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Antifouling properties of marine bacteriocin incorporated epoxy based paint

S. Palanichamy*, G. Subramanian

Offshore Platform and Marine Electrochemistry Centre, CECRI Unit, New Harbour Area, Tuticorin, 628 004,Tamil Nadu, India

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ABSTRACT

Bacteriocin isolated from five marine biofilm forming bacteria viz. *Pseudomonas aeruginosa*, *Bacillus* sp., *Micrococcus* sp., *and Flavobacterium* sp., were evaluated for their antibacterial activities against each other. Among them, the secondary metabolites isolated from Pseudomonas aeruginosa exhibited maximum antibacterial activity. When the pure strains of *P. aeruginosa* and *Micrococcus* sp. were co-cultured in liquid nutrient broth, the *P. aeruginosa* outcompeted the population of *Micrococcus* sp. Further, a field study was conducted to show the antifouling activity of *Pseudomonas aeruginosa* biofilm, developed on stainless steel (SS) coupons in natural seawater for 60 days. Initiation of macrofouling was evident on the control coupon within 30 days of exposure; whereas no such incidence of macrofouling initiation could be observed on the coupon that was pre-biofilmed by *P. aeruginosa* even up to 60 days. Similarly, the bacteriocin incorporated epoxy based paint formulation exhibited antifouling property for 3 months. The bacteriocin of P. aeruginosa which exhibited antifouling activity was characterised using GC–MS and identified the active compounds.

1. Introduction

Biofouling is a process wherein any submerged substrata, natural or artificial, in the marine environment are colonised by micro and macro marine organisms. Marine biofouling, though a natural process, can impact shipping and other industries, causing serious economical consequences. The application of toxic substances like copper or organotin compounds [1–3] has been the most effective method of controlling biofouling so far. Since these chemicals are toxic to marine organisms [1,4], development of eco-friendly and nontoxic antifoulants have been considered essential.

Biofilm can enhance or inhibit larval settlement of marine invertebrates [5]. The fact that biofilm forming organisms like bacteria and diatoms produce compounds that could disrupt or prevent biofilm formation could be meaningfully used for the development of eco-friendly antifouling substances [6]. Similarly Evans [4] and Harder et al. [7] demonstrated that some bacteria can produce large waterborne, polar compounds that act as antifoulants. Hence, marine microbes could be the beneficial sources of antifouling compounds. When compared to other marine organisms, microbes have an advantage that there is no need for large quantity from nature, can be easily cultured and the production of compound is much more rapid. Further, field study evaluation of antifouling properties of paint formulations with bioactive compounds derived from marine bacteria from tropical region has not found a place in the literature. Hence, the present study was designed to delineate the antifouling properties of bacteriocin produced by marine biofilm bacteria by incorporating in to epoxy based paint.

2. Materials and methods

2.1. Collection of bacteriocin

Marine biofilm forming Pseudomonas aeruginosa was isolated from stainless steel (SS304) coupon. 250 ml of 24 h cultured nutrient broth of *Pseudomonas aeruginosa* was centrifuged and the supernatant was precipitated with ammonium sulphate. It was centrifuged again at 6000 rpm for 15 min and the supernatant was discarded. The precipitate was dissolved in 10 ml of 50 mM Tris-HCl and used for antagonistic activity studies [8].

2.2. Antibacterial assay

Well diffusion method was employed for the study of antibacterial activity of the gram-negative and gram-positive isolates. Sterile Zobell marine agar medium was used for the preparation of agar

* Corresponding author.
E-mail address: spscientist18@rediffmail.com (S. Palanichamy).

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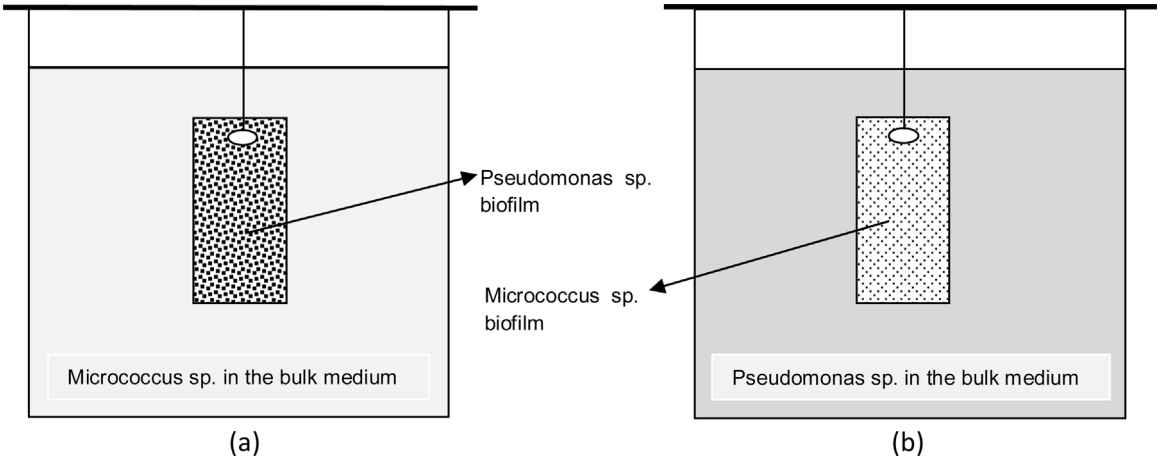

