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Anti-biofilm ability of garlic extract on *Pantoea agglomerans* and application to biosand filter

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ABSTRACT

A biosand filter (BSF) is used as one of the appropriate technology to get purified water in water-poor countries and developing countries. BSF purifies water to the level of cleanness necessary to be used in daily life and is widely used worldwide, especially given its producibility and simplicity. However, there is a circumstance that pathogens may be included in the formation of schmutzdecke (biofilm) that helps with internal water purification. The environment may be exposed to the pathogens located inside the BSF, causing complications. *Pantoea agglomerans* found in the biofilm of the BSF were selected as an opportunistic pathogen, and research was conducted to identify and inhibit the biofilm formation of opportunistic pathogens. The natural substances used to inhibit the biofilm formation were garlic extract and moss extract. Moreover, indole, which acts as a signaling molecule in microbes, was also used as a control. The biofilm growth curve of *P. agglomerans* in vitro was measured and the biofilm formation inhibition abilities of indole and natural extract were confirmed. Subsequently, genes related to biofilm formation and quorum sensing, a method of bacterial communication, were selected, and their expression levels were compared in relation to each other. BSF was produced and applied in an actual filter and water was poured in for 4 weeks to form biofilm. Garlic extract and moss extract were added to each filter, and the gene expressions and colony-forming unit (CFU) counts of the biofilm layer, effluent water, and the source water were compared. Furthermore, 16S rRNA sequencing was carried out. Indole and garlic treatment groups inhibited biofilm formation by 85.37% and 76.99% respectively and when incubated for 18 h, the biofilm formation was reduced to as low as 20%. Each treatment significantly affected gene expression in indole and garlic treatment groups: in both groups, *pagI*/*R* was reduced; in the indole treatment group only, *bssS* increased; and in both groups, *ompW* increased only at the early stages of biofilm formation. Indole and garlic treatment group's *pagI*/*R* increased in correlation to the duration of incubation. Moss-treated group inhibited biofilm formation rates, CFU counts, and gene expression levels, but to a lower extent than the other groups. *P. agglomerans* biofilm was inhibited by garlic or indole. The application on the actual filter confirmed the antibacterial effect and anti-biofilm effects of garlic and that the total variety of microbes was also reduced.

Keywords: Biosand filter; Biosand filter; Biofilm; *Pantoea agglomerans*; Garlic; Moss

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1. Introduction

A biosand filter (BSF) is a water distillation system and is used widely in developing nations. According to the 201 Annual Address of the Centre for Affordable Water and Sanitation Technology (CAWST), 5,981,000 people received welfare from water distillation systems including BSFs. Furthermore, according to CAWST, more than 200,000 BSFs have been composed, 12,346 institutions are concerned with water sanitation and 37 countries are benefiting from BSF systems. According to CAWST, BSF's purification rates of reducing organisms such as bacteria, viruses, cryptosporidium and giardia reach up to 99.99% [1].

Biofilms are structured clusters of bacterial ingredients enclosed in the extracellular matrix which is made up of extracellular polymeric substance [2] and stick to other cells or surfaces. Bacterial biofilms can lead to problems. Once a biofilm has been formed, the antibiotic resistance of the bacteria in it are enhanced by 500 to 1,000 times [3]. There is research that indicates that a biofilm can be a factor in microbial infections [4]. On the other hand, a biofilm built from the bacteria contained in BSF is believed to serve a water purifying function [5]. However, biofilm in BSF consists of bacteria from the initial source water which includes the environmental bacteria, including opportunistic pathogens. Biofilm-integrating opportunistic pathogens may also serve as the water purification function. However, when dumping the BSF, pathogen and opportunistic pathogen from the biofilm can cause environmental issues. Moreover, there is no systematic standard guideline dumping the BSF.

Bacteria can communicate with each other using their signal molecules which is called quorum sensing [6]. In bacteria, gram-negative bacteria utilize acyl-homoserine lactones (AHLs) as signal molecules and gram-positive bacteria utilize autoinducing peptides as signal molecules in quorum sensing [7]. Once bacteria concentration increases and reaches a threshold, enhanced signal molecules can influence specific actions including microbial luminescence virulence and biofilm formation [8,9]. Consequently, managing the quorum sensing system is one of the methods for biofilm regulation, especially its inhibition [10].

