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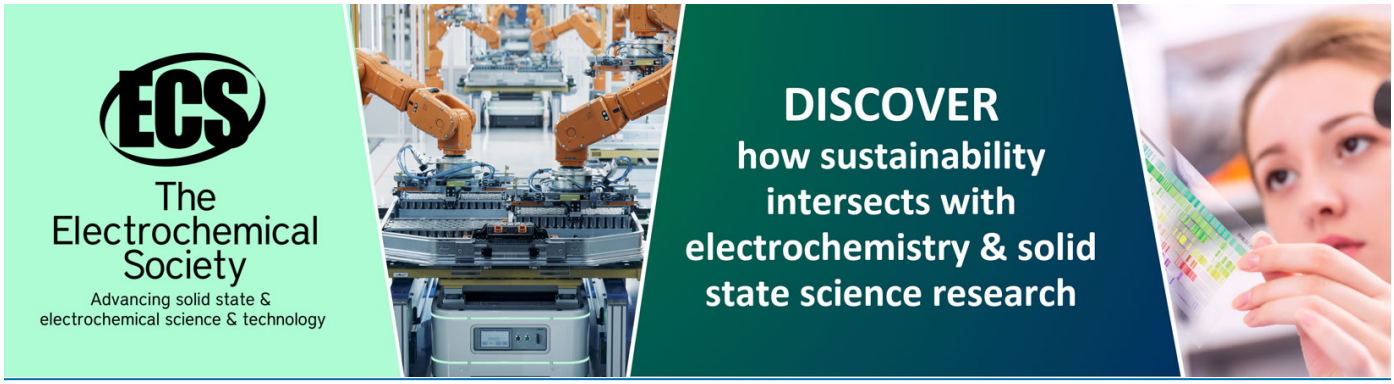

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Biofouling of various metal oxides in marine environment

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Abstract. Biofouling has induced serious problems in various industrial fields such as marine structures, biomaterials, microbially induced corrosion (MIC) etc. The effects of various metals on biofouling have been investigated so far and the mechanism has been clarified to some extent(1,2), and we proposed that Fe ion attracted lots of bacteria and formed biofilm very easily(3). In this study, we investigated the possibility for biofouling of *Pseudomonas aeruginosa* on various metal oxides such as Fe_2O_3, TiO_2, WO_3, AgO, Cr_2O_3 etc. And in addition of such a model experiment on laboratory scale, they were immersed into actual sea water as well as artificial sea water. As for the preparation of metal oxides, commercial oxide powders were used as starting material and those whose particle sizes were under 100 micrometers were formed into pellets by a press. Some of them were heated to 700 ℃ and sintered for 10 hours at the temperatures. After the calcinations, they were immersed into the culture of P. aeruginosa at 35 ℃ in about one week. After the immersion, they were taken out of the culture and the biofouling behaviors were observed by optical microscopy, low pressure scanning electron microscopy (low pressure SEM) etc. Biofouling is generally classified into several steps. Firstly, conditioning films composed of organic matters were formed on specimens. Then bacterial were attached to the specimen's surfaces, seeking for conditioning films as nutrition. Then bacteria formed biofilm on the specimens. In marine environment, more larger living matters such as shells etc would be attached to biofilms. However, in the culture media, only biofilms were formed.

1. Introduction

Biofouling is defined as the unwanted accumulation of biological material on man-made surfaces[1]. It produces various failures and drawbacks. For example, biofouling onto inside walls of cooling pipes in various factories along sea sides or in nuclear power plant etc., where sea water is mainly used, decreases the cooling capability, which leads to the increase of carbon dioxide in atmosphere finally. Biofouling is classified into microfouling and macrofouling mainly and the mechanism can be described schematically, as shown in Figure 1[1]. Firstly, organic compounds are adsorbed to solid surfaces to form thin organic films called "conditioning films". The carbon compounds attract planktonic bacteria in oligotrophic environments and they attach to solid surfaces. They gather around on solid surfaces and excrete polysaccharide due to quorum sensing at the same time, which lead to

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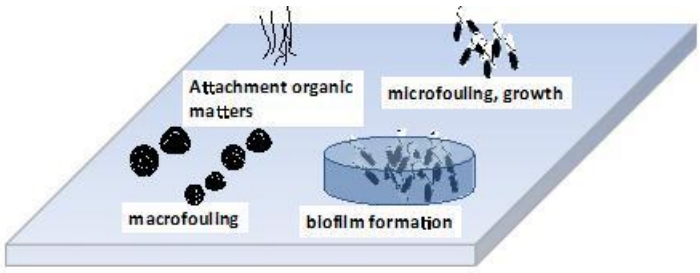

