Comparison of Effect of Nanocoating Against Biofilm Forming Bacteria on Mild Steel

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ABSTRACT

Copper has been known to possess antimicrobial properties since as far back as the Phoenician era where ship hulls were copper sheathed to prevent the inevitable effects of biofouling. As a consequence of evolving scientific research and development, the realization of novel materials and agents has enabled new scientific branches, such as nanotechnology. In this paper, we investigate the performance of different forms of copper coating for application as antifouling materials. Samples were deployed in Tuticorin-New harbour for four weeks and analyzed for evidence of biofouling. It was found that copper in its nanoform, produced the greatest antifouling effectiveness in mild steel compared to other forms ofantifouling coating.

Keywords: Copper, Antifouling coatings, Biofilm, Mild steel

INTRODUCTION

Marine biofouling is one of the chief unanswered troubles at present affecting the shipping industries and industrial equipments. The term 'biofouling' refers to the undesired accumulation of microorganisms, plants and animals on any artificial structures, which are exposed to aquatic environments. The establishment of fouling communities on a wide variety of substrata has been investigated thoroughly and the resulting literatures are vast. Theestablishment of the fouling community has been characterized interms of several stages and some of these stages can overlap oroccur in parallel. In the development of biofouling, any submergedsurface rapidly becomes coated by a conditioning film comprising of organic and inorganic molecules which may act as sourceof nutrients for microorganisms. Formation of this film is immediately followed by the accumulation of microorganisms (bacteria, diatoms), which secrete extracellular polymeric substances (EPS) during attachment, colonization, population growth and the resulting layer is termed as the biofilm (microfilm). The biofilm maypave for the settlement of larvae of higher organisms such as barnacles, mussels and tubeworms which constitute macrofouling.These organisms cause serious technical problems by settling onship hulls, power plant cooling systems, aquaculture systems, fishing nets, pipelines, submerged structures and also oceanographic research instrumentation thereby leading to huge economic losses. In ships, the friction between the hull and water increases, which indirectly increase in fuel consumption (up to 40-50% with

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low-density biofouling) and decrease the speed and manoeuvrability(International Maritime Organization, 1999).

In order to avoid economic losses, associated with accelerated deterioration of the artificial structures in contact with seawater, different types of protections have been used overtime. Among them, it is necessary to specify the copper coatings that began to be used by the Phoenicians, continued to be successful and used on wood ships until the 18th century. When iron ships were first built, paints widely known as "patents" was used, in which the copper sulphate acted as a biocide. The copper based mixture works well for short term and serves as an ideal antifouling agent at least for three years after application (Clare, 1995). When copper is used in nano level, the impacts on the environment is much lesser. The results of scientific studies have revealed that the nanocoatingsprevent biofilm formation, bacterial adhesion besides the attachment of macro foulers. (Szewczyzk, 2010).The present investigation is based on a nanocoating method byincreasing the surface smoothness, which prevents the settlement of bacterial species which are considered to be effective fouling species and responsible for primary film formation

MATERIALS AND METHODS

Test panels

Mild steel of size 15 cm height, 8 cm breadth, and 12mm thickness and weight of 1kg, respectively were prepared and mounted with the help of 4mm polypropylene rope in an iron frame having a dimension of 106.5cm length and 106.5cm width. With the help of a loop provided on the top, the frame was tied with a 12mm polypropylene rope and suspended in the CECRI jetty, inside the Tuticorinnew harbour area at a depth of 1.5m.

Sampling schedule

After immersion in seawater, the study panels were sampled periodically for microbial studies. For microbial analysis, the samples were drawn after 24h of immersion and subsequently after the1st week, 2nd week, 3rd week and the 4th week.

Antifouling coatings

Steel was encasedby adopting Physical Vapor Deposition (PVD) and Spray methods. For comparative studies,the selected boat-building materials were painted with commercially available antifouling paint (NOAH marine paints, Cochin).Various nanocoating methods exposedfor the study are described below.

Physical Vapor Deposition (PVD)

Copper powder was taken in a conicalcontainerwith a flat bottom having a size of 29mm (dia) x 13mm (h)with a capacity of 7CC. The pellet die assembly was then compressed under a pressure of 15 tonto form a pellet within five minutes duration.After pelletformulation, it was placed over the substrate heater i.e. electron gun. The copper pellet targets the panels once thevacuum havecreated inside the chamber arc gas will released. The copper powder is heated above 1789° Celsius, until it gets vapourized. The vaporized atoms get deposits to the panels. The mild steel substrate to be used was cut into proper dimension (75mm x 25mm), so as to

properly fit it into the substrate holder of the sputtering machine. The surface of the mild steel was polished and smoothened with emery sheets of various fineness followed by cleaning with acetone. The respective prepared composite pellets were then placed in the sputtering machine (Hind Hivac, Bengaluru) as targets and the mild steel substrates were coated using RF sputtering in argon atmosphere.