Pantoea agglomerans is a gram-negative *Bacillus* widely dispersed on plant surfaces, in soil and in human feces. It acts as an opportunistic pathogen to humans, plants, and as well as other crop pathogens, which serves as a plant pathogen competitor [11]. Because it is a ubiquitous bacterium and causes crop infections, in the agricultural environment especially, its inhibition is especially important to improve agricultural output.

Indole is generally distributed in the natural environment and is particularly known as one of the signaling molecules of bacteria [12]. As a signaling molecule, indole may have an impact on bacterial action such as endospore, antibiotics resistance, pathogen's expression and biofilm formation [13]. Most bacteria can produce indole, but not all of them [14]. Certain bacteria including *P. agglomerans* cannot produce indole. However, they have genes associated with indole such as indole transport or indole receptors [15].

Recently, natural products exhibited antibacterial effects on pathogens such as *Escherichia coli* or *Staphylococcus aureus* [16]. Natural products' substances are safer than chemical antibacterial products [17]. Moreover, the earlier study shows how natural product extracts from garlic, radish and chives exhibited not only antibiotic effects but also anti-biofilm activities. Garlic had the most impact on both gram-negative and positive bacteria [24]. Moss, along with garlic, has recently been studied for antibiotic effects and anti-biofilm effects [18]. In one study, moss extract may have shown biofilm inhibition effects on *Legionella pneumophila* which causes legionellosis [19].

In this study, anti-biofilm effects of indole and natural product extracts from garlic (*Allium sativum*) and moss (*Sphagnum cristatum*) on *P. agglomerans* were investigated *in vitro* and on biofilm of the BSF. In the earlier study [20], *P. agglomerans* were found in BSFs from Chunmaji Lake which used original source water. Anti-biofilm effects of indole and natural product extract on *P. agglomerans* were examined using crystal violet biofilm formation assay [21], the comparison and quantification of gene expressions associated with the biofilm formation, bacterial quorum sensing of *P. agglomerans* and colony-forming unit (CFU) in biofilm. This study can propose a guideline to remove biofilm pathogen in BSFs and quorum sensing may also be one of the concepts of removing biofilm.

2. Materials and methods

2.1. Bacterial culture condition and media

P. agglomerans (ATCC 33261) was offered in a lyophilized state from the Rural Development Administration Republic of Korea. Luria-Bertani (LB) and plate count agar (PCA) for biofilm CFU counting from Difco (USA) were used. Lyophilized *P. agglomerans* were subcultured in LB broth and cultivated at 30°C.

2.2. Garlic, moss extraction and minimum inhibitory concentration value determination

Natural products' extraction procedure from the Lee and Lee [22] was used. Garlic was purchased from Hanaro Market, Pogang, Korea. It was washed 3 times and ground with the same volume of distilled water (DW). After grinding, it was centrifuged at 10,000 RPM at 24°C for 30 min and the supernatant was filtered with Whatman No. 1 filter paper. Moss was purchased from Creative Water Solution, New Zealand. Dried-Up moss was doused with Tryptic soy broth (TSB) from Difco (USA) for 4 h. After being soaked, moss with TSB was filtered through Whatman No. 1 filter paper. Subsequently, it was centrifuged at 10,000 RPM at 24°C for 30 min and the supernatant was filtered with Whatman No. 1 filter paper again. All extracts were kept at –70°C.

The method of determining the minimum inhibitory concentration (MIC) value from the Clinical and Laboratory Standards Institute was followed [23]. MICs value test for garlic and moss extract referred to by Kang et al. [24] were used. 100 ul of each stocked garlic and moss extract was injected in the well of a 96-well plate and the concentration of each extract ranged from 100% to 0.78%. After that, *P. agglomerans* suspension (10^7 CFU/mL) was injected into each well, the overall volume is set to 200 ul. Ciprofloxacin was used as a positive control and LB broth was used as

a negative control. The prepared plates were incubated overnight at 30°C. All 96-well plates were prepared in triplicates.

