alloys are widely used in modern industries especially to manufacture light cellular telephones, computer parts, medical and paramedical devices etc. Its relative abundance in seawater and the low density and high strength/weight ratio of its alloys are the promising properties reckoning them as alternative material for aluminum alloys[1,2,3]. However, corrosion susceptibility restricts their application[1]. On that account, contemporary research is largely focused on the electrochemistry of magnesium and its alloys while in contact with an aqueous medium and the ways to improve their corrosion resistance[1-6,2,7].

While magnesium and its alloys attracted the attention of electrochemists, microbiologists and nutritionists had a different side of the story to tell ie about its essentiality for life. Magnesium is the molecular key that activates many important enzymes responsible for many biochemical reactions in a living cell[8,9]. Mg is regarded as one of the intracellular bulk elements[10] and is involved in enzyme catalysis, which performs a variety of roles such as structure stabilization, charge neutralization, and control of osmotic pressure.

Attachment of bacteria on material surface may lead to microbiologically influenced corrosion[11,12]. This has been the focus of major research activities in the

recent past[13-17]. Mg is used as sacrificial anodes to protect the underlying metal from corrosion and is thus significant in the field of corrosion protection. In addition, as this metal corrodes easily while in contact with water, it might act as an additional nutrient released into the environment. While acting as sacrificial anodes or in use for other applications, bacterial attachment and MIC could be a possible threat for Mg deterioration. In spite of this significance, reports addressing the bacterial colonization of Mg alloys are lacking. Therefore, it was thought appropriate to study the metal microbe interaction occurring at the surface of magnesium alloys, which would enable us to formulate a suitable strategy for their better utilization especially in aquatic and humid environments.

2. Materials and Methods
2.1 Material tested

The elemental composition of AZ31B, the magnesium alloy used in the experiment is given in Table 1.

Table 1 Chemical element composition (%) of Magnesium alloy used in the study (Rem. = remaining portion)

Alloy	AZ31B
Al	3.0
Mn	0.46
Zn	1.0
Si	<0.01
Cu	<0.01
Ni	0.001
Fe	0.003
Ca	<0.01
Mg	Rem.
Others total	0.30

Coupons of size 30x10x1.5 mm were cut and polished with emery papers to a surface finish of 1200-grit. They were degreased, washed, dried and stored in a desiccator until the experiment.

2.2 Bacteria tested

A biofilm forming bacteria *Pseudomonas* sp. isolated from a corrosive ground water environment in Japan was used as the test strain. This strain was reported to be a prolific colonizer on stainless steel surfaces causing severe pitting corrosion[18,19]. The bacteria were sub-cultured twice repeatedly in Nutrient Broth (Difco: Bacto peptone 5 g l^{-1} and Bacto beef extract 3 g l^{-1}, distilled water 1 l) and the log phase (14-16h) culture was used for the experiment.

2.3 Experimental procedure

Bacterial adhesion on these coupons was observed for six days. The medium used was a dilute Nutrient Broth (0.1% v/v in micro filtered distilled water, Cl$^-$ concentration < 0.1 ppm). Twelve flasks with 200 ml of the experimental medium and four flasks with 200 ml of distilled water were taken and sterilized. One ml each of inoculums was added to 8 flasks containing 0.1%v/v nutrient broth. The rest were kept as sterile controls to study the effect of medium (medium control) and distilled water (distilled water control) on Mg coupons. Coupons were degreased, sterilized and were dried under UV inside a laminar flow chamber before introducing them aseptically into the experimental flaks. The flasks were kept in an incubator shaker at 28°C with a shaking rate of 90 rpm. Coupons were retrieved after 1,2,3 and 6 days of incubation. The experiment was not continued further because, the number of bacteria in the medium with coupons reduced considerably by this time that the experiment could not be continued. The whole experiment was repeated three times.

2.3.1 Coupon analysis

One set of the coupons retrieved from the experimental flasks were lightly rinsed with sterile distilled water, air dried inside a sterile chamber and were stained with acridine orange, a fluorescent nucleic acid dye (0.01% w/v in sterile distilled water). The coupons were observed under an Epifluorescence microscope (excitation 490nm and emission 530nm). Fifteen fields on each coupon were observed and the images were recorded using a CCD camera. These images were analyzed for the area of bacterial adhesion using image-processing software. Area of adhesion is expressed as percentage of the area of field of observation.