Spray coating

The nanocoating was accomplished for steel by spray method. The synthesized copper nano powder was mixed with enamel paint with amagnetic stirrer at 60^0c for 6 hrs. Subsequently the paint mixed with nano powder was coated with 2-4 bar / 30 psi pressure and dried for 24 hrsat room temperature.

Isolation and Enumeration of biofilm forming bacteria from the test Panels

For the isolation of biofilm forming bacteria, a template of size 5 x 3 cm^2 was placed on each test panel and the bacteria were scraped using ascalpel and placed in a test tube containing 10 ml of sterile saline. Serial dilutions were done with the same diluent. Appropriate dilutions were then inoculated in specific media (Zobell Marine Agar) for the enumeration of biofilm forming bacteria. About 0.1ml of the appropriate dilution was inoculated on the sterile medium and spread plated uniformly with sterile glass spreaders. The plates were left at the room temperature for about 30 min till the sample is completely absorbed by the medium. The petri plates were then inverted, stacked in lots and incubated at a temperature of 37˚C for 24 h. The colonies were counted as biofilm forming bacteria and expressed as CFU/cm^2.For purification of the selected colonies, the predominant colonies were streaked on Zobell agar medium, and the single isolated colonies were picked up and maintained in Zobell agar slants and stored in a refrigerator.

DNA Extraction

From the purified colonies, genomic DNA was extracted (HiMedia DNA extraction Kit). Approximately 1.5ml of overnight growth broth culture was taken in a 2 ml microfuge tube,180 µl lysis bufferand 20 µl of proteinase K were added. After homogenization, the tubes were incubated at 55°C for 30 min in a water bath. Then, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The contents were mixed gently and centrifuged at 9200 rpm for 10 min. The top aqueous layer was then transferred to a new 1.5 ml microfuge tube. The DNA was precipitated by the addition of equal volume of isopropanol and 0.2ml volume of 10M ammonium acetate and by inverting the tube several times. The tube was centrifuged at 13,200 rpm for 10 min. The supernatant was removed and the pellet was washed in 500 µl of chilled 70% ethanol, air-dried and resuspendedin 100 µl sterile water.

Polymerase Chain Reaction

The 16S rDNA region was amplified by PCR from the isolated genomic DNA using the universal primers. Primers used for PCR analysis were, Forward: 5'-AGAGTTTGATCMTGG-3'and Reverse: 5'-ACCTTGTTACGACTT-3'

The amplification was carried out in 25 µl of reaction mixture containing 2.5µl buffer 0.25µl dNTP, 19 µl of molecular grade water, 0.25µl of Taq DNA polymerase, 1µl of each forward and reverse primers and 1µl of template DNA. The PCR protocol comprised of initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 sec, annealing at 52°C for .30 sec, extension at 72°C for 45 sec and afinal extension at 72°C for 10 min. The number of cycles was 35.

Agarose gel electrophoresis

The PCR amplifiedproduct (4 µl) was mixed with 1 µl of 6X loading buffer and subjected to electrophoresis on a 2% agarose gel containing ethidium bromide at a concentration of 0.5 µg/ml in TAE (1X) buffer. The gel was observed under UV transilluminator and photographed in a documentation system.

Purification and sequencing of DNA Samples

Amplified PCR product was purified using column purification kit as per manufacturer's guidelines and used forsequencing. (Unibiosys Lab, Cochin, India) Phylogenetic analysis was done using theMEGA software.

RESULTS AND DISCUSSION

Effect of antifouling coating on Microbes- Steel

In steel panels, initially the biofilm forming bacteria in control, antifouling painted nanocoated and spray coated were 4.3,4.64, 0 and 4.22, logcfu/cm^2, respectively. The counts increased with the duration and reached 4.66, 5.7, 4.65 and 4.98 logcfu/cm^2, respectively after the 4th week.Overall analysis on antifouling, showed that nanocoatedvapor deposition worked on first three weeks and it showed arapid decrease in its antifouling activities due to changes in surface topography. Next to vapor deposition, spray coating shows reduction in colonies formation compared to antifouling painted (Table1).