2.3. In vitro biofilm formation and inhibition rates

Biofilm formation assay of O'Toole [21] was used for *in vitro* biofilm formation and some modifications were made. To specify the time-dependent biofilm inhibition rates, 6 h intervals of up to 36 h were set up. *P. agglomerans* were injected in 96-well plates with indole, garlic and moss. Indole concentration referred by Feng et al. [25] was used, at 1 and 2 mM. Garlic and moss concentrations set at MIC values of *P. agglomerans* were used. After confirming *P. agglomerans* growth curves with each reagent, the CFU, optical density (OD), and gene expressions were measured at two biofilm phases: the early biofilm formation phase (incubated for 8 h) and beyond the middle biofilm formation phase (incubated for 18 h). To verify the indole and garlic and moss extracts' capacity to inhibit biofilm formation, not their antibacterial properties, all reagent was injected for 6 h before biofilm collection time. 100 ul of *P. agglomerans* suspension (10^7 CFU/mL) was added to each well and each reagent was treated at a specific point in time (Fig. 1). Indole concentration was set at 1 mM since there was no significant difference in biofilm growth rate between 1 mM and 2 mM. Garlic and moss compound concentrations were set at their individual MICs values of *P. agglomerans*. At each biofilm collection time (8 h, 18 h) all the suspension in the plates was removed and washed three times with PBS (phosphate-buffered saline) to remove the remaining planktonic cells. After washing, 225 ul of 0.1% crystal violet was added to each cell and was incubated for 15 min. The incubated crystal violet was then removed, and the wells were washed with PBS three times and dried up for 8 h. Thirty percent acetic acid was added to each well after being dried, the wells were incubated for 15 min and the OD at 600 nm was measured using a microplate ELISA reader (Thermo, USA). 30% acetic acid was used as a blank and LB broth was used as a negative control. The inhibition rates were calculated by using the formula below [26].

$$\text{Percentage inhibation} = \left(\frac{\text{OD}_{\text{NEGATIVE CONTROL}} - \text{OD}_{\text{EXPERIMENTAL}}}{\text{OD}_{\text{NEGATIVE CONTROL}}}\right) \times 100 \quad (1)$$

2.4. Biofilm CFU comparative assessment

To verify whether the biofilm inhibition effects were due to biofilm inhibition or were caused by antimicrobial effects, the viable biofilm proportion percentage was calculated against total viable cells. Depending on the previous biofilm OD detection experiment, biofilm and planktonic states were separated. Biofilm state was separated by washing each plate well with PBS and vortexing for 5 min. The washing step was repeated twice. For planktonic state, the supernatant was moved from each well to new wells. Prepared biofilm and planktonic states were cultured on PCA. Each plate was replicated three times. Biofilm proportion rate was computed from the formula below [27].

$$\text{Biofilm proportion percentage} = \left\{\frac{\text{CFU}_{\text{biofilm}}}{\text{CFU}_{\text{biofilm}} + \text{CFU}_{\text{planktonic}}}\right\} \times 100 \quad (2)$$

2.5. Real-time quantitative polymerase chain reaction

Three genes were chosen to confirm the biofilm inhibition rate on gene expression. First was *pagI/R* which produces AHLs and their receptors in *P. agglomerans* [28]. The second was *bssS* which transfers indole in bacteria and influence bacterial biofilm formation [15]. Last was *ompW* which is related to biofilm debris expression [29]. *pagI/R* and *bssS* expressions were assessed for indole treatment, whereas all three genes were measured when garlic and moss were treated. 200 ul of *P. agglomerans* suspension was injected into 20 mL LB broth in 50 mL falcon tube and was incubated at 30°C for as long as the two biofilms' collection time. Each reagent was treated 6 h before the collection, the same as the previous experiment. To make a pellet, the suspension was centrifuged at 13,000 rpm at 26°C for 30 min. Pellet was homogenized with 1 mL of TRIzol (Life Technology, USA). TaKaRa MiniBEST Universal RNA Extraction kit (TaKaRa, Japan) was used for RNA extraction. The RNA concentration was set to 100 ng/mL and cDNA was synthesized for real-time quantitative polymerase chain reaction (RT-qPCR). PrimeScript 1st cDNA Synthesis Kit (TaKaRa, Japan) was used for cDNA synthesis from the RNA. Synthesized cDNA was magnified throughout RT-qPCR with StepOnePlus Real-Time PCR System (Thermo Fisher, USA). 16s rRNA was used as a reference gene and SYBR master mix (Thermo Fisher, USA) was used. Primer information recorded in Table 1 was used. The comparative

