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The Efficacy of Bacteriophage as a Method of Biofilm Eradication

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The ability of bacteriophage and their associated polysaccharide depolymerases to control enteric biofilm formation was investigated. Bacteriophages specific for *Enterobacter* strains were isolated from primary effluent sewage. Combinations of three phages were required before complete eradication of single species biofilms of *Enterobacter cloace* occurred. Attempts to eliminate a susceptible bacterial population within a dual species biofilm were unsuccessful. It was thought that the structural heterogeneity of the biofilm produced pockets of unattainable, susceptible bacteria. These results suggest that phage and bacteria can co-exist stably within a biofilm. Bacteriophage, would, therefore, make poor tools for the control of biofilm formation. However, the results suggest that combined treatment with bacteriophage polysaccharide depolymerases and disinfectant may provide an alternative control strategy.

Keywords: biofilm; bacteriophage; biocontrol

INTRODUCTION

With the resistance of pathogenic bacterial strains to traditional antibiotics becoming an increasing problem, there has been interest in the development of alternative methods of control. Bacteriophage and their associated enzymes have been suggested as alternative agents to chemical treatments. This technology was developed, and is still employed within the former Soviet Union, where bacteriophage are reportedly used to treat both primary and nosocomial infections, alone or in conjunction with traditional antibiotics (Holzman, 1998). Non-medical uses of bacteriophage include the selective removal of pathogenic or problematic species from contaminated surfaces. Kudva *et al.* (1999) described the isolation of *Escherichia coli.* O157 antigen-specific bacteriophage. These phages could play a role in biocontrol of *E. coli* in fresh foods, without compromising the viability of other normal flora or food quality. The bacterial spot pathogen of tomato plants, *Xanthomonas campestris* pv. *vesicatoria* was successfully controlled with bacteriophage (Flaherty *et al.*, 2000). A viral polysaccharide depolymerase was also evaluated as a potential tool to control fire blight (Kim & Geider, 2000).

The majority of studies have focused on host: phage dynamics in planktonic cultures, particularly *E. coli* phages (Bohannan & Lenski, 2000) and, more recently, marine bacteriophages (SimeNgando, 1997). There has been very little published on the interactions of bacteriophage within biofilms. Bacteriophages are capable of infecting bacterial biofilms. Indeed, a biofilm may be the preferred site for phage reproduction when compared to the less accessible bacteria found in liquid cultures (Wiggins & Alexander, 1985). Doolittle *et al.* (1996) examined the infection of *E. coli* and *Pseudomonas aeruginosa* biofilms by phage T4 and E27, respectively. Full infection of the thinner *E. coli* biofilm occurred, but the thicker biofilms of *P. aeruginosa* were only infected at the surface layers; access to the deeper layers was restricted. Hughes *et al.* (1998a; 1998b) have shown that phage possessing specific polysaccharide depolymerases were able to degrade a pathway through the biofilm EPS to gain access to the bacterial surface. It was shown that the majority of bacteria were being removed from the biofilm due to the action of the enzyme before the cells had a chance to lyse. Adding phage to biofilms containing

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a phage-resistant strain of *Enterobacter agglomerans*, and again where only soluble enzyme was added to the biofilm, the action of the phage enzyme alone still removed a substantial quantity of biofilm material. It was hypothesised that if the host bacteria produced exopolysaccharide, phages with polysaccharide depolymerase activity could be used to selectively remove the polysaccharide from the biofilm.

The aim of this study was to examine the efficacy of bacteriophage and their polysaccharide depolymerases as a method of biofilm eradication. In addition, the high specificity of phage may make them particularly useful tools in the selective removal of a potentially pathogenic species within a mixed species biofilms. However, in a synergistic dual species biofilm of *E. agglomerans* and *K. pneumoniae*, addition of a phage enzyme specific for the EPS of the *E. agglomerans* strain removed both the strains from the biofilm (Skillman *et al.*, 1999). The inability of a strain-specific phage to eradicate one strain in this mixed species biofilm was thought to be due to the close confinements of the strains within the biofilm. In addition, the interactions between the extracellular polysaccharides produced by the two strains, which resulted in changes to the physical properties of the polymers, may also have affected the selective removal of the *E. agglomerans* strain. Further studies in the authors' laboratory have indicated that this removal of both species in a dual species biofilm by a strain-specific phage may also have been due to the high titres of phage used. It was indicated that smaller titres of phage might be more successful in selectively removing one species (Napier & Sutherland, unpublished observations). Smaller titres of bacteriophage were, therefore, used to selectively remove one species from a dual species biofilm. The action of combined treatments of disinfectant and phage enzyme as a potentially effective control strategy was also investigated.

