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Enzymes Enhance Biofilm Removal Efficiency of Cleaners

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Efficient removal of biofilms from medical devices is a big challenge in health care to avoid hospital-acquired infections, especially from delicate devices like flexible endoscopes, which cannot be reprocessed using harsh chemicals or high temperatures. Therefore, milder solutions such as enzymatic cleaners have to be used, which need to be carefully developed to ensure efficacious performance. *In vitro* biofilm in a 96-well-plate system was used to select and optimize the formulation of novel enzymatic cleaners. Removal of the biofilm was quantified by crystal violet staining, while the disinfecting properties were evaluated by a BacTiter-Glo assay. The biofilm removal efficacy of the selected cleaner was further tested by using European standard (EN) for endoscope cleaning EN ISO 15883, and removal of artificial blood soil was investigated by treating TOSI (Test Object Surgical Instrument) cleaning indicators. Using the process described here, a novel enzymatic endoscope cleaner was developed, which removed 95% of *Staphylococcus aureus* and 90% of *Pseudomonas aeruginosa* biofilms in the 96-well plate system. With a >99% reduction of CFU and a >90% reduction of extracellular polymeric substances, this cleaner enabled subsequent complete disinfection and fulfilled acceptance criteria of EN ISO 15883. Furthermore, it efficiently removed blood soil and significantly outperformed comparable commercial products. The cleaning performance was stable even after storage of the cleaner for 6 months. It was demonstrated that incorporation of appropriate enzymes into the cleaner enhanced performance significantly.

Endoscopes are widely used as a valuable diagnostic and therapeutic tool; however, it has been reported that health care-associated outbreaks of infections can be more frequently linked to contaminated endoscopes than to any other medical device (1, 2). Endoscopes are in contact with different body fluids, and the channels provide an ideal surface for bacterial adhesion. Viable bacterial cells can be detected on many endoscopes even after cleaning and disinfection processes (3–6). The main reason for this is that under natural conditions, most bacteria occur in the form of biofilms. They adhere to surfaces and are embedded in a self-produced layer of extracellular polymeric substances (EPS) (7, 8). EPS provide structural integrity to biofilms and protect the bacteria against environmental influences such as UV irradiation, antibiotics, and disinfection and make them much more tolerant to these stresses than planktonic cells (9–11). It is a huge challenge to avoid and remove biofilms, especially in moist environments such as used endoscope channels.

The long and narrow endoscope channels are difficult to reach by mechanical devices, and the use of harsh chemicals or high temperatures could harm the sensitive materials built into endoscopes. For reprocessing of endoscopes, mild cleaning agents are needed to combat biofilms. One effective approach is to destabilize the biofilm EPS, which contain proteins, polysaccharides, lipids, extracellular DNA, and other substances. Some enzymes such as protease (12, 13), DNase I (12, 14), alginate lyase (15, 16), amylase (13, 17), and cellulase (18, 19) have been reported to support biofilm removal. Therefore, inclusion of these enzymes in cleaning agents can improve the efficiency of biofilm detachment. A few enzymatic cleaners are commercially available, but they often failed to show the expected biofilm removal efficacy in practice (20). One of the reasons for failure is the use of inappropriate test parameters during the cleaner development process, which might lead to an overestimation the cleaning performance, e.g., relevance of the used microorganisms, biofilm formation conditions, or readout of biofilm removal.

Here we describe a process for the development and evaluation of novel enzymatic cleaners targeting endoscope biofilms. We selected a biofilm quantification method to assess the cleaners based on methods described in a previous study (21). The performance of newly formulated enzymatic cleaners in the removal of biofilms formed by clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* was first screened and optimized in a 96-well-plate system. Afterwards, standard methods were used to evaluate the efficacy of biofilm removal from endoscope surfaces and cleaning of coagulated blood. A new cleaner (deconex Prozyme Active) containing four enzymes in a novel base formulation was developed and appeared to perform better than nine comparable commercial products.

MATERIALS AND METHODS

Terms and abbreviations. Base formulations (abbreviations starting with B) are cleaner solutions, including surfactants and other ingredients, without enzymes (B1A and B2B, etc.). Abbreviations starting with E refer to different enzymes, including proteases, polysaccharidases, lipases, and DNases (E1 and E2, etc.). Cleaners (abbreviations starting with C) are commercially available endoscope-cleaning solutions (C1 and C2, etc.). High-level disinfectant is a solution that should achieve complete elimination of all microorganisms in or on an instrument.

Chemicals and reagents. Chemicals and reagents were purchased from Sigma-Aldrich (Switzerland) if not mentioned otherwise. Enzyme

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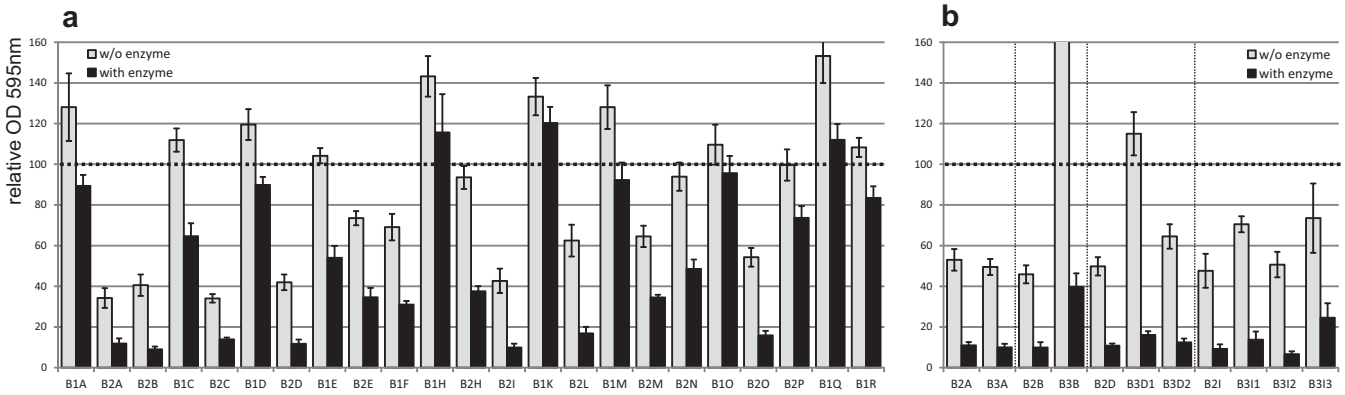

FIG 1 Screening for novel base formulations. Shown is the amount of *P. aeruginosa* biofilm remaining after treatment with enzyme-free base formulations (light gray) compared to that remaining after treatment with the same base formulations containing a mixture of seven enzymes (dark gray). The *y* axis represents the biofilm amount quantified by crystal violet staining relative to the negative control (biofilm treated with WSH containing no detergents or enzymes). Error bars represent data from 6 individual replicates. In a first round of screening, 4 base formulations were selected (a) and further optimized in a second round of screening (b). B3A is a derivative of B2A, B3B is a derivative of B2B, B3D1/2 is a derivative of B2D, and B3I1/2/3 is a derivative of B2I.

solutions were obtained from Novozymes (Denmark), and cleaner base formulations were provided by Borer Chemie AG (Switzerland).

