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EFFICACY OF IODOPHOR TO INACTIVATE *AEROMONAS HYDROPHILA* BIOFILM ON TWO SUBSTRATES

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The multiple day old biofilms of *Aeromonas hydrophila* on two substrates, high density polyethylene (HDPE) plastic and *Eichhornia crassipes* leaves were evaluated for its susceptibility to bactericidal action of iodophor at two dose levels (20 mg/l and 40 mg/l). The biofilm on HDPE was highly resistant to the killing action of iodophor on the days 2, 4 and 6 tested at 20 and 40 mg/l for 10 min. The iodophor was effective in inactivating the biofilm cells on *Eichhornia* at 40 mg/l for 10 min, whereas at 20 mg/l for 10 min, the 4 and 6 days old biofilm on *Eichhornia* showed resistance to iodophor. The planktonic cells were completely killed at 20 mg/l of iodophor. The study indicates that biofilms of *A. hydrophila* were resistant to iodophor in comparison to planktonic cells.

INTRODUCTION

A. hydrophila is a ubiquitous and highly heterogeneous bacterium, frequently associated with diseases in carps, eels, milkfish, channel catfish, tilapia and ayu (Amin *et al.*, 1985; Miyazaki and Jo, 1985; Das and Mukherjee, 1997; Rahman *et al.*, 1997; Nayak *et al.*, 1999; Samal *et al*., 2008). It causes the disease known as motile aeromonad septicaemia (MAS), a common cause of fish mortality in warm water fishes under stress conditions or in concert with infection caused by other pathogens. Like certain bacteria, *A. hydrophila* is capable of forming biofilms on surfaces (Costerton, 1984). However, the colonization factors of the bacterium are not fully understood. It has been suggested that the presence of wavy pili (*w*-pili) or 'flexible pili' correlates with the adherence of *A. hydrophila* (Ho *et al.*, 1990; Hokama *et al*., 1990). Merino *et al.* (1997) reported that the presence of flagella is essential for some *A. hydrophila* strains in adhesion and invasion. Lipopolysaccharide (LPS) and 43 kD outer membrane protein (OMP) have also functions as adhesin in some virulent strains isolated from fish (Merino *et al.*, 1996; Fang *et al.*, 1998).

Weeds are commonly encountered in aquatic environment in floating, submerged or rooted forms. Water hyacinth is a common problem in most of the freshwater ponds. Dried and sterile forms of water hyacinth were used in the present experiment as biofilm substrate to make it possibly contamination free from other sources.

Unlike planktonic cells of bacteria, the biofilm cells are resistant against sanitizers (Karunasagar *et al.,* 1996; Venugopal *et al.,* 1999; Jeyasekaran *et al.,* 2000), antibiotics (Anwar and Costerton, 1990), phagocytosis and the killing effect of whole blood and serum (Anwar *et al.,* 1992). According to Karunasagar *et al.* (1996) the biofilm of *Vibrio harveyi* on cement, HDPE and steel surfaces exhibited differential sensitivity to chlorine; the maximum resistance being found on the cement surfaces followed by HDPE and steel surfaces. Similar reports are available for *V. parahaemolyticus* and *Listeria monocytogenes* (Venugopal *et al.,* 1999; Jeyasekaran *et al.,* 2000). This is because of the glycocalyx layer, a physical barrier and strongly anionic layer that serve to protect the bacterial microcolonies from adverse conditions such as the presence of antibiotics and disinfectants (Costerton *et al.,* 1981). Despite the potential threat of *A. hydrophila* as a bacterial pathogen in freshwater aquaculture practices in the tropics, the biofilm formation of this bacterium is not studied according to the changing environmental conditions. With this background the present work was carried out to investigate the biofilm formation of *A. hydrophila* on two different substrates and its susceptibility to iodophor, a common disinfectant.

MATERIALS AND METHODS

A. hydrophila used in this study was isolated from epizootic ulcerative syndrome (EUS) affected *Channa* sp. The isolate was identified using Enterobacteriaceae identification kit (Personal computer with Microsoft™software of Microsoft Corporation, Redmont, W.A. 98073-9713 along with Multiscan, 340/MCC, Flow Laboratories Ltd. U.K.). The HDPE coupons were washed with commercial detergent solution and rinsed in double distilled water. The *Eichhornia* leaves were sun dried for two days and oven dried at 100 °C for 2 h to remove the excess moisture. The biofilm of *A. hydrophila* was grown in *Aeromonas hydrophila* (Ah) broth containing maltose (3.5 g), yeast extract (3.0 g), L-cysteine hydrochloride (0.3 g), bile salts (1.0 g), sodium chloride (5.0 g) and bromothymol blue (0.03 g) in one liter of distilled water (pH 7.0±0.05) following Frank and Koffi (1990). Fifty ml beakers containing 20 ml of Ah broth and HDPE or *Eichhornia* were sterilized at 121 °C (15 psi) for 15 min. A 24 h culture of *A. hydrophila* was inoculated into the media and incubated at 30 ^{0}C for six days. Observations were made on day 2, 4 and 6 by taking the samples in triplicate.