Another set of coupons (both experimental and controls) was observed under a Scanning Electron Microscope (SEM). Experimental coupons were kept overnight in gluteraldehyde at 4°C for fixation of biofilm. They were dehydrated using gradient concentrations of ethyl alcohol, freeze-dried and kept in a dessicator until observation. The surface preparation was done by gold palladium coating. Control coupons were observed as such without any surface preparation.

2.3.2 Total viable count (TVC) in the medium

The TVC in the medium was determined by standard plate count method. This was simultaneously done with the coupon retrieval.

2.3.4 Estimation of biofilm TVC and surface pH

A separate set of experiment was conducted to determine the total viable count of biofilm formed on the Mg coupons over the period of time viz. 1,2,3 and 6days. Eight flasks with 200ml of 0.1% (v/v) nutrient broth each were inoculated with *Pseudomonas* sp. Mg coupons were introduced and two coupons each were retrieved

after the intervals mentioned above. The biofilm formed on the coupon surfaces was scrapped out with a sterile nylon brush using 5ml sterile distilled water. The TVC was estimated by standard plate count method. The coupon surface pH was estimated using pH papers. Sterile medium with coupons served as the control.

2.3.5 Estimation of total Mg^{2+} concentration

Total magnesium ion concentration in the experimental medium, medium control and distilled water control were estimated during each sampling. 50 ml of the liquid medium from the test flasks was drawn and the concentration of Mg^{2+} was estimated using Inductively Coupled Plasma Mass Spectrometry (ICPMS). The total magnesium ion concentration was expressed as ppm.

2.3.6. Variation of medium pH

The pH of the medium was measured using a portable pH meter (accuracy + 0.1, Horiba, Japan). The pH of distilled water control and medium control was measured along with the experimental samples during 1,2,3, and 6 days of samplings. The pH of the medium inoculated with *Pseudomonas* sp. devoid of Mg coupons also was measured for comparison.

2.3.7 Effect of pH on growth of *Pseudomonas* sp.

In another set of experiments, media with different pH varying from 7-10 were prepared and the growth of *Pseudomonas* sp. was monitored for six days. The pH of the culture medium was adjusted using 0.1N NaOH (controlling the quantity of HCl for fine adjustment to traces so as the chloride ions do not interfere with the bacterial growth). The total viable count in the medium was determined by plating on nutrient agar plates after 1, 2, 3 and 6 days.

2.3.8 Statistical analysis

The results were analyzed using simple regression analysis and one-way ANOVA. The area of adhesion data was arcsine transformed prior to the analysis of variance and the homogeneity of the variance was tested using Cocheran's test for homogeneity[20]. The hypothesis tested for the ANOVA was that the bacterial attachment did not vary over the study period.

3 Results
3.1 Bacterial adhesion and total viable count

The adhesion of bacteria increased distinctly during the initial period of the experiment. By the second day, the % area of adhesion reached 33.69+10.25, henceforth the attachment rapidly reduced to reach a minimum value of 2.71+1.36 by the 6th day (Figures 1 & 2). This however showed similarity with the fluctuation of the total viable count of bacteria in the experimental medium (Figure 3). The TVC in the medium without coupons fluctuated between 10^{7-8}/ml during the experimental period. The results of the analysis of variance showed a

significant variation in the % area cover with time (ANOVA, F=11.624, p=0.0002)
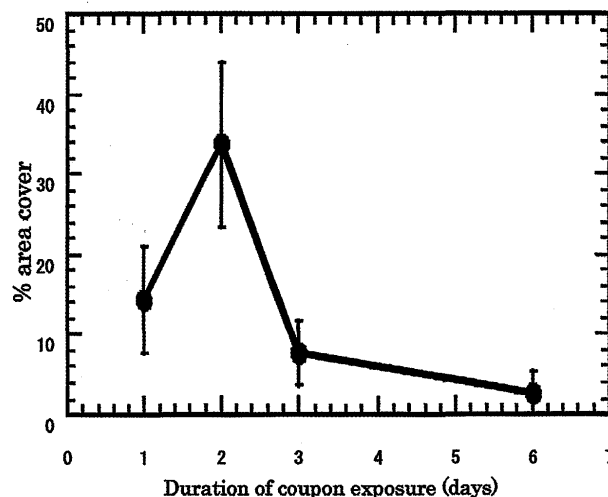