Bacterial strains and growth conditions. Bacterial strains were obtained from the Leibniz Institute German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). *Pseudomonas aeruginosa* (DSM 1117) and *Staphylococcus aureus* (DSM 20231) were grown on tryptic soy agar at 37°C. Liquid cultures were grown in 30% tryptic soy broth (TSB) (9 g/liter, which corresponds to 30% of recommended concentration) supplemented with 2.5 g/liter glucose at 37°C and 160 rpm.

Biofilm removal assay using 96-well plates. Bacterial cultures grown overnight were diluted to an optical density at 600 nm (OD_{600}) of 0.2 in 30% TSB supplemented with 2.5 g/liter glucose. Two hundred microliters of the bacterial suspension per well was added to transparent (for absorbance) or white (for luminescence) flat-bottom polystyrene 96-well plates (BRANDplates pureGrade). The biofilm in the wells was washed once with 350 μl of a 0.9% NaCl solution before cleaner treatment. All cleaners were used at a concentration of 1% in freshly prepared water of standardized hardness (WSH) containing 1.25 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 3.33 mM $NaHCO_3$ in deionized water. Each column (6 wells with bacteria and 2 wells with medium only) was treated with a different cleaner. A mixture of 1% SDS, 1% EDTA, 1% NaOH, and 0.1% NaClO was used as a positive control, and WSH was used as a negative control. Treatment was done with 250 μl cleaner per well for 40 min at 25°C. To determine the staining background, two rows of the microplate were filled with medium without bacteria. Plates were incubated for 24 h at 33°C with shaking at 40 rpm. For biofilm quantification, crystal violet staining and a BacTiter-Glo assay were applied as described previously (21).

Cleaning performance against artificial blood soil. TOSI (Test Object Surgical Instrument) slides (Pereg, Germany) were used by immersing them without a plastic cover in the cleaning solutions to be tested. No mechanical force was applied. The slides were removed from the cleaning solution after 15, 30, and 45 min of incubation; photographed; and then immersed again. At the 60-min time point, the slides were removed, photographed, gently rinsed with deionized water, and photographed again. The resultant cleaning kinetics was judged visually.

Cleaning performance using EN ISO 15883. Biofilm was formed in Teflon tubes (Karl Storz, Germany) according to Annex F of part 5 in EN ISO 15883 (version 2005) (https://www.iso.org/obp/ui/#iso:std:iso:ts:15883:-5:ed-1:v1:en). Treatment with a cleaner or WSH (negative control) was done at a flow rate of ~200 ml/min for 15 min at 25°C. If stated, disinfection after cleaning was done with deconex HLD PA/PA20. The tubes were cut into small pieces, and detachment of biofilms was done by vortexing in a NaCl solution. The following quantifications were conducted: (i) the OD_{600} of the suspension was measured, (ii) viable cells were quantified by determination of CFU on agar plates, (iii) protein levels were quantified using the Lowry assay (22), and (iv) polysaccharide levels were quantified by the phenol-sulfuric acid method (23).

Statistical analysis. For each sample, the biofilm value was calculated by subtracting the mean value for the 2 wells with medium only from the arithmetic mean for 6 wells with biofilm. Sample standard deviations were calculated from the values for the 6 similarly treated wells. Statistical significance was determined by using the unpaired, parametric, two-tailed Student *t* test. The value of the negative-control (WSH) wells was set to 100%, and the other values were calculated accordingly. Three independent experiments with six repeats per condition were performed for comparison to commercial products in the 96-well-plate biofilm removal assay.

Further details. More detailed materials and methods are provided in the supplemental material.

RESULTS AND DISCUSSION

Screening for enzyme-supportive base formulations. It was found that a small amount of protease alone was sufficient to completely remove *S. aureus* biofilms, almost independent of the base formulation (data not shown). Therefore, the use of *S. aureus* as a model microorganism is not appropriate for the selection of a base formulation since the bacteria found on endoscopes include many other species (3), of which, for example, the predominant species *P. aeruginosa* could not be easily removed with only protease (21).

A first round of screening with 23 novel base formulations (surfactants and other ingredients without enzymes) was performed based on the prerequisite that the desired compositions should support enzyme activity and display maximal biofilm removal. For this purpose, *P. aeruginosa* biofilm was treated with base formulations mixed with or without an enzyme cocktail (Fig. 1a). The mixture contained seven enzymes, including proteases, polysaccharidases, lipases, and DNases, and was expected to degrade the major components of biofilm EPS. Some base formulations (e.g., B1K and B1O) did not remove biofilm with or without enzymes. Others (e.g., B2L, B2H, and B2O) also did not remove much biofilm without enzymes (<50%), but the addition of enzymes allowed a strong increase in biofilm removal but only up to a maximum of ~85%. The best base formulations (B2A, B2B,

