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A Novel Biofilm Control Agent that Inhibits Quorum Sensing

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1. Introduction

In industrial spaces, living areas and medical settings, it is well known that water-containing environments are suitable for microbial growth where microbes proliferate to form “Biofilm” [1]. Biofilms are viscous, membranous deposits composed of bacterial aggregates and metabolites such as polysaccharides that they produce. They grow on various solid surface such as sinks, bathrooms, drains, medical devices, reverse-osmosis membranes (RO membranes), chemical stock tanks, and cooling towers. They cause not only physiological discomfort but also considerable economic and hygienic harm, such as corrosion of metal components, decrease in productivity, product contamination, bad odors and causes of infectious diseases. For example, economic losses in metal corrosion involving biofilm are estimated to be approximately $1.1 trillion per year in the United States, which is recognized as a key issue in various fields (2016, NACE International Institute).

Compared with bacteria which are dispersed in water as individual cells, biofilms are difficult to control because they are resistant to bactericides or physical cleaning, and thus, a fundamental solution is poor. When using chemicals, conventional measures include "sterilization by bactericides" and "detachment by cleaning" [2]. However, it is clear that these are insufficient, and biofilm issues are more pronounced especially when the use of bactericides is limited or physical cleaning is difficult to keep.

On the other hand, a promising new approach is “to suppress the biofilm formation itself”. Recent studies have revealed that the biofilm formation is induced by chemical interactions between bacteria, i.e., signal transduction called quorum sensing (QS), and attempts have been growing [3] to physiologically suppress the biofilm formation by inhibiting and disturbing QS in various ways [4) 5)].

However various compounds have been revealed to inhibit QS, most of them have not come to practical use. For example, biological compounds are difficult to utilize in cost or stability, and synthetic compounds are difficult to produce in large quantities. In such circumstances, we have developed a novel type of agents (Biofilm control agents: BFC agents) from the basic study, including screening of active compounds available, then succeeded in commercializing QS inhibitory technology. In this report, we show an overview of BFC agents and introduce various application examples.

2. Overview of BFC agents

2-1. Overview and characteristics of BFC agents

Unlike conventional agents which depend on bactericidal effect, BFC agents have the ability to physiologically suppress the biofilm formation. Specifically, BFC agents inhibit the production of QS signaling molecules and suppress the accumulation of biofilms moreover can solve disadvantages of conventional bactericides. QS is a physiological function mediated by interbacterial signaling. When biofilm forming bacteria come to a certain density, and the concentration of QS molecules reach a threshold

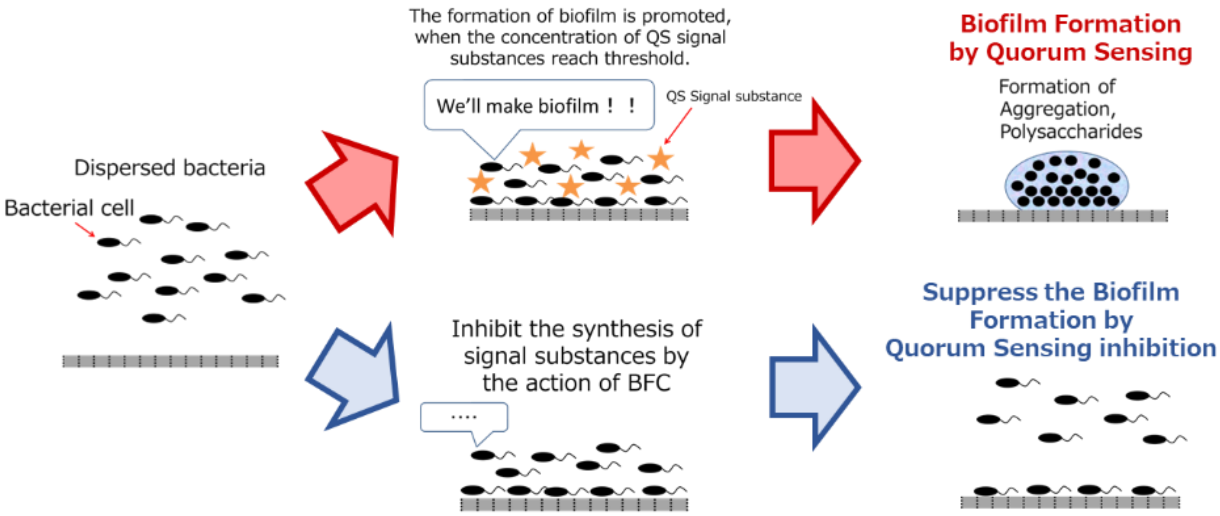

Fig. 1. Mechanisms of QS mediated biofilm formation and inhibition (Upper row: Untreated; Lower row: BFC treated)

level, then the biofilm formation is triggered.