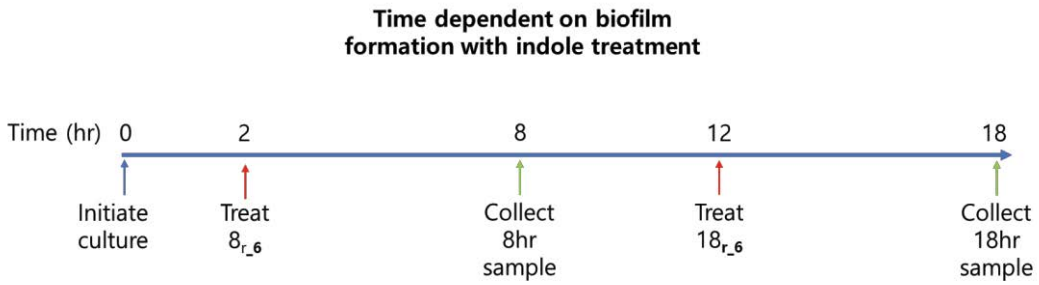

Fig. 1. Biofilm growth and reagent treatment timetable depending on the treatment time. Abbreviation: 18_{r_6}, 8 h biofilm reagent treatment for 6 h; 18_{r_6}, 18 h biofilm reagent treatment for 6 h.

Table 1
Primer sequence used to RT-qPCR

Gene	Orientation	Tm (°C)	Sequence (5′-3′)
pagI/R	F	57°C	GCACCACATAGCAACTTCC (19 mer)
	R		TCCGCCAGAATTAGCAACC (19 mer)
bssS	F	59°C	TGGGCTGGGATATCAGTACC (20 mer)
	R		TGACTCTATCTTGGCGATGC (20 mer)
ompW	F	59°C	TGACTGGATGCTGAATGCGT (20 mer)
	R		TCAGAAACGGTAACCCGCTC (20 mer)
Universal	F	66°C	ACTCCTACGGGAGGCAGCAGT (21 mer)
	R		GTATTACCGCGGCTGCTGGCAC (22 mer)

expression level was calculated by calculating 2^{-ddct} method and expressed using RQ (Relative Quantification) value [30].

2.6. Application to garlic and moss extract of BSF

BSF was constructed using the process that was used in Park et al. [20]. Biosand filter was filled with pebbles of 5 cm or less diameter and 0.35~0.7 mm width sand. Two biosand filters were prepared and 300 mL of DW was poured into each for a week to stabilize the sand filter. Chunmaji Lake (Pohang, South Korea) water was collected at the same place every day, and 300 mL was poured at 9 am and 6 pm every day for four weeks to make biofilm [31]. Additionally, 50 mL of *P. agglomerans* suspension (10^5 CFU/mL) was poured into each filter. After biofilm formation, 50 mL of garlic or moss concentrations set at the MIC for *P. agglomerans* were poured into each filter for a week. 20 g of biofilm samples were collected 5 cm below the top surface of the biofilm. The samples were vortexed with 20 mL DW for 10 min to detach bacteria and biofilm debris from the soil. Then, the samples were filtered with Whatman No. 1 filtered paper and then centrifuged at 13,000 RPM for 30 min to make a pellet which was used for RNA extraction. The samples from the inside of the filter were used to count CFU and measure each selected gene expression. Additionally, bacterial 16s rRNA sequencing of water from Chunmaji Lake, filters inside of non-treatment, garlic or moss treated pellets, and purified water of each filter was performed. Biofilm and planktonic samples were plated on PCA and classified according to morphology. For sequencing, samples were sent to Macrogen (Seoul, South Korea).