To enumerate the biofilm cells, the biofilm grown substrates were rinsed in sterile phosphate buffered saline (PBS) to remove unattached cells if any and the biofilm cells on the substrates were released by vortex-mixing for 4 min in 10 ml PBS. The serially diluted samples were plated onto nutrient agar (HiMedia, Mumbai, India) and incubated at 30 °C for 24 h. The colony counts were expressed in log cfu per cm^2. To determine the bactericidal action of iodophor (Asian Catalyst and Chemicals, Mumbai, India) on biofilm cells, the biofilm grown substrates were rinsed in sterile PBS and incubated at 30 °C for 10

min in 20 or 40 mg/l of iodophor. The viable counts after iodophor treatment were estimated as described above. The control substrates were incubated in PBS for 10 min instead of iodophor and processed similar to iodophor treated ones.

To determine the susceptibility of planktonic cells to the iodophor, a 24 h culture of *A. hydrophila* was centrifuged at 6000 xg for 10 min. The cell pellet was resuspended in sterile PBS; an aliquot of this was serially diluted and plated onto the nutrient agar. To the same suspension, iodophor was added to give a final concentration of 20 or 40 mg/l and it was incubated at 30 ^{0}C for 10 min. The serially diluted aliquots were plated as described above. Data were expressed as means of three samples. The level of significance of the iodophor concentrations against each sampling day was determined by analysis of variance (ANOVA) (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

The cell densities of the biofilms of *A. hydrophila* on HDPE and *Eichhornia* are shown in Table 1 and Table 2, respectively. The cell counts on HDPE on all the days tested were slightly higher compared to that *Eichhornia* substrate. Table 1 shows that the biofilm on HDPE was highly resistant to the killing action of iodophor on the three sampling days tested at 20 and 40 mg/l for 10 min. The biofilm formation and the concentrations of iodophor were significantly different ($p \leq 0.05$) for HDPE. The biofilm cells on *Eichhornia* showed mixed results in order of their susceptibility to iodophor as shown in Table 2. The iodophor was effective in eradicating the biofilm cells on *Eichhornia* at 40 mg/l for 10 min, whereas at 20 mg/l for 10 min, the 4 and 6 days old biofilm on *Eichhornia* showed resistance to iodophor. The biofilm formation and concentrations of iodophor were not significantly different ($p > 0.05$) for *Eichhornia.* However, the planktonic cells of *A. hydrophila* cells were completely killed at 20 mg/l iodophor for 10 min.

Table 1. Logarithmic growth of *A. hydrophila* biofilm (log cfu/cm^2) on HDPE and the viability of the same at different concentrations of iodophor. Values are mean ± SD; data in parenthesis is indicated as percentage variation over control. Means bearing common superscript are not significant at 5%

Days	Control	Concentration of iodophor	
		20 mg/l	40 mg/l
2	4.392±0.012^a	4.336±0.01^a (1.275)	4.297±0.009^b (2.163)
4	4.828±0.009^a	4.716±0.009^a (2.319)	4.394±0.024^b (2.319)
6	4.898±0.008^a	4.758±0.007^a (2.858)	4.462±0.013^b (8.901)

The present study shows that *A. hydrophila* is capable of forming biofilms on surfaces and exhibits resistance to iodophor when compared to planktonic cells of the same species. This study also shows that the biofilm formation of *A. hydrophila* on two surfaces is different, in the same way its susceptibility to iodophor was different on the two substrates tested. This might be due to the variations in the physico-chemical properties of the substrates used. As the age of biofilm progresses, the thickness of the biofilm also increases and renders it more resistant to iodophor. Thus, the results have great significance on aquaculture practices, especially in hatchery operations of freshwater fish and shellfish, where *A. hydrophila* is autochthonous microflora of water, fish and shellfish. The routine prophylactic measures adopted might not be efficient in inactivating the already attached cell population on hatchery equipment such as fibreglass, cement cisterns, plastic wares and water distribution systems, despite the fact that the incoming water is disinfected. On the other hand, the biofilm formation of *A. hydrophila* in grow-out culture systems or in natural aquatic environment on available surfaces such as dead and decaying matter, aquatic plants, artificial substrates introduced (bamboo poles, hide-outs) cannot be ruled out due to the persistence of this bacterium and its ability to sustain wide range of physico-chemical limits (pH 5.2-9.8, temperature < 10-45 ^{0}C) (Doukas *et al*., 1998).

Table 2. Logarithmic growth of *A. hydrophila* (log cfu/cm^2) biofilm on *Eichhornia* and the viability of the same at different concentrations of iodophor. Values are mean ± SD; data in parenthesis is indicated as percentage variation over control. Means bearing common superscript are not significant at 5%.

Days	Control	Concentration of iodophor	
		20 mg/l	40 mg/l
2	3.999±0.011	ND	ND
4	4.262±0.0372	4.041±0.0157^a (5.185)	ND
6	4.745±0.462	4.528±0.0335^a (4.573)	ND

ND = not detected,

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