TABLE: 1 Effect of antifouling coating on Microbes- Steel

Panel type	In weeks (log CFU/cm^2)			
	1st	2nd	3rd	4th
Steel Control	4.37	4.66	4.44	4.3
Vapor deposition	0	0	0	4.65
Antifouling painted	4.64	5.07	4.69	5.7
Spray coated	4.54	4.22	4.66	4.98

Isolation of biofilm forming bacteria

A total of three isolates were selected from the test panels (H_1, A_2, and A3). Two of the isolates (H_1, A_3) were present in all the test panels. Secondisolate (A_2) was isolated particularly from the nanocoated panel (Table 1).

Table-2: Details of BLAST analysis, percentage of similarity and NCBI accession numbers of marine biofilm forming bacteria isolated from different kinds of coated panels.

S.NO	Assigned Code	Sequence length (bp)	Similarity (%)	BLAST results	NCBI's accession
1.	*H1*	977	99	*Pseudomonas aeruginosa*	JQ659528
2.	*A2*	989	99	*Ferrimonasfuttsuensis*	JQ799090
3.	*A3*	1007	99	*Vibrio alginolyticus*	KJ872832

Amplification of 16s rDNA from biofilm forming bacteria

16S rDNA gene amplification of the isolates H, A_2, and A_3 amplified the gene in all the bacterial species resulting in a 1200 bp product, which was clearly visualized in the agarose gel electrophoresis. The first isolate and the third isolate was identified as *Pseudomonas aeruginosa* (H_1) and *Vibrio alginolyticus* (A_3) based on 99% similarity with the available sequence in the NCBI data base. These two species were present in all type of antifoulingcoated panels. The second isolate was identified as *Ferrimonasfuttsuensis* (A_2), which was present in steel control and nanocoated panels

Phylogenetic analysis methods

The overall phylogenetic analysis, of the selected sixteen biofouling bacterial sequences showed two major divergences found among the species in Minimum Evolution method (Fig3). Inner level divergences were observed in all the bacterial species. Bacterial species, such as *Pseudomonas aeruginosa, Ferrimonas futtsuensis, Vibrioalginolyticus* are significantly diverged from other twelve species. These three diverged species have closeness measured as less 0.1 in divergence scale and *Vibrio alginolyticus*were found in same branch whereas *Pseudomonas aeruginosa, Ferrimonas futtsuensis*are diverged in two distinct inner nodes. Apart from the subjected sequences, *Proteus mirabilis, Halomonasaquamarina, Halteleaalkalilenta*are diverged from the first node and further diverged internally. The other distinct divergence observed was *Arthrobactersp, Micrococcus sp, Micrococcus luteus. Exiguo bacteriumaestuarii, E. arabatum, Exiguobacteriumsp, Jeotgali bacillusalimentarius, Bacillus Flexus*and *Bacillus sp*. are diverged in the same node and further diverged in different branches due to course of evolution. From the overall phylogenetic analysis reveals the *Pseudomonas aeruginosa, Ferrimonas futtsuensis, Vibrio alginolyticus*are shown the distantly related with other selected species of bacteria involved in biofouling activities.

Figure-1: Effect of antifouling coating on Microbes- Steel

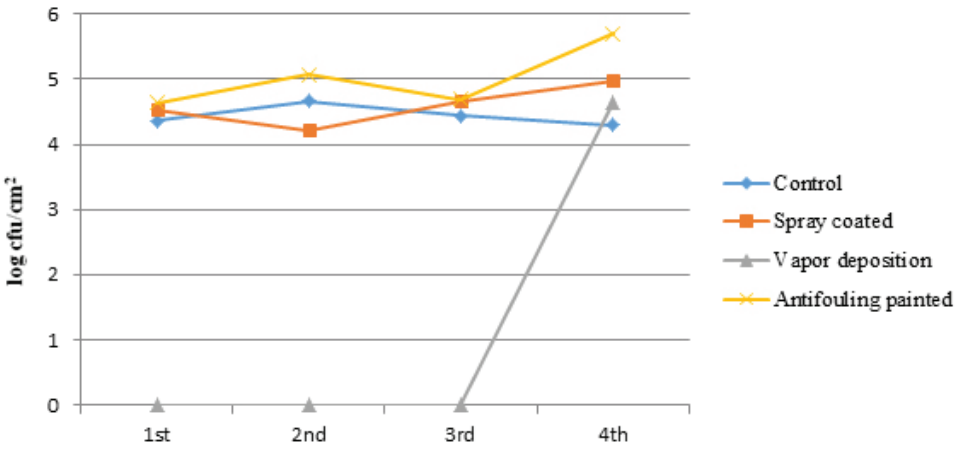


Fig 2.Ethidium bromide stained 2% agarose gel showing results of electrophoretic analysis of amplified 18S rRNA gene eukaryotic specific primer obtained from different bacterial species. Lane M- 1200 bp DNA marker; Lane A- *Pseudomonas aeruginosa*; Lane B – *Ferrimonasfuttsuensis*; Lane C-*Vibrio alginolyticus*