Fig. 1. Schematic showing the antibacterial and antifouling properties of marine bacteria, (a) *Pseudomonas* sp. and (b) *Micrococcus* sp.

plates. Sterile swab was used to inoculate the culture on surface of nutrient agar plates to ensure even distribution of the medium. The isolated pure cultures of *Bacillus* sp., *Micrococcus* sp., *Flavobacterium* sp. from the biofilm were swabbed on separate nutrient agar plates. On the agar plates, wells of the size 6 mm were cut using sterile well cutter. Each 0.1 ml of bacteriocin of *different bacteria was* added in the wells. A well added with 0.1 ml of 50 mM Tris-HCl was served as control. All the plates were incubated at 37 °C for 24 h and after incubation period, the zone of inhibition was measured using a millimeter scale [9].

2.3. Antibacterial activity by co-culture method

Each 1 ml fresh cultures of both gram-negative, *P. aeruginosa* and gram-positive *Micrococcus* sp. were inoculated in 100 ml of 50% nutrient broth taken in a 250 ml conical flask. The flask was plugged with cotton and kept in an orbital shaker (160 rpm) at room temperature. After 24 h incubation, the culture broth was subjected to gram staining to observe the existence of the isolates.

2.4. Antibacterial activity of biofilm

P. aeruginosa was allowed to form biofilm on SS coupon of size 10 mm × 40 mm for 48 h and immersed in 400 ml of 24 h old pure broth culture of *Micrococcus* sp. in 500 ml glass beaker. Similarly, *Micrococcus* sp. was allowed to form biofilm on SS304 coupon for 48 h and immersed in pure broth culture of *P. aeruginosa* as shown in Fig. 1. The effect of bacteriocin produced by biofim on the other bacteria in the bulk medium was studied for 24 h in terms of population enumeration by pour plate method. Then the biofilm developed coupons of both *P. aeruginosa* and *Micrococcus* sp. were examined by Epi-fluorescence microscope (Nikon Eclipse 80i fluorescence microscope fitted with type D-FL fluorescence attachment and filter block type B-2A in combination with a calibrated eyepiece reticule. Randomly chosen fields were photographed on an Evolution MP camera).

2.5. Antifouling study

PVC coupons of the size 10 mm × 50 mm × 1 mm were cut from a sheet, degreased using methanol and immersed in pure cultures of *P. aeruginosa* for a week time as to develop biofilm. Care was taken as to form biofilm on both the sides of the coupons by ensuring upright position of the coupons with the help of twine. The culture flasks were cotton plugged and incubated at room temperature. The culture medium was renewed daily. The coupons on which biofilm of *P. aeruginosa* developed and bare coupons in triplicate were fixed on PVC frame using insulated brass bolt and nut. The frame was then immersed in natural seawater at 2 m depth below the mean sea level at Tuticorin. The coupons were observed periodically for recording initiation of macrofouling, if any.

2.6. Composition of coating formulation

The coating formulation was prepared with the following composition, epoxy equivalent of 425–450 grade was used as binder, polyamide as hardener and pigment such as zinc phosphate, iron oxide, talc and mica were used for the development of primer to a thickness of 80 ± 5 μm. The primer was formulated with a pigment volume concentration (PVC) of 35%. The middle coat comprises of epoxy as binder, polyamide as hardener and pigment such as micacious iron oxide, talc and silica, to a thickness of 65 ± 5 μm with a pigment volume concentration (PVC) of 35%. The top coat comprises of epoxy as binder, polyamide as hardener and pigment such as, TiO_2 (Anatase), talc and mica as extender pigments and 2% (w/v) bacteriocin of Pseudomonas as antifoulant, to a thickness of 45 ± 5 μm with a pigment volume concentration (PVC) of 20%.

2.7. Coupon preparation and application of coatings

Commercially available metal sheets of mild steel (C: 0.16%, S: 0.4%, Mn: 0.7%, S: 0.04%, P: 0.04% and Fe: balance) of 2 mm thickness supplied by M/s. Lawrence Metal Industries, Chennai were used in this study. Sheets of mild steel were cut into required number of coupons of sizes, 25 mm × 75 mm for field evaluation. The coupons were pickled [10], sandblasted [11], and three coats of the paint formulation were applied by brush and allowed to dry for 24 h between each application, with a final dry film thickness of 190 ± 5 μm. A set of coupons coated with the above formulation without bacteriocin was served as control. All the coated coupons were allowed to get cured at room temperature for 7 days and preserved in desiccators with proper packing for conducting lab and field studies.

2.8. Evaluation of coatings for antifouling properties in natural seawater

Sufficient numbers of coated coupons (25 mm × 75 mm) of paint formulations along with control (without biocide) coupons (25 mm × 75 mm) were mounted on wooden rafts using PVC washers and insulated brass bolts & nuts. The rafts were immersed at a water depth of 2 m below the mean low tide level in the Gulf of