MATERIALS AND METHODS

Bacteria

The strains *Enterobacter cloace* NCTC 5920 and *E. agglomerans* were used. *E. agglomerans* strain *Ent* was isolated from biofilms on industrial surfaces (Dr M V Jones, Unilever Research Laboratory, Port Sunlight, UK).

Bacteriophage Isolation, Purification and Concentration

Primary effluent sewage was centrifuged at 10000g for 20 min to remove particulate material, then passed through a 0.45 μm filter (Millipore) to remove bacteria. The filtered sewage (50 ml) was added to an equal volume of prewarmed double strength YE medium (Sutherland & Wilkinson, 1965), and 20 ml of overnight bacterial culture added. The optical density (600 nm) of the culture was monitored over a 12 h period. A fall in OD due to cell lysis indicated the probable presence of an infectious phage. The culture was centrifuged and the supernatant (containing the phage particles, phage components and the contents of the lysed bacterial cells) was collected. When dialysed, concentrated and filtered (Millipore, 0.45 μm pore size), the supernatant acted as an impure source of bacteriophage and bacteriophage proteins (including any polysaccharide depolymerases).

To purify the bacteriophage, the supernatant was serially diluted in phage buffer (100 mM TRIS, 50 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$) and 0.1 ml added to 3 ml sloppy agar to which 0.1 ml of overnight culture had been added. The sloppy agar mixture was poured onto a YE agar plate and incubated overnight at 30°C. Individual plaques were picked off and inoculated into flasks of exponentially growing susceptible bacteria, and again incubated overnight at 30°C. The above process of centrifugation and filtration was then repeated. The supernatants were concentrated against polyethylene glycol (PEG 6000) for 16 h, filtered (Millipore, 0.45 μm pore size) and stored at 4°C.

Screening of Bacteriophage Activity on Bacteria

For experimental purposes, a phage was required that would selectively lyse only one species of bacteria in a dual species biofilm. The phages were screened for lytic and polysaccharide depolymerase enzyme activities on strains *Ent* and 5920. A lawn of each bacterial strain was spotted with 10 μl of each concentrated phage solution. The plates were incubated overnight at 30°C, and the lawns examined for formation of plaques and haloes of decapsulated bacteria, which is indicative of the presence of phage possessing a polysaccharide-degrading enzyme (Lindberg, 1977). The bacteriophage isolated (phage 11229, ϕEnt, Blackburn and Philipstown), and their phage and enzyme activity on the strains *Ent* and 5920 are shown in Table I. The phage ϕ1.15 had previously been isolated (Hughes *et al.*, 1998b).

Biofilm Susceptibility Assays

The biofilm batch culture vessel contained glass coverslips supported by a stainless steel stand (Hughes *et al.*, 1998a). The stand was capable of supporting 12 22 mm × 22 mm glass coverslips. The vessel held 250 ml of YE medium and was incubated

TABLE I The bacteriophage isolated and their phage and enzyme activity on *Ent* and 5920

Phage name	Phage activity	*Ent*	5920
11229	Phage	−	+
	Enzyme	−	+
ϕEnt	Phage	+	+
	Enzyme	+	+
ϕ1.15	Phage	−	+
	Enzyme	−	+
Blackburn	Phage	−	+
	Enzyme	−	+
Philipstown	Phage	+	−
	Enzyme	−	−

at 30°C. The medium was agitated using a magnetic stirrer (120 rpm).

Single and dual species biofilms were obtained by inoculation of the culture vessel using 24 h planktonic cultures. The biofilm cultures were incubated o/n (16 h) at 30°C. Biofilm cell numbers were estimated by viable count. Slides were removed from the culture vessel and washed in 3 changes of sterile phosphate buffered saline (PBS) to remove any unadhered cells. The slide was then placed in a sterile 10 ml beaker containing 1 ml sterile PBS and mechanically crushed to a fine residue to remove the adhered cells. Viable counts were made of the solutions of mechanically removed cells and the number of CFU cm^{-2} estimated. In the case of the dual species biofilm, the strains *Ent* and 5920 could be easily distinguished by colony morphology. Three slides were randomly selected from three culture vessels to obtain a mean number of attached cells in the single and dual species biofilm.