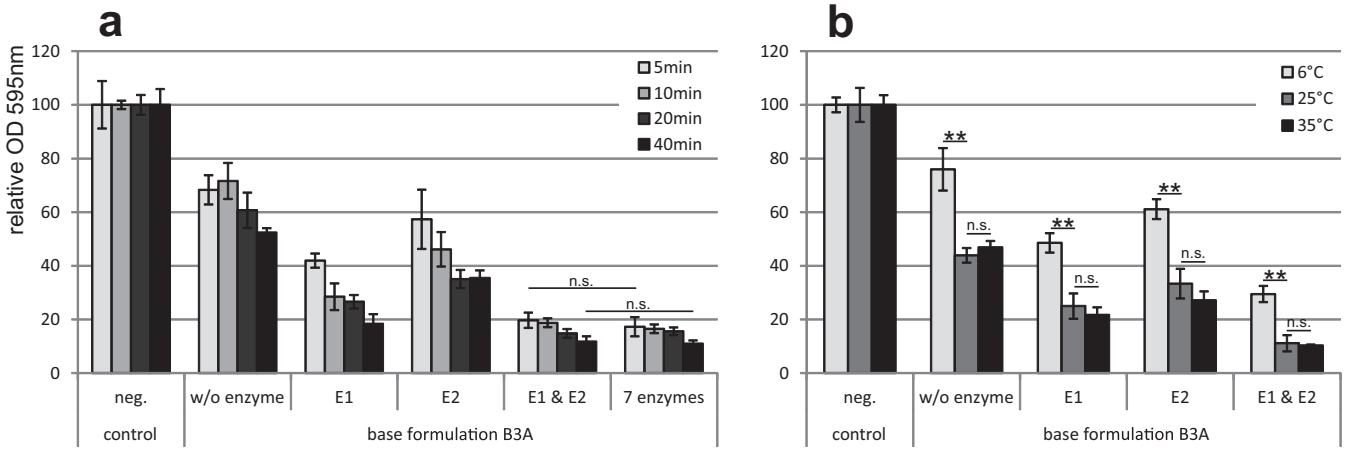

FIG 2 Biofilm removal under different conditions. *P. aeruginosa* biofilm was treated for different time periods at 25°C (a) or at different temperatures for 40 min (b) with base formulation B3A containing no, one, or two enzymes. The *y* axis represents the biofilm amount quantified by crystal violet staining relative to the negative control. Error bars represent results from 6 individual replicates. Enzyme E1 represents a polysaccharidase, and enzyme E2 is a protease. A *t* test was applied to calculate statistical significance (not significant [n.s.; $P > 0.05$] or highly significant [**, $P < 0.001$]) for comparisons, as indicated by lines in the graph.

B2D, and B2I) displayed ~60% removal without enzymes and 90% removal in combination with enzymes. Other base formulations that exhibited good efficiency of >60% removal without enzymes (e.g., B2C) were not further investigated due to the lesser effect of the enzymes (only 86% removal in combination with enzymes).

The selected base formulations were further optimized by a slight adaption of the detergent composition. In some cases, performance was slightly increased (e.g., B3A compared to B2A), while in other cases, less biofilm was removed (e.g., B3B compared to B2B) (Fig. 1b). B2A, B2B, B3A, B3D2, and B3I2 were identified as the most promising formulations and were further investigated. To study the capacity of the base formulations to support enzyme activities to remove biofilm at reduced enzyme numbers and concentrations, single enzymes (E1 to E8 [one protease, one lipase, one DNase, and five different polysaccharidases]) were individually added at three different concentrations. While all tested base formulations containing E1, E2, or E8 displayed strong biofilm removal ability, only certain formulations supported E3, E4, E5, and E7 activities, and E6 did not remove biofilm in any formulation (see Fig. S1 in the supplemental material). Base formulations B3A and B3D2 allowed significant biofilm removal with six out of the eight enzymes at low enzyme concentrations (0.5% enzyme solution in the cleaner concentrate), whereas the other base formulations supported fewer enzymes. With an increase of the enzyme concentration to 2.5%, biofilm removal was increased for several enzymes in B3A but not in B3D2. Thus, B3A was selected for the following optimization of cleaner composition.

Optimization of enzyme composition. Since enzyme activities are strongly dependent on treatment duration and temperature, different conditions were investigated. For base formulation B3A with single enzymes, a clear increase in the biofilm removal efficiency was observed with an increase of the incubation time from 5 to 40 min (Fig. 2a); thus, an incubation time of 40 min was selected. Treatment at both 25°C and 35°C resulted in good biofilm removal, while at 6°C, the cleaner was clearly less efficient (Fig. 2b). This demonstrates that a temperature of 25°C is sufficient for good performance in biofilm removal, which is important for a manual cleaner that is usually used at room temperature.

Investigation of different combinations of enzymes selected from a total of 13 individual enzymes (2 proteases, 9 polysaccharidases, 1 lipase, and 1 DNase) in base formulation B3A revealed that a mixture of 2 enzymes (1 polysaccharidase, E1, and 1 protease, E2 [0.5% {vol/vol} of the concentrate each]) was sufficient to remove 90% of the *P. aeruginosa* biofilm within 40 min, with 80% removal already after 5 min (Fig. 2a). This performance was similar to that of the seven-enzyme mixture. The addition of further enzymes to the two-enzyme mixture did not increase biofilm removal significantly. Enzyme mixtures missing either E1 or E2 were not able to reach similar levels of biofilm removal. However, based on the performance against artificial blood contaminations, two additional enzymes were included in base formulation B3A, and enzyme concentrations were increased. For example, improvement in cleaning of TOSI slides was observed with increasing concentrations of E2, representing a protease (see Fig. S2 in the supplemental material). The final cleaner containing four enzymes (1 to 2% each) is named deconex Prozyme Active.

Comparison of different cleaners. The novel formulation deconex Prozyme Active was compared with 9 comparable commercially available cleaners from different manufacturers (see Table S1 in the supplemental material). Staining of the total biomass with crystal violet revealed that *S. aureus* was removed easily by most commercial cleaners, including deconex Prozyme Active, that contain a protease (Fig. 3a). This is consistent with data from previous reports (24, 25). The tested nonenzymatic cleaners (C5 and C7) were not able to remove *S. aureus* biofilms under the static conditions used, and the positive control also only partially (60%) removed the biofilm.

