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Development of polyvinyl chloride biofilms for succession of selected marine bacterial populations

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Abstract

Present investigation was made to bring out the pattern of biofilm formation by heterotrophic bacteria on non-toxic material, polyvinyl chloride (PVC) sheet fitted wooden rack that was immersed in seawater and the study was conducted in Tuticorin coast. Samplings were made over a period of 7 days with the following time period intervals: 30 min, 1, 2, 4, 24, 48, 72, 96, 120 and 144 hr. Bacterial enumeration was made by spread plate method on nutrient agar medium and characterization of bacterial isolates up to generic level was done. Gram-negative bacteria like *Pseudomonas* sp., *Enterobacter* sp., *Aeromonas* sp., *Cytophaga* sp. and *Flavobacterium* sp. were found to be the pioneer in colonizing the surface within 30 min and seven genera were represented in the biofilm. Among them two genera were found belonging to Gram-positive groups which included *Micrococcus* and *Bacillus* sp. The early stage biofilm *i.e.* up to 24th hr was wholly constituted by Gram-negative groups. However, the population density of *Pseudomonas* sp. was found to be higher (315 CFU) when compared to other Gram-negative forms. Occurrence of Gram-positive group was noted only at 48th hr old biofilm (28 to 150 CFU). The period between 48 and 96th hr was the transition where both the Gram-negative and Gram-positive groups co- existed. After 96th hr, the biofilm was found constituted only by Gram-positive groups. The isolates of early stage biofilm were found to produce allelopathic substance like bacteriocin.

Key words

Marine biofilm, Bacterial succession, Biofouling

Introduction

Biofilms are defined as microbial communities of cells that are irreversibly attached to a substratum, to an interface, or to each other and are embedded into a matrix of extracellular polymeric substances that they have produced (Donlan and Costerton, 2002). Biofilm formation is a complex biological phenomenon and has been generally described as a temporal process involving a succession of distinct stages: a reversible and then irreversible attachment of planktonic bacteria onto a surface, the formation of microcolonies either by the colonel growth of attached cells or by the active translocation of cells across the surface, the coalescence of growing microcolonies to form a macrocolony and cell dispersal. It should, however, be noted that this developmental model still requires further experimental validation, especially concerning the possibility of a hierarchical order of genetic pathways (Monds and O'Toole, 2009). Materials immersed in seawater are acted upon by a series of physical, chemical and biological events which result in the formation of a biofilm complex depends on polluted nature of the environment (Srivastava *et al*., 1990; Abarzua and Jakubowski, 1995). The adhesion which is a basic cause of marine fouling is also a basic property of bacterial cells and is manifested both in marine and terrestrial environments (Balakrishnan Nair, 1995). The bacterial biofilm changes the topography and chemistry of the surface. A number of other microorganisms including fungi, diatoms, cyanobacteria, and microalgae as well as macroalgae and invertebrates settle and attach to the substance to form a complex

structure known as biofouling (Callow and Callow, 2002). Fouling organisms are known to cause serious problems by settling on man-made structures such as ship hulls, cooling system pipes of power stations and other maritime industries. These organisms induce severe corrosion on oil rigs and pipelines and affect aquaculture nets by increasing the hydrodynamic drag and increasing the expenses for cleaning (Lewis, 1994). Thus, the main objectives of the present study was to know the pattern of bacterial (Gram-positive and Gram-negative) succession in a PVC biofilm up to 7 days.

Materials and Methods

Preparation of PVC sheet and bacterial characterization: Six PVC (polyvinyl chloride) sheet was cut in to the dimension of 12" ×12" and the sheets were degreased using acetone and the sheets was mounted on a wooden rack having the total size of 75" × 15" using brass bolt and nut. The rack was immersed at 2 m depth from the mean surface seawater below the offshore platform of Central Electro Chemical Research Institute at Tuticorin unit (Fig. 1) during January, 2009. Biofilm samplings were made for a period of seven days with the following time period intervals *viz.* 30 min, 1, 2, 4, 24, 48, 72, 96, 120 and 144 hr, respectively. At every sampling period, one PVC sheet was removed for biofilm collection. The biofilm was scrapped using sterile brush in a glass tube containing sterile seawater. Bacterial enumeration was done by pour plate method. Nutrient agar medium was used to enumerate the total heterotrophic bacteria. Average bacterial counts of the replicates were recorded. Morphologically dissimilar colonies were randomly selected and isolated and were maintained in slants at 4°C for bacterial characterization. Gram staining, biochemical and motility tests were performed for preliminary identification of the bacterial isolate (Allegrucci and Sauer, 2007) was given in Table. 1.