Figure 1 Process of microfouling and macrofouling.

the formation of biofilm. Excretion of EPS is usually induced by quorum sensing[3]. The biofilm formed on solid surfaces attracts larger creature such as oyster and acorn shell, and macrofouling occur continuously. Therefore, biofouling is related directly to biofilm formation. To understand the correlation between metallic materials as carririers and biofouling much more, it would be the best way to investigate the correlation between biofilm formation and metallic materials. Therefore, we have searched for metal elements inhibiting biofilm formation and found some of them so far[4]-[5]. Kanematsu et al[5]reported that some metal ions from the metallic surfaces would inhibit the growth of bacteria attached to the surfaces and the biofilm formation. On the other hand, metal oxides are generally hard to be ionized in aqueous solutions due to the high bonding stability. It would be explained that metal oxides are made by the covalent bonding between metallic and non-metallic components. It would be interesting to clarify how metal ions as cation would affect biofilm formation and biofouling. In addition, it might lead also to the possibility that ceramics materials could be applied to marine structures, cooling pipes, biomaterial etc.

In this study, we focused on some general metal oxides and to investigate their inhibition capabilities of biofilm formation. Various tableting metal oxides were immersed into ordinary bouillon medium and investigated how they would affect bacteria and biofilm formation.

2. Experimental

2.1 Specimens

Various metal oxide powders such as Ag_2O, CeO_2, Cr_2O_3, Fe_2O_3, TiO_2, ZnO and WO_3 (Wako Pure Chemicals, Japan Aerosol Co.) were broken up by agate mortar. Then agglomerated particles were removed by sifter and the particles whose average diameters were below 100 micrometers were collected. Those fine particles were tableted by a tableting machine (JASCO). They were pressured step by step and finally, they were compressed in a single direction at 400kgf/cm^2 for 20 minutes. Finally, the tablets of 10 mm diameters were formed. They were sintered at 700 ℃ for 10 h in ambient atmosphere.

2.2 Characterization of specimens

All of the specimens were subjected to X-ray diffraction (XRD) analysis to identify and confirm the structures. XRD was carried out by using a general purpose machine for the purpose (RINT-2100, Rigaku). A copper electrode was used as target electrode. The voltage of X-ray tube was 40kV and the current was 20 mA.

2.3 Culture media and immersion tests

Specimens were immersed into ethanol and dried for 4 hours. Then they were immersed into cell suspension of *Pseudomonas aeruginosa* strain PAO1. The suspension was made in the following way. Strain PAO1was cultured in ordinary bouillon (meat extract 10 g, peptone 10 g, NaCl 15 g in distilled water 1L, pH = 7.2) at 35 ℃ for 24 h and then diluted with ordinary bouillon to give a cell density of 10^5 cells/ml. Concentration was checked by CFU (Colony Forming Unit) method. Metal oxide tablets sterilized with ethanol were put in a 24 holes well plate each of which was filled with 2 ml of cell suspension. The plates were kept at 35 ℃ for 7 days in darkness. Then each solution was taken as sample and diluted, and that of 0.1 ml was inoculated into an ordinal agar culture media (1.5 wt% agar was added to ordinary bouillon). It was kept in an incubator at 35 ℃ for 24 h. After the culture procedure, the number of colonies formed on the agar was measured and calculated. As for the biofilm,

bacteria in biofilms were stained with crystal violet and the absorbance intensity was measured for the evaluation how many bacteria existed in them. Concretely, each oxide tablet was immersed into 0.2 wt% crystal violet solution after the culture and rinsed out by phosphoric acid buffer solution. Then the crystal violet attached to the specimen was washed out by ethanol and the absorption strength of the solution was measured by a microplate reader (Model 550, BIO-RAD).

3. Results and discussion

3.1 Identification of oxide specimens by XRD

Those specimens such as WO_3, CeO_2, Fe_2O_3, Cr_2O_3 and ZnO showed quite the same structures with those of starting materials. The results of XRD analysis of these oxides are shown in Figure 2. They indicate clearly that the structures after the sintering at 700 ℃ did not change from the starting points at all. On the other hand, Figure 3 (a) and (b) shows the exceptional results. Figure 3 (a) is the XRD result for Ag_2O specimen. After sintering, silver phase increased in XRD results. It indicates that the silver oxide was reduced by sintering to 700 ℃ to much extent. Figure 3 (b) shows the XRD results for titanium oxide specimen. Before sintering, the main structure of titanium oxide specimen was anatase, while that after sintering was rutile type one. From those results, the specimens except silver oxide were used after sintering. On the other hand, silver oxide was used as non-sintering specimen.