Fig.1 Variation in % area cover of bacterial adhesion on experimental coupons during the study period.
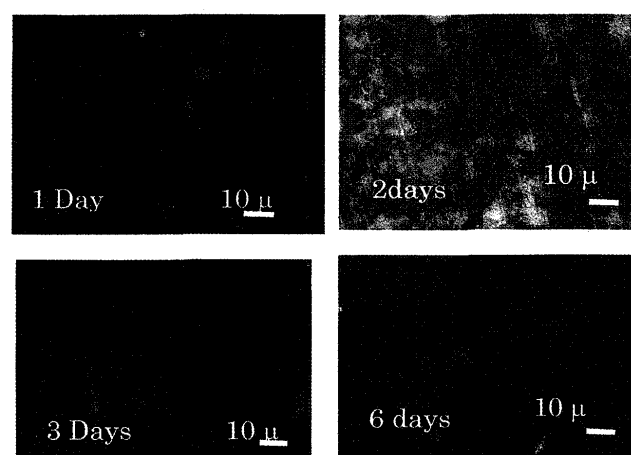

Fig.2 Epifluorescence images of magnesium alloy coupons with attached *Pseudomonas* sp. during the experiment.
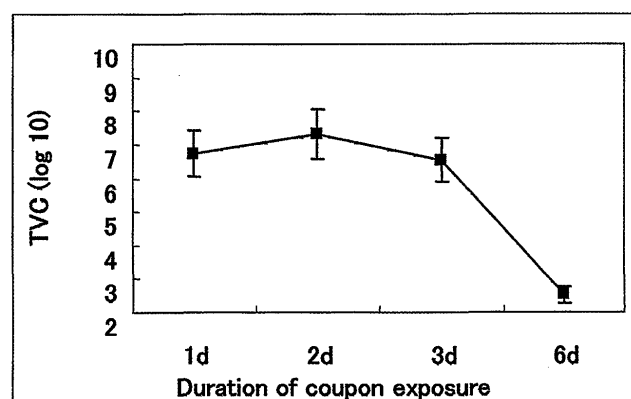

Fig.3 Variation in the TVC of *Pseudomonas* sp. in the experimental medium with magnesium alloy coupons during the period of study.

The biofilm TVC showed a rapid decline from the 2nd day. It varied from 2.6×10^5 cells/cm^2 to 8.3 cells/cm^2 from day 1 to day 6 following a more or less similar pattern as that of the medium TVC (Figure 4).
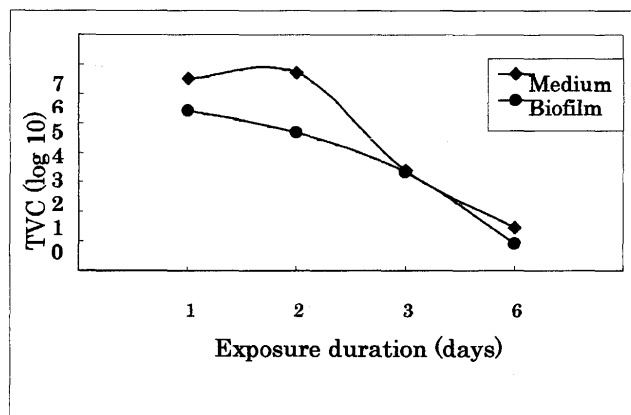

Fig 4 Variation in the medium and biofilm TVC observed on coupon surface during the study period