2.7. Statistical analysis

All experiments except the BSF application test were carried out in triplicate. Statistical analysis has been accomplished using GraphPad Prism, Version 8.0.2 for Window (GraphPad Software, USA). Differences between non-treatment and reagents were analyzed by analysis of variance (ANOVA) with Benjamini's multiple comparison test.

3. Results

3.1. In vitro biofilm inhibition rates

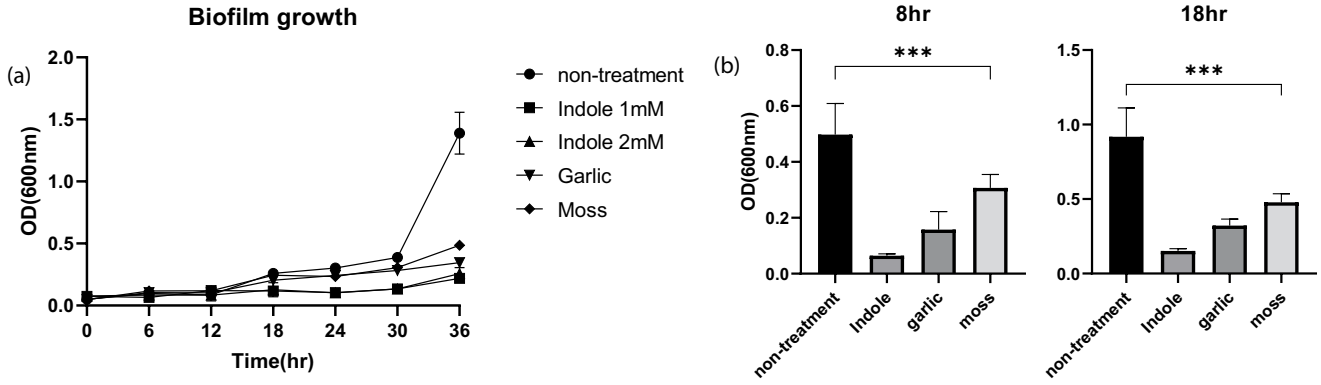

Fig. 2. Biofilm growth curve for 36 h and anti-biofilm effect of reagents treatment on *P. agglomerans*. *Y*-axis unit is OD (600 nm). (a) Indole treated 1 and 2 mM, garlic extract and moss extract treated at MIC values. (b) Anti-biofilm effect of reagents treatment depending on biofilm conformation time.

Table 2
Biofilm inhibition rates on *P. agglomerans* of indole, garlic and moss

Reagents	Inhibition rate
Indole	85.3720 ± 1.4639
Garlic	76.9982 ± 1.5959
Moss	22.9266 ± 0.1680

3.2. CFU counting in biofilm

Table 3
Biofilm's CFU proportion rates of *P. agglomerans* of indole, garlic and moss

Reagents	Biofilm proportion rate (8 h, 18 h)	
Non-treatment	17.9547 ± 4.0792	26.9281 ± 0.0989
Indole	29.6824 ± 6.5245	7.24816 ± 1.8428
Garlic	19.9468 ± 5.0530	6.45833 ± 0.2083
Moss	35.2990 ± 0.4153	26.7561 ± 0.83014

