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STUDYING *IN-VITRO* APPLICABILITY OF HOST-INDEPENDENT STRAIN OF *BDELLOVIBRIO BACTERIOVORUS* AS A BIOFILM REDUCING AGENT

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ABSTRACT

Biofilms are surface-attached microbial communities with phenotypic and biochemical properties distinct from free swimming planktonic cells. The capability of the gram-negative predatory bacterium *Bdellovibrio bacteriovorus* to control and reduce an existing *Serratia marcescens* biofilm was evaluated by colony biofilm assay. A reduction in biofilm biomass was observed as early as 2 h after exposure to the predator, and as much as 75% reduction after 6hrs of exposure. The ability of *B. bacteriovorus* to reduce an existing biofilm was confirmed by reduction in CFUs after exposure of predator.

Keywords: Biofilm, Biofilm reduction, *Bdellovibrio bacteriovorus*

INTRODUTION

Biofilms are surface-attached microbial communities with phenotypic and biochemical properties distinct from free swimming planktonic cells. Biofilm formation is thought to begin when bacteria sense environmental conditions that trigger the transition to life on a surface, followed by a multi step process leading to the formation of a mature biofilm [1]. Biofilms are implicated in a significant amount of human bacterial infections. Bacterial biofilms also cause fouling, product contamination, equipment failure, and decreased productivity due to downtime for system cleaning and replacement. Antibiotic doses which kill suspended cells, for example, need to be increased as much as 1,000 x to kill biofilm cells (and these amounts would kill the patient first!). Disinfection rates for biofilm cells are also far below planktonic kills by antimicrobials.

Biofilms are a concern in the food industry, in that they can arise from raw materials, surfaces, people, animals, and the air. Once food or a surface in a food processing plant is contaminated, the bacteria can form colonies, and eventually biofilms. Other microorganisms may attach to the initially adhered microorganism, and a biofilm could form. Cleansers used to wipe the counter will kill planktonic or single cells of bacteria, but they may not be able to penetrate biofilms. Foods that come into contact with the counter are then susceptible to contamination.

Various techniques have been evaluated for their capability to manage and control biofilms, among them are the use of different materials and coatings to reduce initial cell adhesion to surfaces and a variety of treatments aimed at decreasing or

destroying already existing biofilms, such as heat, cleaning regimens, low-power laser, sonication, chemical treatments, antibiotics, quorum-sensing analogs, and lectins. Recently, there has been a renewed interest in the use of biological control agents against biofilms. These agents include the use of invertebrates and protozoa to reduce biofilms by means of grazing and the use of bacteriophages. *Bdellovibrio* can be used potentially against the biofilm formation as it is predatory in nature. [2]

Bdellovibrio spp. is Gram negative, 0.20–0.4 to 0.5–1.4 □m, motile by means of single, polar, sheathed flagellum. [3] What characterises this bacterial genus as unique is its predatory behaviour. *Bdellovibrio*s attack other Gram negative cells, penetrate their periplasm, multiply in their cytoplasm, and finally burst their cell envelopes to start once again. [4]

Bdellovibrios are largely found in wet, aerobic environments and were first isolated from soil, where they are commonly encountered [5]. However, they can also be found in fresh and brackish water, sewage, water reservoirs, and seawater. Another environmental niche with which bdellovibrios have been associated are biofilms. It is believed that biofilms might offer good conditions for bdellovibrios' survival since these organisms have been found in natural marine biofilms but are not always recovered from the surrounding water. It is suggested that in a biofilm bdellovibrios can benefit from higher prey density, which has been shown to be necessary for *Bdellovibrio* survival [6].

MATERIALS AND METHODS

Bacterial strains, media and culture conditions: HI *Bdellovibrio bacteriovorus* strain (DSMZ 12732) was obtained from DSMZ Germany, and *S. marcescens* (MTCC 97), was obtained from MTCC, Chandigarh, India. HI *Bdellovibrio bacteriovorus* was maintained on YPSC medium [*], while *S. marcescens* was maintained on medium 3[**]. To form biofilm of *S. marcescens* and assay biofilm reduction by 'Colony Biofilm Assay' [7] 25 mm diameter cellulose acetate membranes of pore size 0.22 µm, 180 mm Petri plates, UV light source, Bumper tubes, Saline, Sterile distilled water were used. *S. marcescens* cell grown on NB [***] and were enumerated as CFU/ml on NA [***] plates

*YPSC medium: contains Yeast Extract, Peptone, Sodium acetate trihydrate, L-Cysteine (free base), Agar(1.5%) (supplemented with $MgCl_2.6H_2O$: 3mM and $CaCl_2.2H_2O$: 2mM, after autoclaving and pH is adjusted to 7.6)