Therefore, in environments where biofilms are usually formed, BFC agents can suppress biofilm formation and keep the bacteria dispersed state, then they are readily eliminated by the water flow (Fig. 1).

An overview of the BFC agents developed based on the concept is shown in Table 1. BR-109 is product containing QS inhibiting components only and is intended to use for applications where the use of surfactants is avoided, or for combination with various conventional agents. BR-110 is designed to achieve both suppression and removal of biofilm by combining a surfactant with high permeability and removal effect on mature biofilms. Compared with conventional bactericides, BFC agents have advantages such as high safety to human body, non-oxidizing property which does not damage materials, and effective in a wide range of pH.

Table 1. Overview of BFC agents

Product	Active substances	Effect
BR-109	・Aromatic nitrogen compound ・Aromatic alcohol	Biofilm suppression
BR-110	・Aromatic nitrogen compound ・Aromatic alcohol ・Surfactant	Biofilm suppression & removal

2-2. QS inhibitory effect of BFC agents

Pseudomonas aeruginosa, a type of biofilm forming bacteria, is known to grow in various environments and form difficult-to-remove biofilms. This bacterium secretes green pigment pyocyanin when QS is activated and biofilm formation progresses [6]. Therefore, it is useful for monitoring both the inhibitory effect on QS and the state of biofilm formation, and is widely used as a biofilm assessment species (Fig. 2). Pyocyanin concentration secreted in water can be quantified by extracting with chloroform and measuring the absorbance of 520 nm (Fig. 3). In this analysis, pyocyanin production rate was calculated

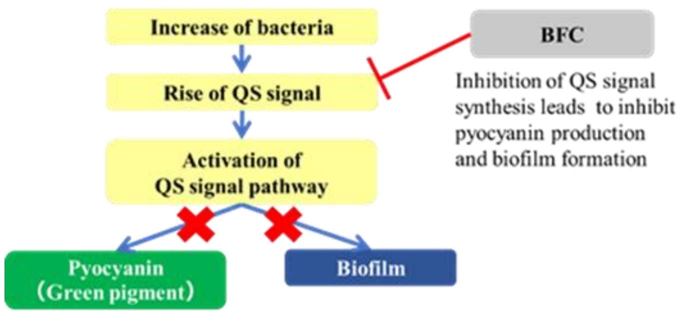

Fig 2. Outline of quorum sensing in *P. aeruginosa*

as pyocyanin concentration per bacterial density, and the inhibitory effect on QS was evaluated. As shown in Table 2, BFC treatment greatly reduced the production rate of pyocyanin, suggesting BFC agent inhibits QS. Additionally, the bacterial density shown as turbidity (OD_{630}) is hardly reduced, which indicates that the inhibitory effect is dependent on changes in physiological status due to QS inhibition rather than bacteriostatic or bactericidal effect.