For the bacteriophage experiments, single and dual species biofilms were grown on glass coverslips overnight. The slides were removed from the cultures, washed in sterile PBS to remove any unadhered cells and placed into fresh YE medium. The cultures were then inoculated with bacteriophage to allow a 1:10 ratio of phage and cells, and also 1:100 and 1:1000 ratios. Numbers of phage were estimated by titration of the phage solution against a susceptible bacterial strain. In the case of the dual species biofilms, the phage:cell ratios were based on numbers of the susceptible species within the mixed species biofilms. Single and dual species controls without phage were also included. Viable counts were estimated after 24 h. In each case, three slides were randomly selected from three culture vessels to obtain a mean number of attached cells in the single and dual species biofilm.

Separation of Phage Enzyme from Phage Particles

The phages were produced in high titre by replication in a susceptible host using the methodology of Hughes *et al.* (1998b). The phage were separated from most bacterial cell debris by centrifugation at 21,000g (8×50 ml tubes) for 20 min. The supernatant was dialysed against phage buffer at 4°C for 24 h, and then concentrated against PEG for 16 h. The concentrated supernatant was re-centrifuged at 21,000g for 20 min, then passed through Millipore Vivacell 70 membrane filters (< 100 kDa cut-off size) to separate the soluble phage enzyme from the phage particles. The filtrate was refiltered (Millipore, 0.22 μm pore size) to ensure no phage remained. The absence of lytic phage was confirmed by spotting on to lawns of susceptible bacteria. The soluble enzyme supernatant was concentrated against PEG and stored at −20°C.

Combined Treatments of Phage Enzyme and Disinfectants

Biofilms of *Ent* were grown overnight and inoculated with ϕEnt polysaccharide depolymerase. Undiluted disinfectant was added to the biofilms and incubated for 5 min. This was followed by a phage enzyme treatment (5 min). Viable counts were then estimated as above.

RESULTS

Effect of Bacteriophage on Dual Species Biofilms

Single and dual species biofilms of *Ent* and 5920 were inoculated with bacteriophage ϕ1.15 to allow 1:10, 1:100 and 1:1000 ratios of phage and biofilm bacteria. Similarly, single and dual species biofilms of *Ent* and 5920 were infected with Philipstown phage. Bacteriophage ϕ1.15 has phage and polysaccharide depolymerase activity against strain 5920, but no effect on strain *Ent*, whereas, Philipstown phage had phage activity against *Ent*, and no activity against 5920 (Table I). Figure 1 demonstrates the effect of the different concentrations of ϕ1.15 phage on single species biofilms of 5920 and dual species biofilms of *Ent* and 5920, and Figure 2, the effect of Philipstown phage on single species biofilms of *Ent* and dual species biofilms of the two strains. As ϕ1.15 phage had no effect on single species biofilms of *Ent*, and Philipstown phage had no effect on single species biofilms of 5920, these data are not shown.

An ANOVA test for the three phage treatments of different concentrations, 1:10, 1:100 and 1:1000, on the single species biofilms revealed no significant difference between each treatment (action of ϕ1.15 on 5920 single species biofilm $p = 0.248$ and for the Philipstown phage infection of *Ent* single species biofilms, $p = 0.46$). In the case of the single species biofilms, the three phage treatments produced similar bacterial removal from the biofilms.