** Medium 3: Beef extract, Yeast extract, Peptone, Sodium chloride, Agar(1.5%) (as given in manual with strain from MTCC)

*** NB and NA: Nutrient broth and Nutrient Agar medium

Biofilm formation: 24 hr grown culture of *S. marcescens* in NB (OD_{600} = 0.25) was taken. Using sterilised forceps, thirteen 25 mm diameter cellulose acetate membranes of pore size 0.22 µm were placed in sterile petri plates. Plates were placed under UV light source for 30 mins, 15 mins for each side. The sterilised membranes were placed on NA plates, supplemented with $MgCl_2.6H_2O$: 3mM and $CaCl_2.2H_2O$: 2mM and inoculated with 20 □l of *S. marcescens* culture. The plate was incubated for 18 hrs at 30°C.

Study of biofilm reduction: Membranes were inoculated with 3 different concentrations of HI *B. bacteriovorus* (40□l on each membrane) as in Table 1. One membrane was kept as control to measure growth of biofilm after 18 hrs incubation. Four membranes (1 control, 3

with each concentration) were assayed after every 2 hrs for sample biofilm growth. Sample biofilm growth assay was done in the following manner: (i) Membrane was aseptically transferred in 10 ml sterile saline, in 15 ml tubes. (ii) Tubes were vortexed for about 2 mins. (iii) Serial dilutions up to 10^{-3} were prepared. (iv) Last dilution was mixed in agar and poured on petri plate. (v) Plates were incubated for 24 hrs and average number of colony forming units (CFU) was measured. CFU was measured using the following formula:

Dilution made * Amount inoculated = Plated Dilution
Plated Dilution^{-1}= Dilution Factor
Dilution Factor * Counted Colonies = CFU/ml [8]

RESULTS

To see effect of HI *B. bacteriovorus* with different concentrations and after particular time period we studied three different concentrations like 10^5, 10^7 & 10^9 CFU/ml and assayed after every 2hrs for 6hrs.

Qualitative examination:

Figure 1 shows difference in intensity of colour of biofilm formed after 6hrs at different concentrations of *B. Bacteriovorus.*

Quantitative assay:

Table 2 shows reduction in CFUs of *S. marcescens* as CFU x 10^9 /ml with different concentration and at different time of action of *B. Bacteriovorus.* Also Figure 2 shows graph in which it is seen that all the concentrations show sharp decrease within 2 hrs of inoculation of HI, and gradual decrease upto 6 hrs of inoculation and that is as significant as 75%. The CFU/ml of biofilm forming organism decreased with increasing concentrations of HI. Also a small and gradual decrease is seen in control run, which is due to prolonged, undisturbed adherence of *S. marcescens* to the membrane.

CONCLUSION

This study proves the ability of HI *Bdellovibrio bacteriovorus* to be successfully used as biofilm reducing agents. These biofilms which are usually formed on abiotic surface such as fermentation vessels, tiles, medical equipments and implants etc. may not be completely eradicated but its growth can be controlled, thus avoiding further building of biofilm.

DISCUSSION

The organism under study, *Bdellovibrio bacteriovorus*, is a Gram negative bacterium. Its uniqueness lies in the fact that it naturally feeds on range of pathogenic gram negative bacteria, while having distaste for mammalian and human cells [10]. It is shown in animal experiments that *Bdellovibrio* only has a weakly immunogenic surface, which does not produce serious life threatening reactions in test animals. These attributes, together with the facts that certain *Bdellovibrio* strains show a very narrow prey spectrum and are capable of penetrating the same tissues as may human-pathogens, gives promise to the development of novel anti-microbial strategies. Thus, *Bdellovibrio* may be developed into a therapeutic agent that could be used as a "**living antibiotic**" [4].

Biofilms have been found to be involved in a wide variety of microbial infections in the body. Infectious processes in which biofilms have been implicated include common problems such as urinary tract infections, catheter infections, middle-ear infections, formation of dental plaque, coating contact lenses and less common but more lethal processes such as endocarditis, infections in cystic fibrosis, and infections

of permanent indwelling devices such as joint prostheses and heart valves. Biofilms can also be formed on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and intrauterine devices.

More recently it has been noted that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds [1].