3.2 Magnesium ion concentration in the medium

The total magnesium ion concentration in the experimental medium with and with out *Pseudomonas* sp. is shown in the Figure 5.
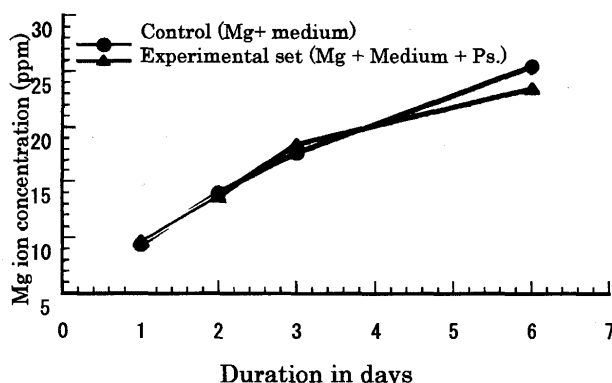

Fig.5 Variation in total magnesium ion concentration in the medium containing coupon but with and without bacteria during the study period.

The total magnesium ion concentration increased with time to reach a maximum value of 23.5 and 25.5 μg/ml in the experimental medium and the control, respectively. The result of t-test showed no significant difference between the final concentrations of Mg ions in the medium with and without bacteria (p=0.15), thus showing no influence of bacteria in the release of Mg ions from the coupons.

3.3 Variation in pH

Variation of medium pH in the experimental and control

flasks is given in Figure 6. The medium pH turned alkaline within a period of one day in flasks with coupons. It varied from an initial value of 6.9 to 9.7 and 9.8 on the 6th day in flasks without and with bacteria, respectively. However, the pH of medium alone, medium with bacteria and that of distilled water did not vary during this period (Figure 6).
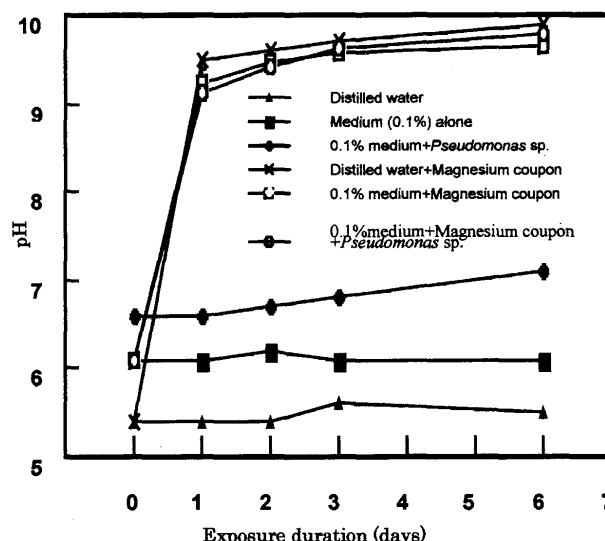

Fig.6 pH variation in the medium with bacteria and coupon, medium and coupon, medium with bacteria, medium alone, distilled water, and distilled water and coupon during the experiment.

The measurement of coupon surface pH showed a rapid increase with in a day of exposure. The coupon surface pH was 8 and 11.6 immediately after immersion and after a period of 6days, respectively (Figure 7).
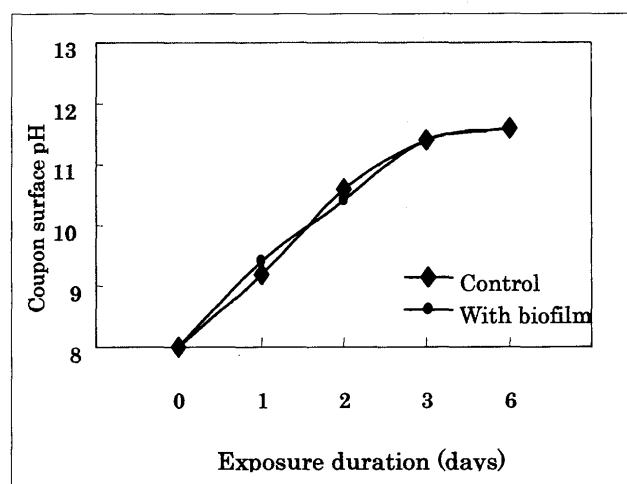

Fig.7 Variation in Coupon surface pH observed during the period of study.