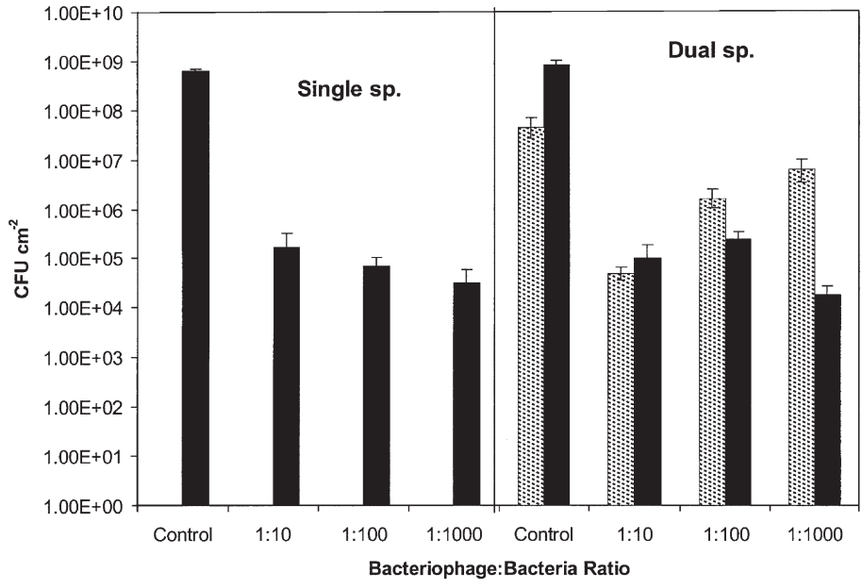

FIGURE 1 The action of ϕ1.15 on single species biofilms of 5920 and dual species biofilms of *Ent* (▨) and 5920 (■). Bacteriophage were inoculated to allow bacteriophage:bacteria ratios of 1:10, 1:100 and 1:1000. Bars = SE; n = 9.

This trend was also evident in the dual species biofilms. For both Figure 1 and Figure 2, it can be seen that the numbers of the susceptible bacteria within the dual species biofilms for each phage treatment remained comparatively constant (ANOVA for action of ϕ1.15 phage, $p = 0.71$ and $p = 0.96$ for the Philipstown phage experiments).

Figure 1 also demonstrates that there was an overall decrease in the numbers of *Ent*, the unsusceptible bacterium with the pair when compared with the dual species control biofilm. The phage ϕ1.15 was an enzyme that is capable of depolymerising the 5920 extracellular polysaccharide (Table I). The action of this enzyme presumably shears off fragments of 5920 polysaccharide, and if *Ent* and 5920 cells were in close proximity, this would result in removal of both strains from the biofilm. This trend is not as evident with the dual species biofilms infected with Philipstown phage. The dual species biofilms inoculated with 1:100 and 1:1000 ratios of bacteriophage:bacteria contained similar numbers

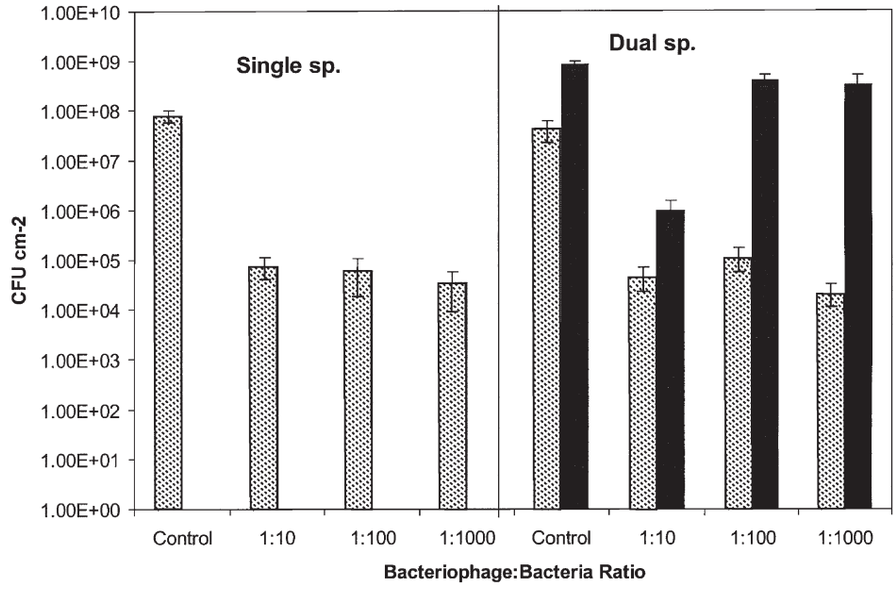

FIGURE 2 The action of Philipstown phage on single species biofilms of *Ent* and dual species biofilms of *Ent* (▨) and 5920 (■). Bacteriophage were inoculated to allow bacteriophage:bacteria ratios of 1:10, 1:100 and 1:1000. Bars = SE; n = 9.

of 5920 cells to the control, untreated biofilms. Philipstown phage does not have polysaccharide depolymerase activity (Table I). The action of this phage might be more localised to areas of *Ent* colonisation within the biofilm, reducing the sloughing off of larger fragments of biofilm. The unsusceptible bacterial population within the dual species biofilms also increased with decreasing phage inoculum size. This was particularly evident with the ϕ1.15 phage data. This suggests that the higher phage inocula were more destructive and less selective in their effect on the biofilms.