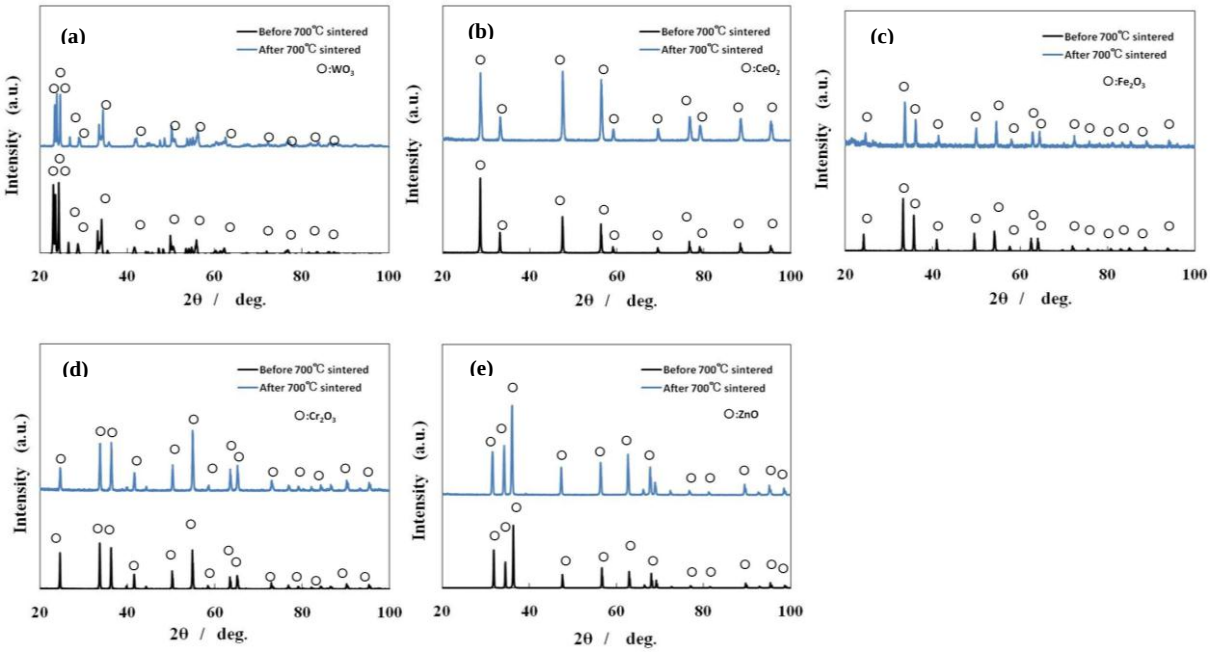

Figure 2 XRD patterns of metal oxide with sintered.
(a)WO_3, (b)CeO_2, (c)Fe_2O_3, (d)Cr_2O_3 and (e)ZnO.

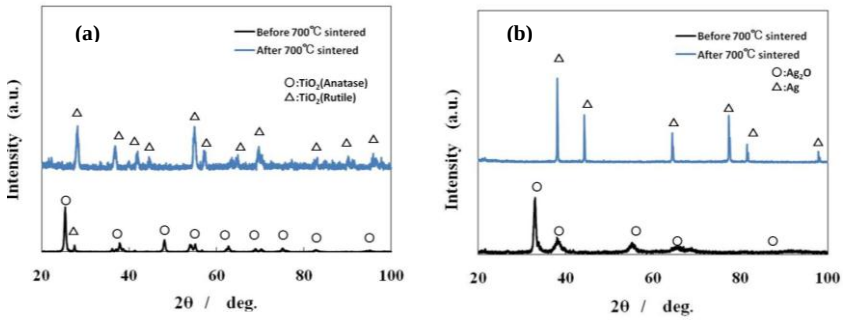

Figure 3 XRD patterns of metal oxide with sintered. (a) TiO_2 and (b) Ag_2O.

3.2 Immersion tests

All of the oxide specimens were immersed into the bacteria cell suspension. Then, a portion of the suspension was sampled and cultured on agar medium to check bacterial viability. Figure 4 shows the suspension diluted one hundred times. As for WO_3, Fe_2O_3, TiO_2, ZnO and CeO_2, the numbers of colonies were too much to be counted, while those for Cr_2O_3 and Ag_2O were almost zero. It suggests very clearly that the latter two oxides have very strong antibacterial effects for *Pseudomonas aeruginosa*. Figure 5 shows the viability of P.Aruguinosa in the metal-oxide-suspensions as revealed by plate counting. Those results made it possible to differentiate the antibacterial effects among other oxides. In addition of silver oxide and chromium oxide, cerium oxide (CeO_2) also showed the relatively strong antibacterial effect. On the other hand, other oxides such as WO_3, Fe_2O_3, TiO_2 and ZnO did not show any remarkable antibacterial effects. Generally, oxides have antibacterial effects, when photoexcited, since the light-excited oxides could produce radical hydroxide ions. However, the series of experiments were carried out under dark conditions.