In contrast, the positive control was effective against the *P. aeruginosa* biofilm, but most cleaners were less efficient in removing this biofilm (Fig. 3b). A mixture of several enzymes in combination with an effective base formulation was required to reach appropriate removal. deconex Prozyme Active removed up to 90% of the *P. aeruginosa* biofilm, which is slightly better than the

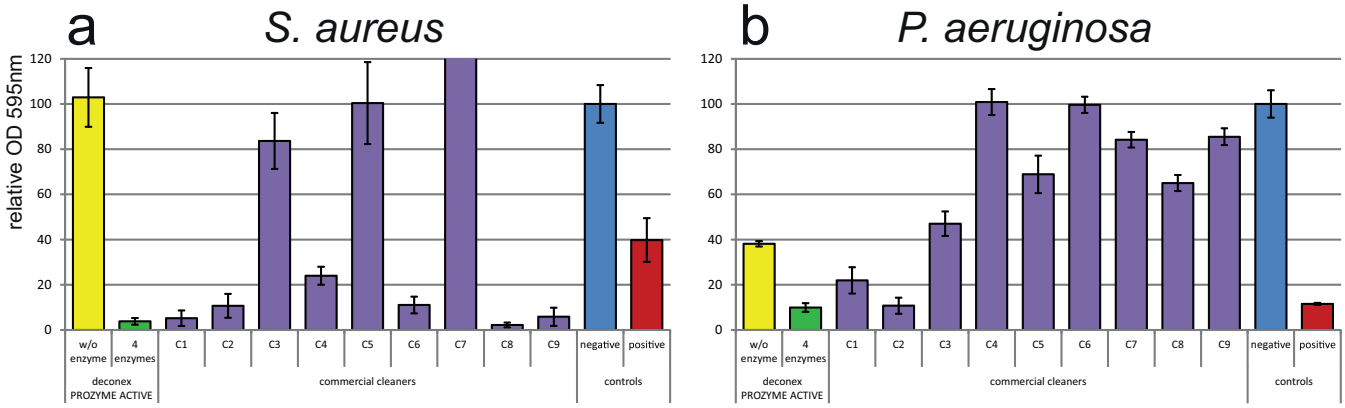

FIG 3 Removal of *S. aureus* (a) and *P. aeruginosa* (b) biofilms with different cleaners. Nine commercial cleaners (purple) were compared to deconex Prozyme Active (green) and its corresponding base formulation B3A without enzymes (yellow). The *y* axis represents the biofilm amount quantified by crystal violet staining relative to the WSH-treated negative control (blue). A mixture of 1% SDS, 1% EDTA, 1% NaOH, and 0.1% NaClO was used as a positive control (red). Error bars represent results from 6 individual replicates.

positive control and similar to the best commercial cleaner, C2. With 80% biofilm removal, C1 was also efficient, but the rest of the products removed <50% of the biofilm. To differentiate the cleaning and killing activities of the cleaners, the viability of the remaining cells after cleaning was further analyzed by using the BacTiter-Glo assay. Remaining viable cells were at levels similar to remaining biomass after treatment with most cleaners (<0.8-log difference between viable cells and biomass [indicated by dashed bars in Fig. S3 in the supplemental material]). Thus, these cleaners did not possess substantial biocidal activity. In contrast, it was found that after treatment with C3 and C7, substantially fewer viable cells (in percentage) were found than the remaining biomass (>1.8-log difference). These cleaners displayed disinfecting properties and rather killed bacteria instead of removing the biofilm.

The efficiency of deconex Prozyme Active in removing artificial blood contamination was also compared to that of commercial products (see Fig. S4 in the supplemental material). Only C2 performed slightly better than and C1 and C8 performed similarly to the new formulation, while all the other commercial cleaners required longer incubation times to reduce and remove the soil. Two nonenzymatic cleaners, C5 and C7, displayed the lowest activity against this artificial blood soil.

Performance against biofilm in endoscope channels. So far, there is not a standard procedure for testing biofilm removal with manual cleaners. In the technical specification Annex F of part 5 in EN ISO 15883 (version 2005) (https://www.iso.org/obp/ui/#iso:std:iso:ts:15883:-5:ed-1:v1:en), a method for biofilm formation and evaluation of biofilm removal from endoscope channels is described. This method is used for testing cleaners and disinfectants in automated processes at elevated temperatures (usually 35°C to 55°C). Acceptance criteria for this method for biofilm cleaning efficacy are set at 90% removal of proteins and polysaccharides. This procedure was used for testing deconex Prozyme Active and other manual cleaners for their biofilm removal capabilities during 15 min of treatment under a continuous flow of 200 ml/min.

With deconex Prozyme Active, the CFU of the *P. aeruginosa* biofilm were reduced by >2 logs (99%) compared to WSH-treated control tubes (see Fig. S5 in the supplemental material). Clearly, more biofilm was removed if the cleaner contained enzymes than when the cleaner contained an enzyme-free base formulation, demonstrating the beneficial effect of the enzymes. Commercial cleaners C4 (0.28-log reduction) and C6 (0-log reduction) did not sufficiently reduce CFU, while C1 (1.62-log reduction) and C2 (1.51-log reduction) were slightly less effective than the novel formulation (2.11-log reduction). After treatment with C7, almost no viable bacteria were recovered (5.57-log reduction). The results regarding the efficiency of removal of EPS compounds were similar to those for the remaining CFU, except for C7, where neither protein nor polysaccharide levels were significantly reduced (see Fig. S6 in the supplemental material). This suggests that C7 killed the bacteria rather than removing the biofilm, which is consistent with observations from the 96-well-plate biofilm removal assay. These results are summarized in Table 1.

Another important criterion for the standard assay is that cleaning should allow complete killing of all bacteria by subsequent disinfection. Therefore, the biofilm remaining on the tube was subsequently treated with a high-level disinfectant (deconex HLD PA/PA20). No viable bacteria were recovered from the disinfected deconex Prozyme Active-treated tubes, while ~1,600 CFU per cm^2 were found on tubes treated with WSH (negative control) prior to disinfection. This demonstrates the importance of an efficient cleaning step to enable the success of the consequent disinfection.

Microscopy analysis was performed to confirm biofilm cleaning. It was observed that large parts of the biofilm were removed by treatment with deconex Prozyme Active but not after WSH treatment. While the control displayed a dense biofilm with multiple layers (99.6% surface coverage), the deconex Prozyme Active-treated sample exhibited much lower surface coverage (11.1%), and almost no aggregates were observed (Fig. 4). deconex Prozyme Active was also found to be superior to the other cleaners regarding biofilm removal from endoscope tubes (see Fig. S7 in the supplemental material). Only C1 (45.8% coverage) and C2 (27.4% coverage) also displayed some bacterium-free areas, but in addition to higher surface coverage, more aggregates were observed. For C7, bacteria appeared blurry, even though the cells were perfectly in focus. This was likely due to killing of

TABLE 1 Reduction of biofilm biomass, numbers of viable bacteria, polysaccharide levels, and protein levels after treatment with cleaners compared to the negative-control (WSH) treatment[d]

Cleaner	% reduction of biomass as determined by OD_{600}	% reduction of bacterial CFU	Log reduction of bacterial CFU[a]	% reduction in polysaccharide levels[b]	% reduction in protein levels[c]
Base formulation B3A	82.3	89.21	0.97	79.2	82.4
deconex Prozyme Active	94.6	99.23	2.11	93.1	97.9
C1	93.0	97.61	1.62	86.2	89.4
C2	91.4	96.89	1.51	84.6	95.1
C6	−10.2	−1.32	−0.01	−31.5	−9.2
C4	19.4	47.81	0.28	19.2	19.0
C7	−34.4	99.99973	5.57	13.1	5.6

[a] Log_{10} reduction compared to the negative control.
[b] As determined by the phenol-sulfuric acid method described previously by Dubois et al. (23).
[c] As determined by the protein quantification assay described previously by Lowry et al. (22).
[d] Negative values indicate a lower level of removal than with WSH.

the bacteria by destroying their membrane integrity, as a similar effect was observed when the WSH-treated tube was disinfected with deconex HLD PA/PA20 (see Fig. S8 in the supplemental material).