3.4 Effect of pH on bacterial viability

The experiment on the viability of bacteria vis-à-vis pH showed a negative impact. The number of bacteria reduced with the increase in medium pH. As compared to pH 7 and 8, the TVC showed a slight reduction in medium with pH 9 while the impact was severe at pH 10. At pH 10, the bacteria showed nearly 100% mortality by the 6th day (Figure 8).
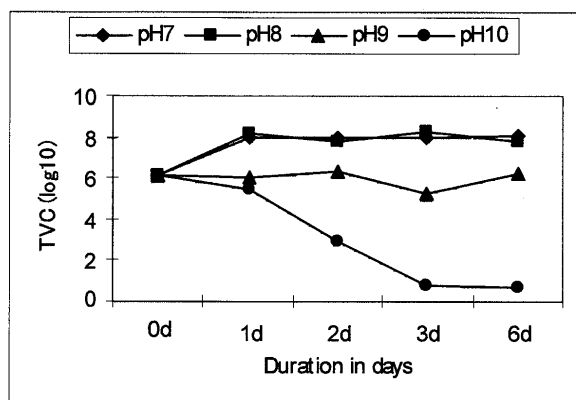

Fig.8 Variation in total viable count of *Pseudomonas* sp. inoculated to medium with different pH during the period of study.

4 Discussion

Magnesium and its alloys are reported to be highly unstable while in contact with water. On contact with water, magnesium starts reacting with it forming Mg $(OH)_2$ and H_2. The overall corrosion reaction of pure Mg as given in Song et al (1997)[2] could be represented as:

$$Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2 \text{ ------------- (1)}$$
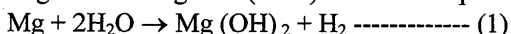
The anodic and cathodic reactions could be written as:

$$Mg \rightarrow Mg^{2+} + 2e \qquad \text{---------------- (2) and,}$$
$$2H_2O + 2e \rightarrow H_2 + 2OH^- \qquad \text{---------------- (3)}$$
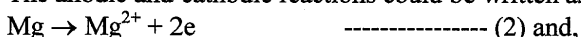
The protective film of magnesium hydroxide is formed according to the following reaction:

$$Mg^{2+} + 2OH^- \rightarrow Mg(OH)_2 \qquad \text{---------------- (4)}$$
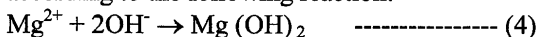
As the metal corrodes, the evolved Mg (OH) $_2$ (also MgO) start depositing over the surface to form a passive film. However, a part of it also dissolves into the liquid there by increasing the concentration of magnesium ions and the pH of the medium. In the present study, the total Mg^{2+} concentration of the medium increased from near zero in the initial day to about 25ppm by the 6th day. According to the literature, the corrosion processes of Mg and its alloys in contact with water are highly complex[3].

As far as bacteria are concerned, Mg^{2+} is one of the major elements of their cellular composition. The intracellular requirement of microorgainsms for Mg $^{2+}$ is about 10-20μM. It is bound to ATP, ADP and nucleic acids while a substantial amount is bound to ribosome. They are involved in stabilizing the structures of nucleic

acids and also to activate enzymes that are concerned with the synthesis of DNA, RNA, and proteins[8]. Its weight percentage in bacterial cell is about 0.5 and is present as inorganic magnesium salts[8]. It is highly significant for all living cells as it acts as cofactors for various enzymatic reactions[10]. Although magnesium salts have to be supplied externally, too much concentration of it would adversely affect the survival of bacterial cells because it is one of the major cell components that help in regulating the osmotic pressure[10]. In the present study, the concentration of magnesium ion was above 12-13 *ppm* by the second day, which went up to 25 *ppm* by the 6th day. Also the pH of the medium increased to above 9 by the second day. Consequently, TVC in the medium as well as the area of bacterial adhesion on coupons suffered a reduction from the 2nd day.