Most importantly, the results in Figures 1 and 2 indicate that the complete eradication of the single species biofilms and of the susceptible bacterial population in the dual species biofilms was not achieved.

Addition of Multiple Phages to Dual Species Biofilms

It was hoped that the addition of two or three phages to dual species biofilms of *Ent* and 5920 would be more successful in selectively removing 5920 cells. From the phage isolated from primary effluent sewage, 11229 and Blackburn bacteriophage had both phage and enzyme activity against 5920, but no activity against *Ent*. The effect of a combination of phage ϕ1.15 and 11229, and of ϕ1.15, 11229 and Blackburn was, therefore, examined and compared with the action of a single treatment of ϕ1.15 phage.

Figure 3 shows the addition of single, double and triple doses of phage to single species biofilms of 5920 and dual species biofilms of *Ent* and 5920. Biofilms were inoculated with phage to allow a 1:100 ratio of phage and bacteria. The bacteriophage inocula contained equal amounts of each phage. Cultures were incubated for 24 h, and viable counts

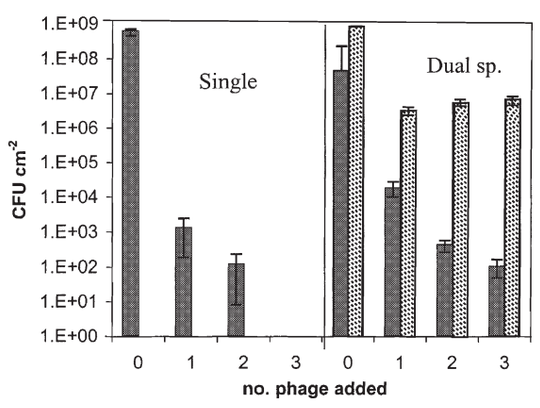

FIGURE 3 Addition of several phages to single species biofilms of 5920 and dual species biofilms of *Ent* (▨) and 5920 (▩). Combinations of ϕ1.15 and 11229 ('2 phage') and ϕ1.15, 11229 and Blackburn ('3 phage') were compared to the action of a single dose of ϕ1.15. Bars = SE; n = 9.

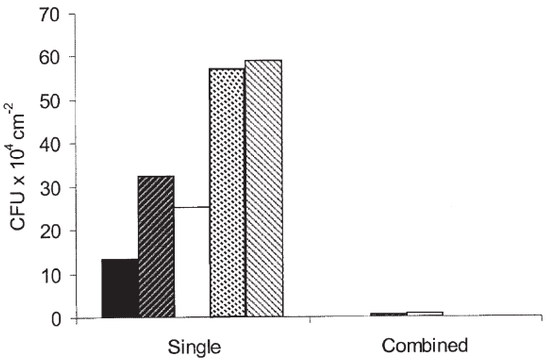

FIGURE 4 Combined treatments of bacteriophage polysaccharide depolymerase and disinfectant. *Ent* single species biofilms were treated with a hypochlorite based disinfectant (▨), a non-ionic disinfectant (□), an amphoteric based disinfectant (▩) or a quaternary ammonium compound (▧), or phage enzyme (■), or a combination of the two. All samples were the mean of 3 replicates.

estimated. The results indicate that the inoculum containing three phage types was required to eradicate the single species biofilm of 5920. Although this combination was also most successful in selectively removing 5920 cells from the dual species biofilm, again complete removal of 5920 cells from the biofilms was not achieved.

Combined Effects of Phage and Disinfectant

The action of combined treatments of disinfectant and phage enzyme as a potentially effective biofilm control strategy was investsigated. Figure 4 demonstrates the removal of single species biofilms of *Ent* using a polysaccharide depolymerase and a selection of disinfectants, and compares this with combinations of both disinfectant and enzyme. Enzyme was added to the biofilms before the addition of disinfectant, as this was thought to remove cells from the biofilm, giving better access to the remaining attached cells. The combined effects of phage enzyme and disinfectant were more effective than either of these alone.