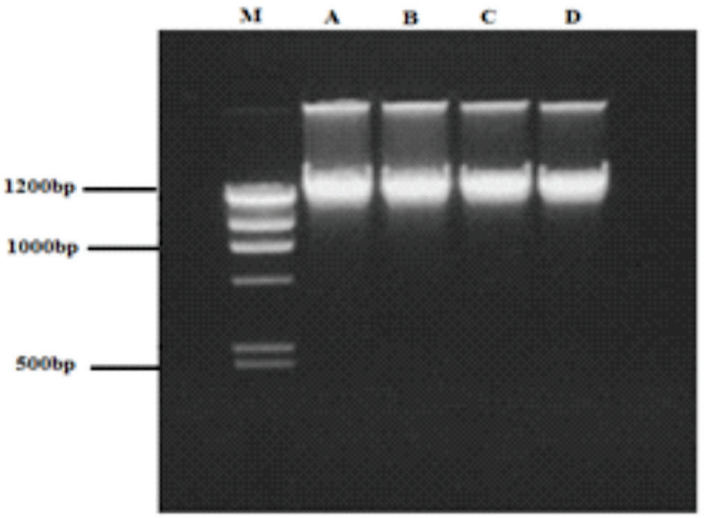
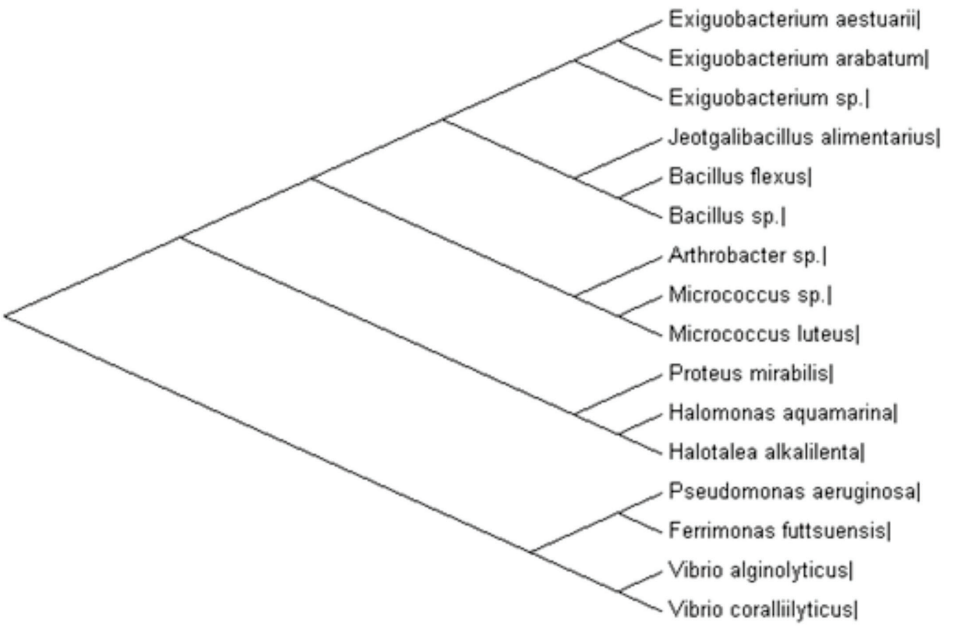


Fig-3: Phylogenic tree constructed using the Minimum Evolution Method



Sequencing of the amplified 16S rDNA gene region

The bacteria isolated identified based on the amplification 16S rDNA fragments and sequencing are presented in Table 2. All the three speciesviz., *Pseudomonas aeruginosa, Ferrimoasfuttsuensis, Vibrio alginolyticus,* showed more than 99% homogeneity based on the entries available in the NCBI databases.

Effect of antifouling coating on biofilm forming bacteria on various test panels

Deposition of microorganisms on surfaces and biofilm formation is an important bacterial survival strategy. Biofilms occur spontaneously on both inert and living systems, being of concern to a wide range of scientific disciplines. In industry, biofilms can have a detrimental impact because of accumulation at interfaces (Characklis,1983; Cooksey, 1983). Competition for living space is more intense in themarine environment; hence all submerged surfaces in the marine environment are rapidly colonized by bacteria and they form the important component in the development of a fouling community (Mitchell and Kirchman, 1984). Biofilms are formed by microbial cells embedded in an exopolymeric matrix. The extracellular matrix is mainly composed of polysaccharides and proteins, along with compounds such as DNA and humic substances (Nielsen *et al.,* 1997; Jahn*et al.,* 1999).