Stability of the formula regarding cleaner performance. To investigate the stability of the formulation, the cleaner concentrates were stored at room temperature (25°C) and, for accelerated aging, at increased temperatures. After storage for 24 weeks at 25°C, the new formulation still removed >90% of the *S. aureus* biofilm and >85% of the *P. aeruginosa* biofilm (see Fig. S9 in the supplemental material). Storage of the cleaner concentrates at 40°C did not affect the performance significantly. Even after incubation at 50°C for 24 weeks, ~75% of the biofilm was removed, being clearly more effective than the enzyme-free version, which removed <50% of the biofilm. Additionally, artificial blood contaminations were removed effectively by deconex Prozyme Active stored at 25°C and 40°C for 24 weeks, while at 50°C, its activity was impaired slightly after 12 weeks and slightly more after 24 weeks (data not shown). This confirms that the product keeps its activity during storage at room temperature and even survives short periods at higher temperatures, e.g., during transport.

Conclusions. The 96-well-plate biofilm removal assay and the endoscope ISO test led to matching results regarding the efficiency of the novel and commercially available cleaners studied. For total biofilm biomass assessment, the results of optical density, protein, and polysaccharide quantification with the ISO test correlated with the results of crystal violet staining in the 96-well-plate assay, while for viable bacteria, the CFU corresponded to those determined by the BacTiter-Glo assay. This confirms that the 96-well-plate assay represents an appropriate model to screen for cleaners that remove biofilms and to investigate which formulation rather acts as a disinfectant.

The addition of enzymes to the base formulation had a clear beneficial effect on the efficiency of biofilm removal. The *S. aureus* biofilm was removed efficiently if an active protease was present, whereas for *P. aeruginosa*, single enzymes added to the formulation were not sufficient. An optimized enzyme mixture including protease, polysaccharidases, and other enzymes in a selected base formulation was required to achieve efficient removal of *P. aeruginosa*. Therefore, many commercial products displayed good performance against *S. aureus* and blood contamination but had problems with the removal of *P. aeruginosa* biofilms. Nonenzymatic cleaners were not effective in either blood cleaning or biofilm removal but rather worked as a disinfectant, killing the bacteria. However, a cleaner should mainly remove bacteria, as the standard endoscope reprocessing procedure is followed by disinfection. Among the tested high-end enzymatic endoscope detergents, the novel cleaner deconex Prozyme Active demonstrated the best efficiency in biofilm removal. Additionally, it was among the best products in removing blood contamination.

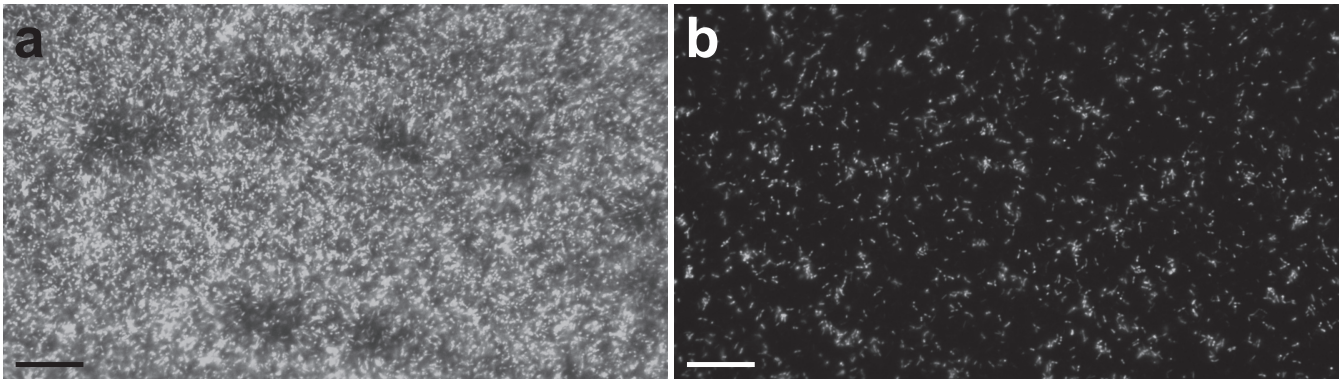

FIG 4 Bacteria remaining on endoscope tubes after treatment. Syto9 staining of *P. aeruginosa* biofilm on tubes treated with WSH (a) or deconex Prozyme Active (b) was visualized with a 20× water immersion objective. Bacterial cells appear as white spots. Bars, 25 μm.

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Efficient Biofilms Eradication by Enzymatic-Cocktail of Pancreatic Protease Type-I and Bacterial α-Amylase

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Abstract: Removal of biofilms is extremely pivotal in environmental and medicinal fields. Therefore, reporting the new-enzymes and their combinations for dispersal of infectious biofilms can be extremely critical. Herein, for the first time, we accessed the enzyme "protease from bovine pancreas type-I (PtI)" for anti-biofilm properties. We further investigated the anti-biofilm potential of PtI in combination with α-amylase from *Bacillus sp.* (αA). PtI showed a very significant biofilm inhibition effect (86.5%, 88.4%, and 67%) and biofilm prevention effect (66%, 64%, and 70%), against the *E. coli*, *S. aureus*, and MRSA, respectively. However, the new enzyme combination (*Ec*-PtI+αA) exhibited biofilm inhibition effect (78%, 90%, and 93%) and a biofilm prevention effect (44%, 51%, and 77%) against *E. coli*, *S. aureus*, and MRSA, respectively. The studied enzymes were found not to be anti-bacterial against the *E. coli*, *S. aureus*, and MRSA. In summary, the PtI exhibited significant anti-biofilm effects against *S. aureus*, MRSA, and *E. coli*. *Ec*-PtI+αA exhibited enhancement of the anti-biofilm effects against *S. aureus* and MRSA biofilms. Therefore, this study revealed that this *Ec*-PtI+αA enzymatic system can be extremely vital for the treatment of biofilm complications resulting from *E. coli*, *S. aureus*, and MRSA.