Serratia marcescens is a human pathogen, involved in nosocomial infections, particularly catheter-associated bacteremia, urinary tract infections due to slime layer formation and wound infections. Most *S. marcescens* strains are resistant to several antibiotics because of the presence of R-factors, which are a type of plasmid that carry one or more genes that encode resistance; all are considered intrinsically resistant to ampicillin, macrolides, and first-generation cephalosporins (such as cefalexin).[9]

In this study, we have successfully demonstrated the *in-vitro* reduction in biofilm of *S. marcescens* using *Bdellovibrio bacteriovorus*. Similar evidence of reduction of a biofilm of *E. coli* and *Ps. fluorescence* by static assay and flow cell experiments has been reported. [2]

Recently a US patent for 'Use of Bdellovibrionaceae as an antimicrobial agent' has been published. [10]

Though *Bdellovibrio bacteriovorus* has been found to be unable to infect human cells, thorough studies regarding its safety and efficacy need to be done. But the potential of this organism is quite great and if things work out, it might be just the answer to the increasing menace of drug resistance in gram negative bacteria. [10]

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Table 1 Additions of different concentrations of HI B. bacteriovorus on preformed biofilm of S. marcescens

Disc	HI inoculum (OD)
C_1(control)	-
D_1	H_1
D_2	H_2
D_3	H_3
C_2(control)	-
D_4	H_1
D_5	H_2
D_6	H_3
C_3(control)	-
D_7	H_1
D_8	H_2
D_9	H_3
C_4(control)	-

Here, H_1= 10^5 CFU/ml, H_2= 10^7 CFU/ml and H_3=10^9 CFU/ml

Table 2 CFUx 10^9/ml of S. marcescence after action of B. bacteriovorus

Disc	~CFU(x 10^9)/ml
C_1(control)	2.1
D_1	1.3
D_2	1.0
D_3	0.722
C_2(control)	2.05
D_4	1.0
D_5	0.881

D_6	0.622
C_3(control)	1.96
D_7	0.865
D_8	0.637
D_9	0.532
C_4(control)	1.8

Here, D-1, 2, 3 are assayed after 2hrs, D-4, 5, 6 are assayed after 4 hrs, and D-7, 8, 9 are assayed after 6hrs.

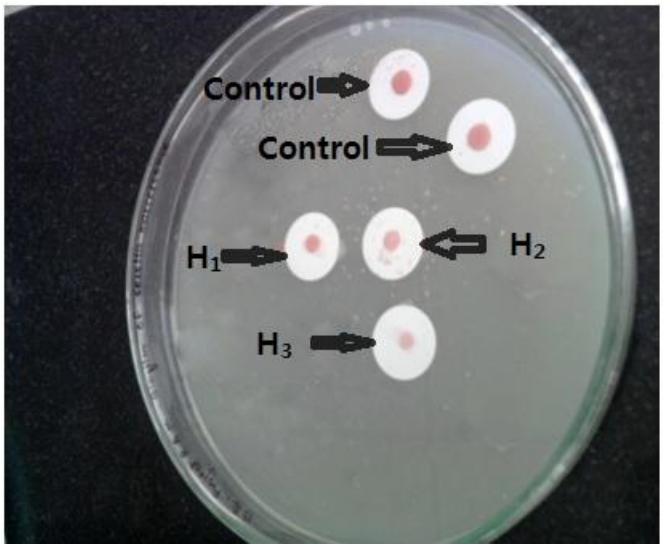

Figure 1 Qualitative analysis, biofilm reduction at 6hrs after inoculation of B. bacteriovorus

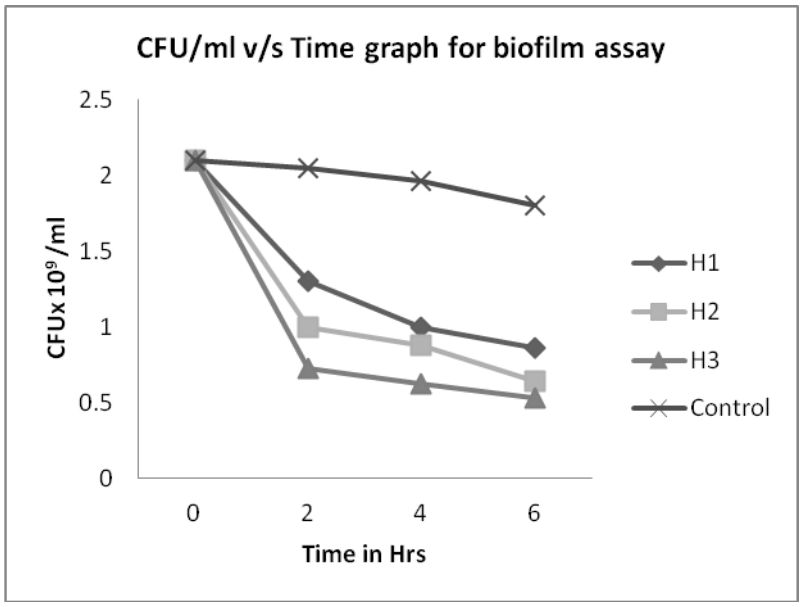

Figure 2 Graph of reduction in CFU's of S. marcescence, H_1, H_2, H_3 are 10^5, 10^7, 10^9 CFU/ml of B. bacteriovorus