Results and Discussion

The heterotrophic bacterial population was enumerated around 117 to 315 CFU m^{-2} (CFU –Colony Forming Unit) within 30 min. A drastic decrease in population density was observed at 1st hr (0 to 52 CFU m^{-2}) and 2 hr (32 to 38 CFU m^{-2}). Again the bacterial population rose drastically from 44 to 272 CFU m^{-2} at 4th hr and continued the similar increasing trend till 48 hr when the population attained their maximum of 39 to 150 CFU m^{-2} and the period of decline followed till 144 hr. A pattern similar to death or decline

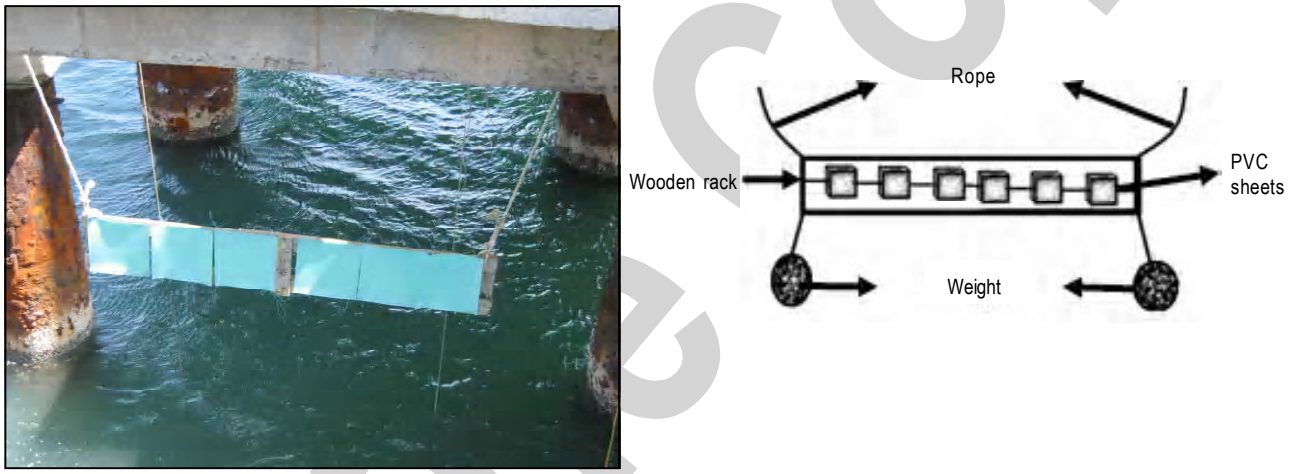

Fig. 1: Immersed PVC biofilm sheets on wooden rack

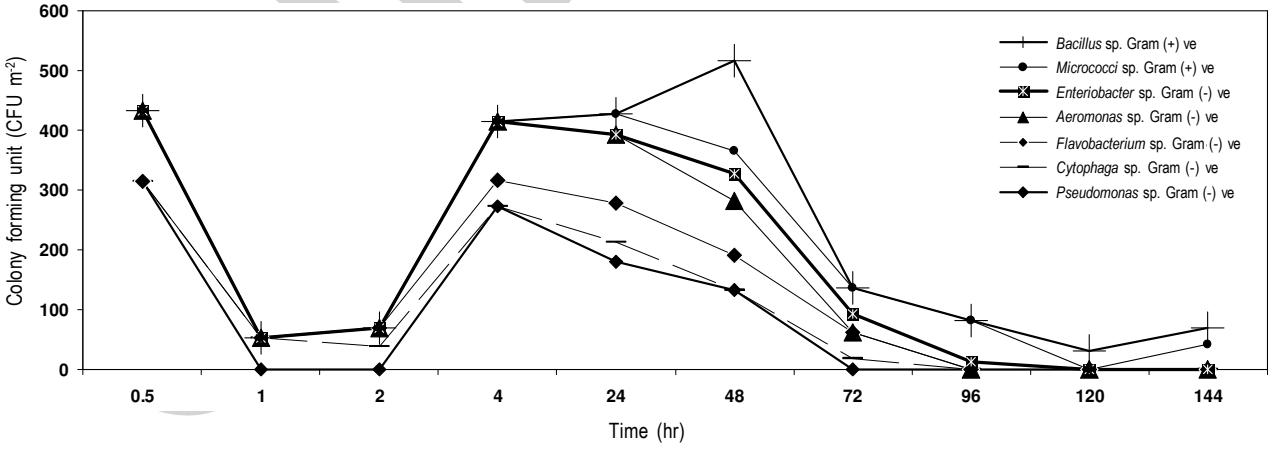

Fig. 2: Pattern of bacterial succession on PVC biofilm during 144 hr

Table - 1: Biochemical characterization of PVC biofilm bacterial Isolates

Biochemical characterization							
Gram staining	**Motility**	**Indole**	**Oxidase**	**TSI test**	**Penicillin sensitivity**	**Pigmentation**	**Suggestive genera**
G(-)ve	+	-	-	-	-	-	*Pseudomonas* sp.
G(-)ve	+	+	+	Acid and gas	-	-	*Aeromonas* sp.
G(-)ve	+	-	-	-	+	Yellow	*Cytophaga* sp.
G(-)ve	+	-	-	-	-	Orange	*Flavobacteria* sp.
G(-)ve	+	+	-	Acid and gas	-	-	*Enterobacter* sp.
G(+)ve	-	-	-	-	-	-	*Micrococci* sp.
G(+)ve	+	+	-	-	-	-	*Bacillus* sp.