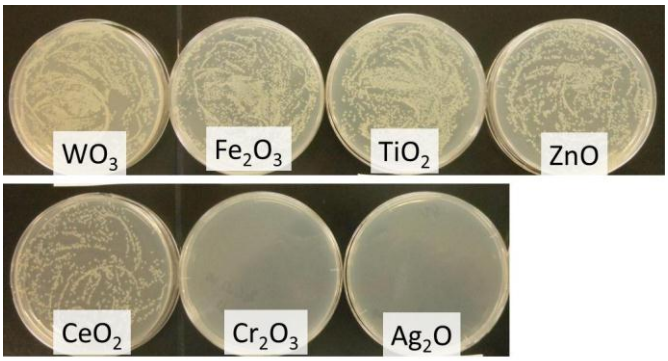

Figure 4 Inoculated solutions diluted a hundred times.

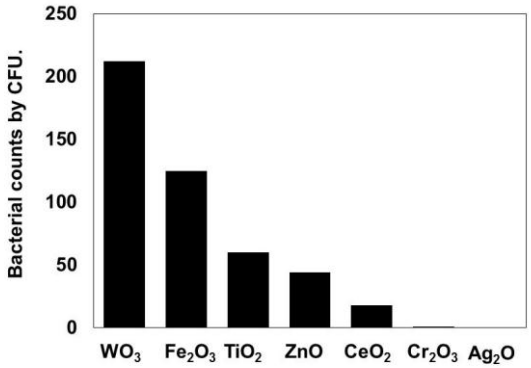

Figure 5 Inoculated solutions diluted a thousand.

Therefore, the antibacterial effects of silver oxide and chromium oxide would be produced due to the dissolutions of chromium and silver as ion, respectively. Originally, both metallic ions have been well-known for their relatively strong antibacterial effects[6]-[7]. In addition to both Cr_2O_3 and Ag_2O, CeO_2 also showed the antibacterial effects. It is also well known that the CeO_2 often shows the effect, particularly as nano particle[8]. Also in this case, dissolved cerium ion would work and show the antibacterial effect. Other oxides did not show so significant antibacterial effects. It could be attributed

to that these oxides were relatively stable as oxide and that they did not produce ions in the solutions, even though titanium and zinc ions often show high antibacterial effects[4].

Usually, antibacterial effects are not always same with the inhibition capability of biofilm formation completely[9]. However, the specimens were generally porous different from metallic specimens and crystal violet could attach to the pores much more easily. Therefore, the absorbance intensity might depend on the porosity of specimens to some extent. In addition to that, crystal violet bound to some specimens specifically. For example, tungsten oxide was stained to much extent, since the dimethylamino group of crystal violet binds to the tungsten oxide surface specifically[10]. On the other hand, some researchers found that the carboxylic acid adsorbed to some metallic oxides such as titanium oxide[11]. However, we have to evaluate the biofilm inhibition capability finally and it requires some staining method by pigments. Therefore, the evaluation method by crystal violet did not work in this investigation. This would be an important topic for the future to find them. In this study, we measured the antibacterial effects by dissolved metallic ions from metallic oxides specimens and evaluated the extent of biofilm formation for those oxides specimens indirectly. The evaluation is based on the premise that the antibacterial effect induced by metallic ions in the bacterial suspension solution could inhibit the biofilm formation. The hypothesis would be absolutely right. However, some other factors might be involved with the biofilm formation. It needs further investigations in the future to clarify the inhibition effects of biofilm formation by oxides much more.

4. Conclusions

The inhibition capability of biofilm formation for th**e** seven metallic oxides, WO_3, Fe_2O_3, TiO_2, ZnO, CeO_2, Cr_2O_3 and Ag_2O, were investigated. The evaluation by the adsorption intensity of crystal violet to biofilms on oxide specimens could not be used, since it also depended on the porosity of specimens and also the specific adsorption of composed organic groups to oxide surfaces. Therefore, we evaluated the extent to measure the colony numbers in bacteria (Pseudomonas aeruginosa) suspension solutions. As results, Cr_2O_3, Ag_2O and CeO_2 showed antibacterial effects and therefore, we presumed that these three oxides would have high inhibition capabilities of biofilm formation due to the effect of dissolved ions at this point. Some new effective pigments are now developed to use the evaluation in the future.

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