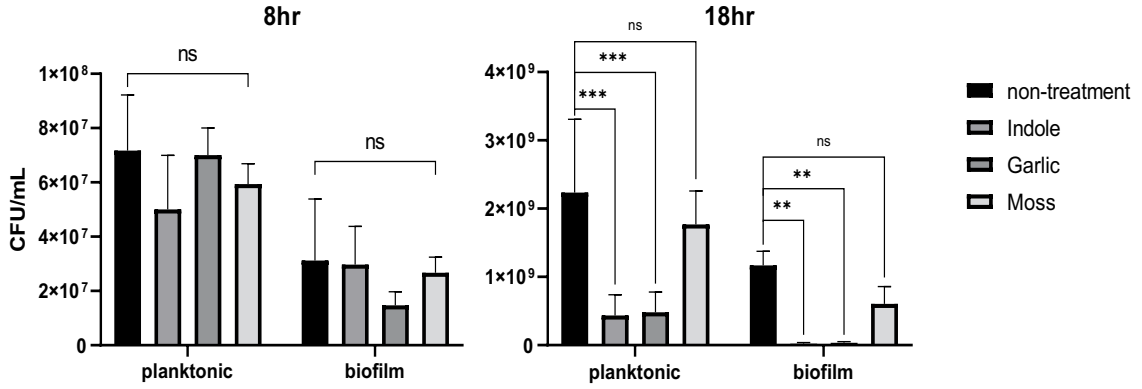

Fig. 3. Comparison between biofilm and planktonic states depending on reagents treatment.

3.3. Quantificative gene expression

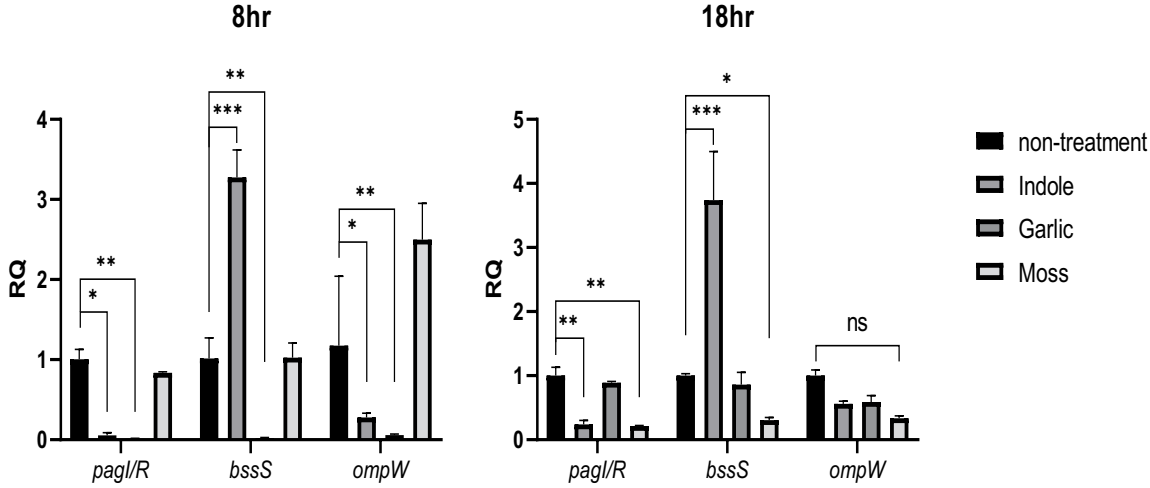

Fig. 4. mRNA expressions of *P. agglomerans* biofilm depending on reagent treatment.

3.4. Application of garlic and moss extract to biofilm on biosand filter

Table 4
Identification of bacteria in the original water, biofilm and purified water for garlic extract and moss extract treatment

Source	Labeling	Genus
Original water	C	*Bacillus*
		Acinetobacter
		Fictibacillus
Non-treatment biofilm	B	*Curtobacterium*
		Pantoea
		Aeromonas
		Arthrobacter
		Micrococcus
		Pseudomonas
Garlic extract treated biofilm	GB	*Bacillus*
		Ralstonia
Moss extract treated biofilm	MB	*Pseudomonas*
Non-treatment purified water	CF	*Acinetobacter*
		Exiguobacterium
		Pantoea
		Aeromonas
		Curtobacterium
Garlic extract purified water	GF	*Enterobacter*
		Leuconostoc
		Pediococcus
		Pseudomonas
Moss extract purified water	MF	*Aeromonas*
		Achromobacter
		Microbacterium
		Salmonella
		Citrobacter
		Exiguobacterium