+ = Positive, - = Negative

phase was observed during 96th hr. Generic composition of the heterotrophic biofilm was found dominated by Gram-negative groups (71.42 %) followed by Gram-positive groups (28.57%). Five among the seven genera was identified as Gram-negative which includes *Pseudomonas* sp., *Cytophaga* sp., *Flavobacterium* sp., *Aeromonas* sp. and *Enterobacter* sp. The two important genera of Gram-positive group include *Micrococci* sp. and *Bacillus* sp. The *Pseudomonas* sp was found to colonize first in a matter of 30 min with a population density of 315 CFU m^{-2} and they were found disappeared in the biofilm samples collected at 1 and 2 hr intervals. However, their recurrence could be noted at 4th hr and thereafter their numerical density gradually decreased and finally leading to complete devastation of this genus after 48th hr. The second dominating bacteria in the biofilm were found to be *Aeromonas* sp. with a population density of 117 CFU m^{-2}. It is interesting to note that the *Aeromonas* sp. was found to be co-aggregating with *Pseudomonas* sp. *i.e.* the period of occurrence and disappearance of these genera is essentially the same as exhibited by *Pseudomonas* sp. The progression pattern of *Cytophaga* sp. varied considerably. Though the occurrence of this genus was recorded till 72 hr, since it's entry at 1st hr, it was found disappeared at 4th and 48th hr samples indicating it's discontinuous existence in the biofilm. A gradual rise and fall of population was observed in case of *Flavobacterium* sp. Colonies began to occur at 2nd hr and attained their peak only at 24th hr and thereafter it showed declining period till 72 hr. Incidence of *Enterobacter* sp. in the biofilm could be seen in the periods between 48th and 96th hr with a poor population density of 12-45 CFU m^{-2}. It is worth mentioning that Gram-positive colonies appeared at later hours of biofilm development. The genera included *Micrococci* sp. which appeared at 24th hr with a population of 38 CFU m^{-2} was found to rise gradually till 96th hr (69 CFU m^{-2}) and after which they disappeared at 120th hr. Again its recurrence could be noted at 144th hr. Similarly, sporadic occurrence of Gram-positive spore former, *Bacillus* sp. could be noted at 48, 120 and 144th hr old biofilm. From the over all observation it is evident that none of the bacteria remain consistently in the biofilm over a period of 7 days (Fig. 2).

Occurrence and distribution of total heterotrophic bacteria in the biofilm harbour different types of bacteria and the motile bacteria rapidly colonize the aggregates (Kirobe *et al.,* 2002). Biofilm bacteria first attach themselves by electrostatic attraction and physical forces. Adhesion varies with bacterial species, substratum and electrolyte type and concentration (Sharron and Madilyn, 1986). There was some evidence for both electrostatic and hydrophobic interaction, but neither interaction could wholly the account for the adhesion. Flagellar motility and adhesion via pilli are important for initiating biofilm formation in *Pseudomonas aueroginosa* under static conditions (O'Toole and Kolter, 1998). The lipopolysaccharide (LPS) of Gram-negative bacteria is an added advantage for the bacterium to adhere and colonize (Davies *et al*., 1998). Accordingly, in the present study also the Gram-negative bacteria *Pseudomonas sp.* was found to be the pioneer bacteria to colonize the surface besides the *Aeromonas* sp. and the *Pseudomonas* sp. was found to be dominant group. In case of simultaneous species deposition, the faster growing organisms rapidly dominate while the slow growing microbe remained established and continue to increase over time (Katherine Banks *et al*., 1991). The development of microbial community in the biofilm particularly by Gram-positive groups were found affected initially, that is up to 24 hr. This could be reason for the drastic decrease of population density at 1 and 2nd hr (Grossart *et al*., 2003a,b), reported that a large fraction of bacterial isolate from marine particle are known to display antagonistic activities against the other bacteria.

The above report is further confirmed by present observation that the bacterial population rose drastically and attained their maximum population at 48th hr. During these periods bacterial diversity was also more. The diversity of bacterial species enhanced biofilm density and population interaction in the development of biofilm (Whiteley *et al*., 2001). In the present study also the dominance of bacterial groups in the biofilm kept changing with time. It was observed that certain bacteria which were found attached in the early stage biofilm got detached in the course of time and again recurred in the biofilm later. A number of reasons have been predicted so far, like lack of available nutrients, shear stress, and production of destructive enzymes like ligase, protease and production of antimicrobial substances like bacteriocin and early development of the bacterial biofilm facilitates further macrofouling (Kiarboe *et al.,* 2002). Adherent bacteria will form biofilms to an extent dictated by nutrients availability in their particular micro niche, but they may not adhere and they certainly will not form biofilm where nutrients are

lacking (Novitsky and Morita, 1976). The settlement of foulers in the marine environment depends on the biofilm produced by bacteria (Henschell and Cook, 1990). To avoid growth of fouling organisms on marine structures, it is necessary to check the proliferation of adhesive microbes. The results of the present study provides an initial step in deciphering the bacterial diversity and bacterial succession pattern with respect to Gram-positive and Gram-negative bacteria on PVC biofilm.

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