Table 2. Pyocyanin production rate

	Untreated	BFC
Pyocyanin Production Rate*	100%	1.2%
Turbidity (OD_{630})	1.8	1.6

***Relative value of the pyocyanin concentration with untreated series as 100%**

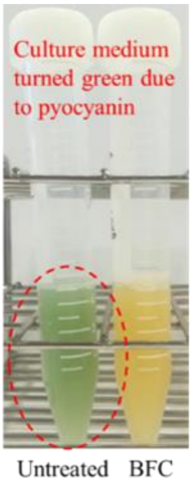

Fig 3. Comparison of pyocyanin levels

2-3. Measurement of biofilm suppression and removal

Table 3 shows efficacy tests of biofilm suppression and removal with polystyrene multi-well plates. Efficacy of biofilm suppression was evaluated by comparing the amounts of biofilm formed on the two series of vessel wall in which *Pseudomonas aeruginosa* was cultured, and BR-110 was added or not (Table 3-1). In biofilm removal tests, biofilms were previously formed on the vessel wall. Then, BR-110 was added, and biofilms were removed from the wall. Then, the amounts of residual biofilm on the vessel wall were evaluated and compared with a series of no BR-110 added (Table 3-2). In both tests, biofilms were stained with crystal violet (CV), and the absorbance of the solution eluted with ethanol was measured at 595 nm, then biofilm forming ratio and the residual ratio were calculated. As a result, by adding BR-110, biofilm forming ratio was decreased to 12% (Biofilm suppression rate: 88%), and biofilm residual rate was 23% (Biofilm removal rate: 77%). The density of dispersed bacteria, measured as the turbidity (OD_{630}) of the culture medium, is little different in the series with and without BR-110, indicating that the effect of BR-110 does not depend on the growth inhibition or bactericidal effect. Therefore, BR-110 is expected not only to suppress biofilm formation by being added in advance but also to remove mature biofilms.

Table 3-1. Biofilm suppression test

	Untreated	BR-110 (0.35%)
Amount of biofilm (absorbance 595 nm)	5.9	0.7
Biofilm forming rate	100%	12%
Turbidity (OD_{630})	1.7	1.5

Table 3-2. Biofilm removal test

	Untreated	BR-110 (0.35%)
Amount of residual biofilm (Absorbance 595 nm)	6.9	1.6
Biofilm residual rate	100%	23%

Biofilm suppression effect of BFC agents has been confirmed against several major bacterial species (Table 4), suggesting that BFC agents have a cross-sectional QS inhibitory effect on wide range of bacterial species, also have a possibility to be used effectively in various fields.

Table 4. Bacterial species sensitive to BFC agents

Bacterial species	Superior classification (phylum)	Gram stain	Main distribution sites
Brevundimonas diminuta	Alpha proteobacteria	Negative	Water, soil, clinical
Acinetobacter baumanii	Gamma proteobacteria	Negative	Water, soil, clinical
Klebsiella sp	Gamma proteobacteria	Negative	Water, clinical
Pseudomonas aeruginosa	Gamma proteobacteria	Negative	Water, soil, clinical
Shewanella algae	Gamma proteobacteria	Negative	Marine, Clinical
Vibrio harveyi	Gamma proteobacteria	Negative	Marine, Clinical
Chryseobacterium sp	Bacteroidetes	Negative	Water, soil, clinical
Microbacterium hominis	Actinobacteria	Positive	Clinical
Staphylococcus epidermidis	Firmicutes	Positive	Clinical

2-4. Visualization of biofilm suppressing effect by confocal microscopy

To visualize the biofilm suppressing effect, biofilms formed on a surface of RO membrane were observed with confocal microscope (Fig. 4). To adsorb the active substances of BFC agent onto RO membrane surface, they were soaked in BFC agent for a defined period of time, then state of biofilms was observed with confocal microscope over time. *Pseudomonas aeruginosa* attached on the RO membrane surface are shown with green fluorescence. In the untreated series, biofilm was found to be greatly thickened over 17 h. On the other hand, in the series of BFC treated, although the amount attached cells on the membrane surface was slightly increased, the growth of the thickness was greatly suppressed. This suggests that BFC agent inhibits QS and reduces synthesis of extracellular polysaccharide which are the main component of biofilm. Comparing both series, about 90% of biofilm suppression was confirmed by BFC treatment.

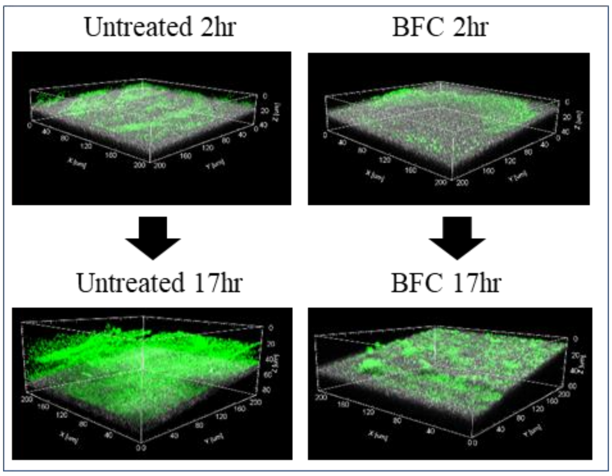

Fig 4. Confocal microscopy of biofilms formed on RO membranes.