Keywords: protease type-I; α-amylase; anti-biofilm enzymes; biofilm eradication

1. Introduction

Bacteria demonstrates versatile-tactics to infect humans [1]. In acute infections, they promptly spread and proliferate as a planktonic/individual form [2,3]. But, when an infection reaches the persistent or chronic stage, they largely colonize the tissues and other body-surfaces in highly-organized patterns of multicellular-aggregates termed as biofilms [1,4,5]. Moreover, the important strategy adopted by bacteria for survival against anti-microbial materials and hostile environmental conditions is the formation of a rigid biofilm [1,2,6]. The microbes in a biofilm community exhibit advanced antibiotic resistance that can be up to 1000 times higher than the corresponding planktonic micro-organisms [7]. The contamination of the medical device and food packaging surfaces with pathogenic bacteria might lead to the biofilm formation, thereby it can cause serious acute and chronic infections to people [8,9]. Biofilm composed of multi-species are difficult to remove through host defense systems or by the antibiotic treatment [5,6,10]. Therefore, recently it has become imperative to advance various treatment approaches for biofilm eradication.

Structurally, biofilms are aggregates of micro-organisms encased in extracellular polymeric substances (EPS) [11–13]. The EPS matrix is mainly composed of polysaccharides, extracellular-polymeric substances,

lipids, proteins, and extracellular DNA (eDNA) [11]. The EPS allows immobilization of cells and retains them nearby, thus permitting for deep interactions, comprising cell to cell communication, and microconsortia formation [11]. Biofilm formation happens in the four main stages: (1) attachment of bacteria to a surface; (2) formation of microcolony; (3) maturation of biofilm; and (4) dispersal of bacterial biofilm [14,15]. In this process the proteins and polysaccharides from EPS play a vital role [14]. Thus, enzymes which can degrade these proteins and polysaccharides are of high importance in biofilm treatment processes [5,16–20]. Enzymatic degradation of EPS induces susceptibility of the microbes to anti-microbial agents. A previous study showed that enzymes induce the anti-biofilm effects that caused the anti-microbial materials to kill the bacteria released from biofilm [21]. Hence, to introduce more enzymes and enzymatic combinations with excellent capacity of EPS removal will be highly encouraging, and it will enhance the efficacy of biofilm infection treatment strategies.

Efficacious removal of complex biofilms needs the usage of multi-enzyme formulations, which are capable of degrading microbial proteins, eDNA, polysaccharides, and quorum-sensing molecules [22]. These include various enzymes such as proteases, amylases, DNAses, β-glucosidases, and lyticases, etc. [5,19,23–25] The protease was found to be more effective compared to amylase for eliminating the *Pseudomonas fluorescens* biofilm [26]. Proteases are of many forms and are well-known as they hydrolyze the peptide bonds and degrade the proteins [27]. Protease induced the degradation of biofilm components and destruction of biofilm backbone [22]. Many proteases from several origins are well studied for anti-microbial and anti-biofilm effects. Bovine pancreatic enzymes are an excellent source for the many therapeutic enzymes [28–31]. However, protease type I (PtI) from the bovine pancreas is still not evaluated for its potential against the biofilms. Looking at the worsening biofilm infection problems and inefficiencies in their treatment, several kinds of protease and their combinations with the other enzymes are highly important [14]. Furthermore, the effect of this important enzyme needs to be evaluated for both the anti-biofilm activities such as "inhibitions of the established biofilm" and "prevention of the biofilm formations" [32]. There is an extreme need of anti-biofilm enzymes which have both of these potentials: biofilm inhibitions and biofilm prevention [5,33,34]. This kind of formulation having both the capacities will help immensely in the available treatment strategies for biofilm infections [33]. Accordingly, the new protease source "PtI" with a combination of α-amylase (αA) for biofilm inhibitions and biofilm prevention of three major bacteria (*Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and methicillin-resistant *S. aureus* (MRSA)) can be a vital study. Hence, this study might add an extremely important and valuable source of multi-enzyme combination to dispersal of the biofilms. Therefore, this kind of enzyme cocktail for biofilm removal is a prerequisite to add better solutions in the treatment of biofilms.

Hence, in this study, PtI from a bovine pancreas was accessed in combination with the αA (denoted as; *Ec*-PtI+αA) against the *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and methicillin-resistant *S. aureus* (MRSA) biofilms for anti-biofilm activities such as "inhibition of established biofilms" and "prevention of biofilm formation". The studied enzymatic combinations were also accessed for the possible anti-microbial properties against *E. coli*, *S. aureus*, and MRSA.

2. Materials and Methods

2.1. Microbial Strains

Escherichia coli (KCCM 11234; *E. coli*), *Staphylococcus aureus* (KCCM 11335; *S. aureus*), and methicillin-resistant *Staphylococcus aureus* (ATCC 33591; MRSA) were purchased from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea) and the American Type Culture Collection (ATCC, Manassas, VA, USA). Protease from bovine pancreas Type I (PtI) having 5 U/mg activity, and α-Amylase from *Bacillus* sp. (Powder form) (αA), having 400 U/mg activity, were purchased from Sigma Aldrich, St. Louis, MO, USA. Each bacterium was incubated on a Tryptic Soy Agar plate (TSA; BD, San Jose, CA, USA) at 37 °C overnight. A colony of the bacterium was inoculated in Tryptic Soy Broth (TSB; BD, San Jose, CA, USA) and incubated at 37 °C 150 rpm overnight.