DISCUSSION

The complete eradication of both single and dual species biofilms was not achieved. Indeed, communities of bacteriophage and bacteria have been shown to be remarkably stable. Horne (1970) reported the coexistence of phage T4 and *E. coli* for periods as long as 52 weeks. Much of the coexistence of phage and bacteria has been attributed to the continual appearance of phage-resistance mutants, and phage mutants unable to overcome bacterial

resistance (Balakshina *et al.*, 1992). Although the emergence of resistance was not evident in this study, the bacteriophage and bacterial biofilms were only incubated for a maximum of 24 h. Given longer periods of incubation, resistance of the bacteria to the bacteriophage would undoubtedly appear. Of more importance to biofilm communities, Schrag and Mittler (1996) provided evidence to suggest that the occurrence of 'spatial refugees', brought about through growth on the walls of culture vessels, might be important in stabilising bacteria-phage interactions. Nutritional limitation is also known to influence stability. Bohannan and Lenski (1997) observed that increasing the input of nutrients led to a large increase in phage numbers, a small increase in bacteria and a reduction in the dynamic stability of both populations. Within a biofilm environment, both spatial heterogeneity and nutritional limitations are common occurrences. These theories suggest growth as a biofilm would increase the stability of bacteria-phage interactions.

Studies by Ripp and Miller (1997) suggest that pseudolysogeny may play an important role in stabilising phage-host interactions. Pseudolysogeny is described as an interaction in which the nucleic acid of the phage resides within a starved host in an unstable, inactive state, allowing the phage to survive for extended periods of time in natural ecosystems. Moebus (1997) provided evidence to suggest the presence of a pseudolysogeny-inducing agent, 'factor X'. Moebus was able to clot pseudolysogenic cells with an antiphage serum, and proved that this was not due to the presence of virions attached to the cell surface. It was hypothesised that the production of an extracellular 'factor X' could block the receptors used by phage to infect a bacterium, thus controlling the number of host cells being infected and ensuring the survival of both host and phage. In a densely packed environment such as a bioflim, this strategy would be extremely efficient in stabilising the interactions between phage and host. This theory could also explain why the use of three phages successfully eradicated the single species 5920 biofilm. If each phage used a different receptor, a cell could still be infected with an alternate phage.

In a study of bacteriophage replication in cultures of *Staphylococcus epidermidis*, *Bacillus subtilus* and *E. coli*, cell densities $>10^4$ CFU ml^{-1} were required before bacteriophage multiplication occurred (Wiggins & Alexander, 1985). In the present study, the three treatments of different phage concentrations produced similar results in the single species biofilms of *Ent* and 5920 (Figures 1 and 2). Again, this suggests that bacteriophage are able to detect the number of cells within a population, and control multiplication in order to achieve a stable phage: bacteria coexistence.

The results in this paper indicate that combinations of three phages are required to completely eradicate a single species biofilm of strain 5920 (Figure 3). Biofilms in natural and industrial environments more commonly consist of complex communities of microorganisms. The removal of mixed species biofilms using bacteriophage would become increasingly complex, and it would seem unlikely that bacteriophage are suitable for removing mixed species biofilms from industrial surfaces. A constant fluctuation between phage-resistant and phage-susceptible bacteria, and also changes to phage-host ranges are likely to occur. It has also been suggested that bacteriophage could be used to remove a pathogenic species, such as *E. coli* O157 from contaminated surfaces (Kudva *et al.*, 1999). These studies reveal that the presence of a nonsusceptible bacterial population within a biofilm could protect bacteriophage-susceptible strains from phage attack, possibly by creating 'spatial refugees' within the depths of the biofilm.

Hughes *et al.* (1998a) demonstrated that treatment to remove EPS using phage polysaccharide depolymerases was effective in removing large quantities of EPS from surfaces. It was thought that the use of phage enzymes in conjunction with disinfectants could provide an effective means of biofilm removal. Figure 4 demonstrates the increased susceptibility of enzyme-treated biofilms to a disinfectant treatment. It is thought that the polysaccharide depolymerase affords better access for disinfection, and consequently, better removal and eradication. This may be used as a semi-specific treatment in the control of biofilm formation.

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