MIC values of garlic extract for *P. agglomerans* were assessed to be 12.5% and 25% for moss extract. Our results show garlic extract exhibited anti-biofilm effects on *P. agglomerans*. In the biofilm assay, biofilm growth curves were assessed at intervals of 6 h for up to 36 h. All reagents treated group reduced the OD values more than the wild-type (Fig. 2). Among the garlic and moss extracts, the garlic extract had better anti-bacterial and anti-biofilm effects (Table 2). Throughout the biofilm formation rates and CFU proportion in each state, the garlic extract's effect was more effective than moss (Fig. 3, Table 3). Both reagents led to decreased expression levels of *pagI/R* and *ompW* biofilm at the 8 h mark. In the case of *bssS*, it was significantly increased in the indole treated group and was decreased in the garlic extract group. Moss did not significantly affect all three genes in the early stage. At the middle stage (18 h), *pagI/R* expression decreased for indole and moss extract, but not in garlic. *bssS* expression on biofilm was similar when indole treated both times. *ompW* expression was not shown to be significantly different in the middle stage of biofilm (Fig. 4).

Bacterial 16s rRNA sequencing results of original water and non-treatment, garlic extract and moss extract were recorded in Table 4. From the original water from Chunmaji Lake, *P. agglomerans* was not found, and instead, *P. agglomerans* were added to each BSF. *P. agglomerans* were found in both BSFs. *Bacillus*, *Acinetobacter* and *Fictibacillus* were observed in the original water from Chunmaji Lake when separated according to morphology. In the non-treatment biofilm inside the filter, *Curtobacterium*, *Pantoea*, *Aeromonas*, *Arthrobacter*, *Micrococcus* and *Pseudomonas* were found. Garlic and moss extract-treated filter exhibited less classes of microorganisms when compared with the non-treatment filter. In the purified water, *Pantoea* was only found in the non-treatment filter. Non-treatment biofilm's CFU was 1×10^6 CFU/mL and garlic extract and moss extract-treated biofilm's CFUs were 1×10^4/mL and 1×10^5/mL respectively. For mRNA expression in the BSFs, only the garlic extract treatment group significantly affected *pagI/R* (Fig. 5). Meanwhile, other genes in the garlic extract and moss extract groups exhibited no significant difference.

4. Discussion

P. agglomerans' MIC value of garlic extract is the same as *E. coli*'s MIC value [24]. Moss extract's MIC value was measured to be twice as much as that of the garlic extract. The cause for the difference in the MIC value of moss extract was believed to be due to the fact that the organic acid of moss extract was not properly absorbed by *P. agglomerans* [32].

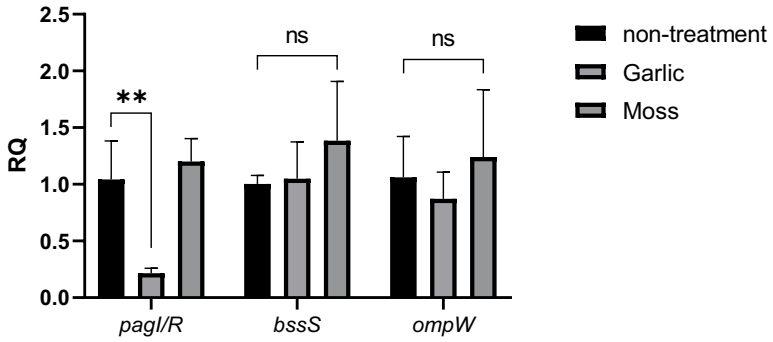

Fig. 5. mRNA expression of BSF. Biofilm formation for four weeks. Reagents treated for one week, 50 mL, MIC concentration to *P. agglomerans*.