The *Pseudomonas* sp. tested in this experiment showed a high rate of settlement in stainless steel (SS) coupons[21]. A comparison of the area of bacterial adhesion between SS 304 L and magnesium alloys showed that these bacteria settled on magnesium alloy more rapidly than on SS 304 L during the initial period of coupon exposure[21]. This species was reported to colonize about 10% area of the field of observation on stainless steel in two days of exposure while on magnesium coupons during the period it showed nearly 34% coverage[21]. In both the experiments, TVC in the medium was in the similar range that is varying between 10^7-10^8/ml during the initial period of coupon exposure[21]. However, the area cover constantly increased in case of SS 304 L coupons, while a reduction of the same could be seen on magnesium alloys. On Mg coupons, the initially attached bacteria got detached probably due to the high level of pH acting at the liquid-substratum interface and H_2 evolution. This is evident from the biofilm TVC data as well, which also showed a reduction from the day 2. In spite of the necessity of magnesium for bacterial growth, which is evident from the sudden spurt in attachment during the initial stage, they could not sustain their growth on the coupon surface for long. Bacterial death might have occurred due to two major reasons. One, excess magnesium ion concentration in the medium, which would have crossed the tolerance limit of these bacteria thereby causing damage to the enzymes and cellular metabolism and crossed the osmotic barrier resulting in their death. As the second possibility, as the concentration of magnesium increased in the medium, the pH also increased and crossed the threshold limit of *Pseudomonas* sp. resulting in their death. In order to find out the effect of pH on the bacterial viability, another set of experiment was carried out.

The experiment on the variation of medium pH showed these bacteria could survive very well up to pH around 8 while the growth reduced at pH 9 and at around pH 10 it showed 100% mortality by the 6th day (Figure 8). This suggests that the bacterial mortality be mainly due to the increase in pH. The tolerance limit of *Pseudomonas* sp. with respect to pH is reported to be around 9[9]. As for the

possibility of Cl⁻ ions influencing the bacterial mortality in the medium, it is thought to be minimum. This is because the Cl⁻ ion concentration was very low in the experimental medium (<0.1ppm) and the volume of HCl (0.1N) used for the fine adjustment of pH was controlled to traces so that it did not interfere with the bacterial growth.

Magnesium and Zinc are widely used as sacrificial anodes for corrosion protection of metallic structures[22]. The vulnerability of magnesium and their alloys to corrosion while in contact with water is utilized here to preserve the underlying structures[6]. The use of magnesium as sacrificial anode is promoted since the dissolved magnesium ion in water is not toxic and the Mg ions are considered as an important cell constituent essential for the microbial growth[9]. However, the present study showed that, since the pH of the surface of magnesium is highly alkaline, reaching around 11 by the 6th day due to the formation of passive $Mg(OH)_2$ film (equations from 1-4), its surface would be protected from the biofilm build up for a longer period than most of the other metallic surfaces do. This property at the boundary layer between metal surface and water makes Mg antibacterial. With this property, the surfaces of its alloys would be bacteria free even when it is used in a highly humid environment, a good sign for many industries that use Mg alloys for making components such as mobile cell phones, computer parts or medical and paramedical devices. However, our experiment is only a laboratory test, but proves a point that this alloy has the property to resist bacterial build up. A more realistic picture could be obtained and the present pattern could be verified by exposing coupons in the real field to study the biofilm buildup on their surfaces.

5 Conclusions

The bacterial adhesion studies on Magnesium alloy AZ31B using Pseudomonas sp. showed an increase in adhesion area up to two days with a sharp reduction thereafter. The TVC in the experimental medium with coupons and TVC in the biofilm showed a similar pattern over the period of time. However, the magnesium concentration in the medium increased gradually during the study period. This was followed by the increase in medium pH, which reached nearly 10 by the third day. The coupon surface pH also showed a sudden increase from 8 to 11.6 during this period. Magnesium reacts with water in the medium to form magnesium hydroxide, which dissolves in water thus increasing the pH. The increase in pH resulted in bacterial mortality and reduced the area cover of bacteria on the coupon surface. This property makes magnesium alloys antibacterial and in real filed conditions it is expected that this property would decrease the bacterial attachment on their surfaces thus preventing the formation of bacterial biofilm.