3. Biofilm suppression on RO membrane

RO membrane is used for water treatment equipment to produce high-purity water industrially used, desalination of seawater and recycling of wastewater, and is a worldwide key technology for ensuring water resources. On the other hand, a major problem in this field is few effective solutions against the phenomena called biofouling in which biofilms are formed on the surface of filtration membrane and the treatment efficiency is deteriorated. For example, hypochlorite, a commonly used as bactericide, cannot be used for ordinary RO membranes because it damages the membrane structure and performance, and organic bactericides generally have low-safety concerns. In contrast, BFC agents can suppress biofilm formation without concerning damage to RO membrane and safety issues.

In this test, we verified the efficacy of BFC agent (suppression of membrane clogging) on actual RO membrane unit in which membrane clogging occurs in a short period due to biofilm. Periodic BFC treatment was performed, and the increasing of inlet pressure of RO membrane was used as an indicator of membrane clogging. The transition of inlet pressure was monitored until it was over 0.8MPa which is the control value for clogging (Fig. 5). Spiral-type of RO modules were used for the water treatment operation, and ground water was supplied to the equipment (Table 5). In order to enhance efficacy of BFC agent, the following was performed as a pretreatment. BR-110 solution diluted to 3.5% was passed through the RO module, kept for 3 hours to absorb the active substances on the membrane surface. During the test, the water treatment operation was stopped once a week, and at the timing, the periodic BR-110 treatment was performed in which 0.35% of BR-110 was passed through the RO module and kept for 1 hour. As a result, BR-110 extended the time until membrane clogging by approximately 7-fold against untreated operations.

The test indicates that BFC agents are capable to

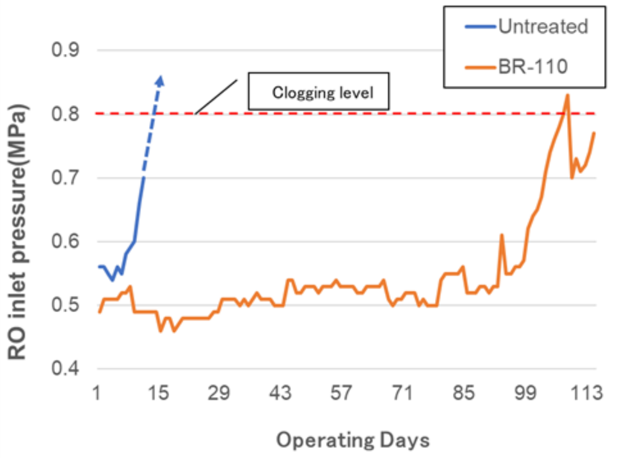

Fig 5. Biofilm suppression test in actual RO membrane unit

Table 5. Operating conditions of RO membrane unit using BFC agent

Supply water	Groundwater
Flow rate	(Water supply) 9300 L/hr, (Treated water) 6000 L/hr
BFC treatment	(Pretreatment) Contact of unused RO membrane with BR-110, 3.5% solution for 3 hours (Periodic treatment) Immersion in BR-110, 0.35% solution once weekly for 1 hour

suppress the membrane clogging of actual RO equipment. Especially, by automating the chemical feed process, it can be expected that efficiency of the water treatment process will be improved, and the maintenance cost will be remarkably reduced.

4. Biofilm suppression in chemical stock tanks

Various issues can arise from chemical dilution tanks in industry due to biofilms formation inside. There are few effective solutions, because, as well as reactive oxidizers, the usage of organic bactericides is also limited for the environmental aspect and safety reasons. For example, it is known that biofilms are formed in dilution tanks of cutting oils and die casting release agents depending on retention time, leading to deterioration of the performance, lowering of recycling efficiency, generation of odors, clogging of flow channels, and cost increase.