In the present study, when the panels coated with copper following three different

methods were exposed to seawater in order to determine their potential quality and recruitment of biofilm bacteria formed over the, biofilm bacterial load (TVC) was found to increase gradually i.e., from 4.3 up to 4.66,4.22 to 4.98, 0 to 4.65 and 4.64 to 5.7 log cfu/ cm^2in the control mild steel, spray coated, nano-coated and antifouling coated, respectively from 24 to 72h intervals. The assessment of bacterial population in the mild steel inferred theslow rate of succession of thebacterial load with respect to thetime interval. This may be due to corrosive nature and changes in ionic charges of the substrates.

PCR method for the identification of bacteria using 16S rDNA gene

Suriya Murthy et *al.,* (2004) studied the biofilm control using plate heat exchangers of surface seawater from the open ocean for the OTEC power plant. Microbiological analysis of biofilms revealed that four distinct types of bacterial colonies were present, the pre dominant bacteria *Vibrio, Flexibacter*, *Pseudomonas and Aeromonas*, were the total viable bacteria in untreated controls were observed and tend to amplified counts with the age of the biofilms. There was a highly significant variation (P = 0.0001) between chlorinated and control biofilms. Feng*et al.* (2000) and Yamanaka *et al.* (2005) reported the mechanism of the inhibitory action of silver ions on microorganisms. They concluded that, when treated with silver ions, microbes their DNA replication ability, expression of ribosomal subunit proteins and other cellular proteins, and inactivation of enzymes essential for ATP production. It has also been hypothesized that Ag+ primarily affects the function of membrane-bound enzymes, such as those in the respiratory chain. However, the mechanism of bactericidal actions of silver nanoparticles is still not well understood.

In our study, the collected bacterial samples were investigated for the isolation, identification, sequencing, characterization bacterial species and their genetic makeup through advanced molecular biological studies including polymerase chain reaction, electrophoresis and sequencing techniques. This study has also helped to predict the mode of binding, genetic materials responsible for biofouling, etc. Polymerase chain reaction a molecular methods to identify the genetic makeup of identified bacterial DNA, which responsible for biofouling activities. Bacterial species, such as *Pseudomonas aeruginosa,* (Sonak and Bhosle, 1995) *Ferrimonas futtsuensis, Vibrio alginolyticus*(Muralidharan *et al.*, 2003) were identified. From the result, *Pseudomonas sp*was observed to be the most dominant bacteria.

The data presented here on the diversity of biofilm bacterial strains in the experimental panels indicated that most of the strain identified were gram-negative in nature. Among biofilm bacterial strains isolated, the most predominant bacterium recorded in all the experimental panels was *P. aeruginosa* (29 – 35%) and next dominant biofilm bacterial strains registered were *V.alginolyticus* with the percentage occurrence range of 17 – 19% respectively. The other biofilm bacterial strains were recorded in lesser proportion (1 – 10%).

From genetic profiling of identified bacterial samples this imperative research will focus and deliver the unknown information about bacterial species and genetic relationship among the different species. knowledge obtained from the bacterial sequence, similarity of this study, will help us to identify the target site of bacteria for the developmentof more potent antifouling materials in future.

CONCLUSION

Rougher surfaces accumulate and retain more colonization, by increasing the surface smoothness, which prevents the settlement of bacterial species which are considered to be effective fouling species and responsible for primary film formation. Copper nanoparticles in expand PVD shows more antifouling potential up to three weeks. This work integrates nanotechnology and bacteriology, leading to possible advances in the formulation of new types of bactericides or antifoulants. Resultsobtained from the present investigation demonstrated the anti microfouling potential of copper nanoparticles. Copper nanoparticles have a high potential to be may assembled or coated on marine industrialsurfaces and to study the possible biofouling control with furtherexperiments for a new anti microfouling material in future.

ACKNOWLEDGEMENT

I wish to express my sincere thanks to **Dr. G. Shakthinathan**, Dean, Anna University, Tirunelveli campus, Tirunelveli, for providing facilities of nanocoating in this study. I would like to express my heartfelt gratitude to **Dr. G. Subramanian**, Scientist, and **Mrs.P. Kalaiselvi** Central Institute of Electro-chemical Research Institute, for providing facilities for hanging of panels and their guidance in the period of study.

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