Reference

1) Maker G L, Kruger J (1990) Corrosion studies of rapidly solidified magnesium alloys. *J Electrochem Soc* **137**: 414-421

2) Song G, Atrens A, St John D, Nairin J, Li Y (1997) The electrochemical corrosion of pure magnesium in 1N NaCl. *Corr Sci* **39**: 855-875

3) Baril G, Pebere N (2001) The corrosion of pure magnesium in aerated and deaerated sodium sulphate solutions. *Corr Sci* **43**: 371-484

4) Gulbrandsen E, Tafto J, Olsen A (1993) The passive behavior of Mg in alkaline fluoride solutions. Electrcochemical and electron microscopical investigations. *Corr Sci* **34**: 1423-1440

5) Nordlien J H, Ono S, Masuko N, Nisancioglu K (1995) Morphology and structure of oxide films formed on magnesium by exposure to air and water. *J Electrochem Soc* **142**: 3320-3322

6) Udhayan R, Bhatt D P (1996) On the corrosion behavior of magnesium and its alloys using electrochemical techniques. *J Power Sources* **63**: 103-107

7) Hollopeau X, Beldjoudi T, Fiaud C, Robbiola L (1998) Corrosion resistance and electrochemical behavior of Mg and AZ91E alloy in aqueous electrolyte solutions. Inhibiting effect of XO_y^{n-} ions. *Corr Reviews* **16**: 27-42

8) Hughes M N, Poole R K (1989) *Metals and Microorganisms*, Chapman and Hall, London/New York, pp 412

9) Kenneth Todar University of Wisconsin, Dept. of Biological Sciences (1997) Nutrition and growth of bacteria, Bacteriology 303 Main page, http://www.bact.wisc.edu/Bact303/NutritionandGrowth p 1-9

10) Beveridge T J, Hughes M N, Lee H, Leung K T, Poole R K, Savvaidis I, Silver S, Trevors J T(1997) Metal-microbe interactions: Contemporary approaches In: *Adv Microbial Physiol* **38**: 177-243

11) Videla H A, Characklis W G (1992) Biofouling and microbially influenced corrosion. *Intl Biodet Biodegrad* **29**: 195-212

12) Scott P J B (2000) Microbiologically influenced corrosion monitoring: Real world failures and how to avoid them. *Mat Perform* Jan. 2000: 54-59

13) Characklis W G (1981) Fouling biofilm development: a process analysis. *Biotechnol Bioeng* **23**: 1923

14) Dexter S C, Duquette, D J, Siebert, O W, Videla H A (1991) Use and limitations of electrochemical techniques for investigating microbiological corrosion. *Corrosion* **47**:

308-317

15) Muller R F, Characklis W G, Jones W L, Sears J T (1992) Characterization of initial events in bacterial surface colonization by two *Pseudomonas* species using image analysis. *Biotechnol Bioeng* **39**: 1161-1170

16) Geesay GG, Gillis R J, Avci R, Daly D, Hamilton M, Shope P, Harkin G (1996) The influence of surface features on bacterial colonization and subsequent substratum chemical changes of 316L stainless steel. *Corr Sci* **38**: 73-95

17) Tide C, Harkin S R, Geesey G G, Bremer P J, Scholz W (1999) The influence of welding procedures on bacterial colonization of stainless steel weldments. *J Food Eng* **42**: 85-96

18) Sreekumari K R, Ozawa M, Tohmoto K, Kikuchi Y (2000) Adhesion of *Bacillus* sp. on stainless steel weld surfaces. *ISIJ International* **40**: (suppl.) S54-S58

19) Yagi K (2000) Studies on the microbiologically influenced corrosion of stainless steel welds (SUS 304 L). M.Sc. Dissertation, Osaka University, pp 68

20) Sokal R R, Rohlf F J (1987) Introduction to biostatistics, 2nd edition. W H Freeman & Co., New York, p 363

21) Sreekumari K R, Nandakumar K, Kikuchi Y (2001) Bacterial adhesion to weld metals: significance of substratum microstructure. *Biofouling* **17**: (in press)

22) Woods Hole Oceanographic Institution (1952) Marine biofouling and its prevention. United States Naval Institute, Annapolis, Maryland, pp 388