In this test, 100 ppm of BR-109 was added to an aqueous emulsion tank in which 20 ppm of an organic bactericide was originally contained, and the effect on biofilm was evaluated. As a result, visible biofilms were formed over the inner wall of the tanks containing only organic bactericide in 2 weeks, whereas tanks containing combination of BR-109 and organic bactericide were able to remain clean beyond 3 weeks (Fig. 6). It is generally known that the effect of bactericides is strong on dispersed bacteria even at relatively low concentration but is greatly reduced on biofilms. In this test, BR-109 suppressed biofilm formation, in parallel, the bactericide exhibited bactericidal effect strongly on the dispersed bacteria, suggesting that the combination effect was favorable. Therefore, BFC agents can be a solution of biofilm issues in chemical tanks, additionally, also have potential to improve the environmental aspects and human safety by reducing bactericide usage.

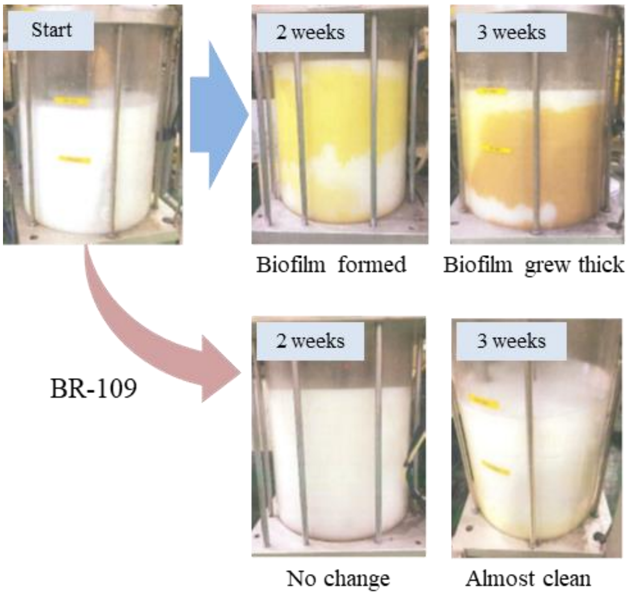

Fig. 6. Biofilm inhibition in aqueous emulsion tanks

5. Applications for coating agents

Recently, the Society of International sustaining growth for Antimicrobial Articles (SIAA) has been working to create anti-biofilm as a new certification system in addition to the conventional antibacterial, antifungal, and antiviral certification [7]. There is a great demand for materials with anti-biofilm property and coating agents that provide similar property in a long term. Therefore, applications of BFC agents are being investigated that improve the performance of equipment and hygienic aspects, such as inside of air conditioners and around water of living areas that are difficult to keep frequent cleaning.

Fig.7 is a biofilm suppression test using test pieces coated with a coating agent containing BR-109. Test pieces were immersed in microbial culture to form biofilm, then stained with crystal violet and quantified them. BFC coated test piece suppressed 84% of biofilm formation. In the application as a coating agent, compared with the use as a liquid agent, since it is possible to localize the active compounds on the surface of materials, the advantage is that the effect can be expressed with a small amount of use. On the other hand, since the persistence of the effect is required, we will continue to focus on improving the performance through demonstration tests.

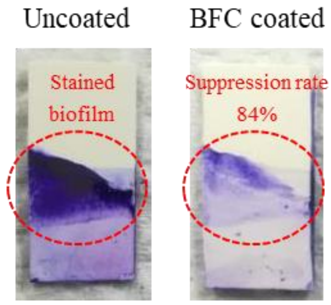

Fig. 7. Biofilm suppression in BFC coated test piece

6. Conclusion

In this report, the overview and applications of BFC agents were introduced which suppressed the biofilm formation by the novel mechanism different from bactericidal effect. Demand for BFC agents is diverse, therefore, trials are being performed for various applications, such as RO membranes, chemical stock tanks, coating agents, cooling towers, and cleaning agent for medical devices, as introduced in this report. Since, biofilm issues are more serious in areas with low water quality and insufficient cleaning, the size of the overseas market is larger than that of Japan, and the growth potential also can be highly expected. We hope to continue to share various challenges with our clients and to propose BFC agents broadly as one of effective solutions for water resources, energy saving, and hygiene issues in the future.

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