There was no biofilm inhibition difference between 1 and 2 mM of indole. All three reagents verified for effectiveness of biofilm inhibition (Fig. 2a). High inhibition effectiveness of the garlic extract and indole compared to moss extract was confirmed (Fig. 2b). According to the biofilm inhibition rate formula, indole and the garlic extract exhibited more than fifty percent inhibition, whereas the moss extract did not reach that level (Table 2). Garlic extract's allicin, which is organic acid known to have antibacterial properties more effectively affected *P. agglomerans* biofilm formation inhibition than the organic acids of moss [33]. Furthermore, the biofilm proportion rate was lower for indole and garlic treatment than for moss extract which exhibited similar rate as that of non-treatment. Indole and garlic extract's biofilm proportion rates were the quarter of the biofilm proportion rate of the non-treatment at the 18 h mark (Table 3).

In the early stage of biofilm, *pagI*/*R* expression was significantly decreased in indole and garlic extract. Garlic extract did not show a significant decrease in *pagI*/*R* expression in the middle stage of biofilm (Fig. 4). The anti-biofilm effect of garlic extract is supposed to be behind the bacterial quorum sensing inhibition throughout the inhibition of *pagI*/*R* expression and the inhibition of AHL synthesis in *P. agglomerans*. Furthermore, it is believed that the antibacterial effects of garlic also affected the bacterial growth system [34]. The indole treated group exhibited a significant difference in *bssS* expression at both stages of biofilm formation (Fig. 4). Even though *P. agglomerans* do not produce indole itself, indole affects *P. agglomerans* growth and biofilm formation by increasing *bssS* expression in both stages. *ompW* expression only showed a significant difference in the early stage of biofilm formation when indole and garlic extract were treated. In the biofilm growth stage, *ompW* expression inhibited by indole or garlic extracts affected the biofilm formation and growth of *P. agglomerans*.

By comparing the biofilm OD and CFU values, the anti-biofilm effects of indole and garlic were quantified. By comparing gene-expression levels in groups incubated for 18 h, the moss group expressed higher levels of *pagI*/*R* than the garlic group (Fig. 4). This difference may be due to the earlier antibacterial effects of garlic than moss.

For mRNA expression of each reagent treatment in the BSF, only *pagI*/*R* expression in the garlic extract-treated group showed a significant difference (Fig. 5). In the BSFs, the other two genes were not significantly affected and the moss extract did not impact any gene expression. Biofilm CFU in the BSF decreased for the garlic extract and moss extract. Particularly for the garlic extract-treated BSF biofilm, CFU decreased two times more than that of the moss extract-treated biofilm. It is believed that the biofilm formation inhibition effect of garlic extract is due to its antimicrobial ability [35].

In the BSF experiments, microorganisms that were not found in the original water were detected in the non-treatment filter (Table 4). This is presumed to be due to the change in the environmental change of the original water source, which was newly collected and poured every day. This phenomenon has been observed in Hwang et al. [31] which constructed a biosand filter and used the water source from a river. *Pantoea* sp was only detected in the non-treatment biosand filter. When garlic and moss extracts were treated, however, *Pantoea* was not found in either biofilm. In the filtered water, furthermore, *Pantoea* was only found in the non-treatment filter (Table 4). This indicated that the garlic and moss extracts can inhibit *Pantoea* growth in the biosand filter.

5. Conclusions

Although biofilm in BSF plays an important role in purifying water, eliminating the opportunistic pathogens that are likely to be included in BSF is also important when dumping BSF. We found that garlic, as a natural reagent, can suppress *P. agglomerans* in biofilm formation in BSFs. Also, we found that the garlic's inhibition of the formation of biofilm on *P. agglomerans* is related to bacterial quorum sensing throughout decreased *pagI*/*R* expressions when forming the biofilms. Throughout the biofilm formation assay, the biofilm formation rates, measured by CFU comparison and quantitative gene expression, of both garlic and moss were confirmed to be sufficient, and garlic was more effective.

When littering the BSF, the pathogen can cause environmental problems. Garlic can be one candidate with antibacterial and anti-biofilm effects. Natural products other than garlic is also thought to be effective in removing pathogens from the BSF. Through these results, a systematic treatment method of BSFs was established and garlic was found to be an effective natural substance. Other natural substances that are easy to acquire in developing countries are also expected to work. Furthermore, research on quorum sensing in a complex microbial environment involving more than single strains will be necessary.

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