2.2. Biofilm Formation and Inhibition Assay

The biofilm formation experiment was referred to by [35]. All bacteria strains were cultured in TSB medium and dispensed into a 6-well plate. OD600 of 1.0 bacteria were seeded and incubated at 37 °C for 24 h. The formed biofilms are washed two times using PBS very carefully without disturbing the biofilm. The inhibition effect of PtI, αA and *Ec*-PtI+αA on biofilms was estimated on developed biofilms in a 6-well plate using Crystal Violet (CV) assay and plate count method (colony forming units/mL or CFUs) [36]. The enzymes were individually PtI (2 U/mL) and αA (2 U/mL), and in combination *Ec*-PtI+αA (2 U/mL each) were treated with each bacterial biofilm formed in a 6-well plate. This plate was incubated for 37 °C for 2 h for the enzymatic treatment. After incubation was completed, the biofilms were washed very carefully two times with PBS and further added with 0.1% CV solution. After having taken a picture of the 6-well plate, 1 mL of ethanol was added to each well, and the absorbance was estimated at 570 nm. The more bacteria remaining in the biofilm, the higher the absorbance of CV at 570 nm [35,36]. This gave the quantitative measure of the biofilm inhibition after the enzyme treatments. Additionally, to confirm the biofilm inhibition assay, the enzyme-treated biofilms in 6 well plates were added with 1 mL of peptone water. The peptone water solution was well mixed with biofilms by micropipette. Then, 0.1 mL of the sample from each well were transferred onto the TSA plates and incubated at 37 °C for 24 h. The more cells remaining in the biofilm, the more CFU will form and vice versa [36]. This gave the quantitative measure of the biofilm inhibition.

2.3. Biofilm Prevention Assay

A bacteria strains of *E. coli*, *S. aureus* and MRSA (OD600 of 1.0) was cultured in TSB medium with the PtI (2 U/mL), αA (2 U/mL) and *Ec*-PtI+αA (2 U/mL each enzyme) into a 6-well plate, respectively. Seeded bacteria with enzymes were incubated at 37 °C for 24 h. The formed biofilms in the presence of enzymes were washed two times using PBS and added with a 0.1% CV solution. After taking a picture of the 6-well plate, 1 mL of ethanol was added to each well, and the absorbance was estimated at 570 nm. Additionally, to confirm the biofilm prevention assay, the biofilms formed in presence of the enzymes in 6-well plates was added with 1 mL of peptone water. The peptone water solution was well mixed with biofilms by micropipette. Then, 0.1 mL of the sample from each well were transferred onto the TSA plates and incubated at 37 °C for 24 h.

2.4. Antibacterial Assay

The anti-bacterial effect of PtI, αA and *Ec*-PtI+αA was assessed against the *E. coli*, *S. aureus*, and MRSA. Each cultivated bacteria (OD600 of 1.0) was incubated with enzymes: PtI (2 U/mL), αA (2 U/mL) and *Ec*-PtI+αA (2 U/mL each enzyme) in TSB at 37 °C for 2 h. After incubation, the microbial viability level was evaluated using the microbial viability assay ELISA kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Moreover, to confirm the anti-bacteria effects, bacteria were seeded on TSA and incubated at 37 °C for 24 h. After that, colony-forming units of each sample were manually counted.

2.5. Statistical Analysis

All results were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). All data were expressed as mean ± SEM. Statistical significance was evaluated by one-way and two-way analysis of variance (ANOVA), followed by Turkey's multiple comparison test at $p < 0.05$.

3. Results and Discussion

3.1. Biofilm Inhibition Study

The biofilms of *E. coli*, *S. aureus*, and MRSA were successfully established in 6-well plates. Then, the PtI, αA, and *Ec*-PtI+αA were accessed their potential for biofilm inhibition of *E. coli*, *S. aureus*, and MRSA. The obtained quantitative data for biofilm inhibition of PtI, αA, and *Ec*-PtI+αA were shown in Figure 1. Photographic images of CV staining given in Figure S1 (Supplementary Information). The αA exhibited 40% inhibition of the *E. coli* biofilm. The PtI enzyme acted on the *E. coli* biofilm and gave very significant 86.5% inhibition. The *Ec*-PtI+αA enzyme combination resulted in the 78.6% inhibition of the *E. coli* biofilm. The obtained results conveyed that PtI works far better for *E. coli* biofilm eradication in comparison with the αA and *Ec*-PtI+αA. The role of the enzyme in anti-biofilm results directly depended on the composition and structure of the biofilm [24]. The slightly lower *E. coli* biofilm inhibition by *Ec*-PtI+αA compared to PtI might be due to the interference caused by αA catalytic activity. The obtained result was supported by the previous report Lim et al., 2019 [17]. Lim et al., 2019 [17] studied EPS-protein degradation by proteinase K to control the *E. coli* O157: H7 biofilm efficiently. In this study, PtI acted better in *E. coli* biofilm, mainly due to the efficient degradation of proteins by PtI from the EPS. The obtained biofilm inhibition by enzymatic combinations can be very significant. This information will provide an additional enzymatic source to eradicate the *E. coli* biofilm.

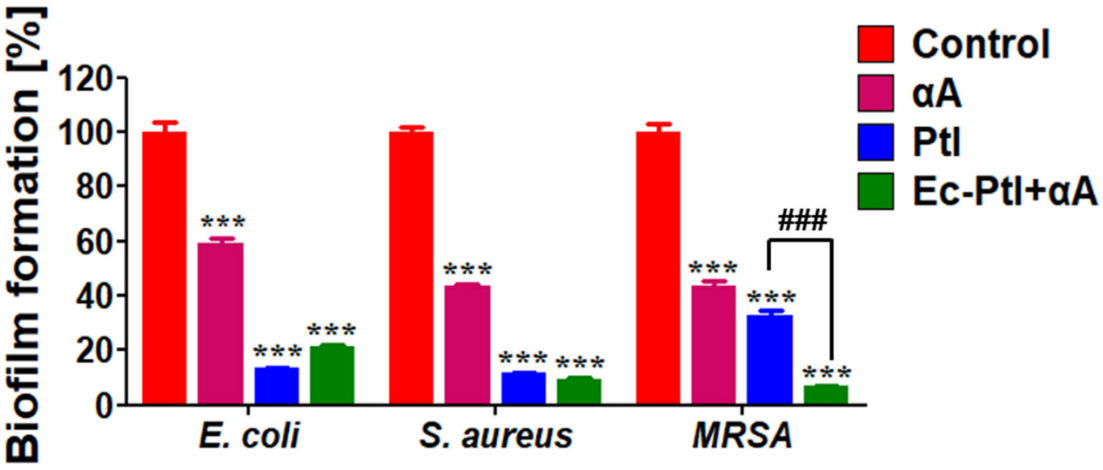

Figure 1. The inhibition effect of PtI, αA, and *Ec*-PtI+αA against biofilm was quantified CV staining at 570 nm. All values are expressed as mean ± SEM (n = 3) and significantly different in comparison to controls (***, $p < 0.001$) and to protease (###, $p < 0.001$) by Tukey's multiple comparison test.

After confirming the anti-biofilm assessments against *E. coli* biofilm, the enzymatic systems were assessed against the *S. aureus* biofilm. The αA, PtI, and *Ec*-PtI+αA were showed 60%, 88.4%, and 90.5% inhibition of the *S. aureus* biofilm, respectively (Figure 1). The photographic images of CV staining were shown in Figure S1 (Supplementary Materials). The PtI and *Ec*-PtI+αA exhibited very high biofilm inhibitions (88.4% and 90.5%) of *S. aureus* biofilm than the αA (60%). Not significant elevation in *S. aureus* biofilm inhibition was observed by *Ec*-PtI+αA than the individual PtI. The culture supernatant of *Pseudomonas aeruginosa* PAO1 containing higher protease activity gave 80% inhibition of *S. aureus* biofilm [37]. Protease aureolysin (Aur) was inhibited 50% of the *S. aureus* biofilm [38]. Protease neutrase from *Bacillus amyloliquefaciens* gave 72% inhibition of the *S. aureus* biofilm [39]. In a comparison of this literature, PtI and *Ec*-PtI+αA exhibited higher biofilm inhibition for *S. aureus* biofilm. Thus, the enzyme PtI and *Ec*-PtI+αA combination can be vital in the treatment of *S. aureus* biofilm.

MRSA is a dangerous pathogen as it encompasses strong resistance against the β-lactam antibiotics [40]. Once the bacteria become resistant to two or more antibiotics, it is usually mentioned as a superbug, multiple-antibiotic-resistant bacteria, or a super-bacterium [41]. Drug-resistant bacteria MRSA (gram-positive) is considered to be a serious threat and is a major challenge to global health [42].

In the biofilm form, MRSA become harder to treat and to handle its consequences [40]. There are limited studies that have investigated the dispersal of MRSA biofilms by applying enzymatic agents [16]. Therefore, in this study, we treated MRSA biofilms with PtI, αA, and *Ec*-PtI+αA. The enzymes; αA, PtI, and *Ec*-PtI+αA showed 60%, 67%, and 93.3% inhibition of the MRSA biofilm (Figure 1). The significant enhancement in the anti-biofilm effect against the MRSA was evident (Figure 1). The EPS composition of the MRSA biofilm might be oriented in such a way that the combination of *Ec*-PtI+αA was able to disperse at a higher rate, rather than the individual enzymes αA and PtI. The obtained anti-biofilm properties of αA, PtI, and *Ec*-PtI+αA against the MRSA biofilm are highly encouraging and could be more suitable for the development of the treatment protocols. It is very important to know the detailed comparison of recently reported proteases for the anti-biofilm effect. The list of recent enzymes applied for biofilm removal was listed in the Table 1. This comparison revealed the newly reported enzyme PtI stand alone, which is worth reporting and is highly efficient at tackling biofilm infections.

Furthermore, the biofilm samples remaining after treatment of enzymes were analyzed for the determination of viable cell numbers by plate count (colony forming units/mL or CFUs) [36,43–45]. The enzyme-treated biofilm mixed well with 1 mL of peptone water by using a micropipette to release the cells from the biofilm. The released cells were placed on the Petri dish to confirm the number of cells remaining in biofilms. Figure S2 supplementary information represents the obtained results of cells remained after the enzymatic treatment. The control Petri dishes of *E. coli*, *S. aureus*, and MRSA showed a higher number of colonies. However, the number of colonies was significantly decreased in the PtI- and *Ec*-PtI+αA-treated biofilms. Thus, the obtained results are in agreement with the CV staining quantitative results (Figure 1). Thus, both CV staining and viable cell numbers by plate count results evidenced the eradication of the biofilms. Thus, in summary, PtI was found to be a new protease source and there is a high possibility of the development of PtI-based treatment of biofilms.

Table 1. The detailed account of several reported protease for biofilm inhibition.

Enzyme	Sources	Biofilms Inhibition (%)	Target Bacteria	Reference
PtI	Bovine pancreas	87 89 67	*E. coli* *S. aureus* MRSA	This work
Flavourzyme	*Aspergillus oryzae*	50	*S. epidermidis*	[39]
Neutrase	*Bacillus amyloliquefaciens*	72 35	*S. aureus* *S. epidermidis*	[39]
Alcalase	*B. licheniformis*	25	*S. epidermidis*	[39]
α-amylase	*B. subtilis*	50 65 70	*Pseudomonas aeruginosa* *Vibrio cholerae* MRSA	[46]
Aureolysin	*S. aureus*	50 33	*S. aureus* *S. epidermidis*	[38]
Dispersin B	*Aggregatibacter actinomycetemcomitans*	50	*S. epidermidis*	[38]
Proteinase K	*Tritirachium album*	5 10 5 75 90	*Pseudomonas aeruginosa* *Vibrio cholerae* MRSA *S. aureus* *L. monocytogenes*	[47]
Papain	Papaya	80	*L. monocytogenes*	[32]
Trypsin	PA clan superfamily	20	*Pseudomonas aeruginosa*	[46]

3.2. Prevention of Biofilm Formation

After accessing the effect of αA, PtI, and *Ec*-PtI+αA on established biofilms, it is necessary to check if there is any role of these enzymes in the prevention of biofilm formation. Hence, to confirm the biofilm-prevention effect, we inoculated the enzymes αA, PtI, and *Ec*-PtI+αA in a growth medium with bacteria *E. coli*, *S. aureus*, and MRSA in the planktonic form before the biofilm formation. The obtained results are shown in Figure 2. The CV staining images showed that PtI and *Ec*-PtI+αA significantly reduced the *E. coli*, *S. aureus*, and *MRSA* biofilm formation (Figure S3, supplementary information). The discoloration was evident in PtI and *Ec*-PtI+αA compared to the control and αA-treated biofilms. Furthermore, the quantitative analysis of the prevention of biofilm was shown in Figure 2. The αA exhibited very low biofilm prevention of *E. coli*, *S. aureus*, and MRSA. The PtI caused 66, 64%, and 70% prevention of the biofilm of *E. coli*, *S. aureus*, and MRSA, respectively. However, *Ec*-PtI+αA exhibited 44%, 51%, and 77% prevention of the biofilm formation of *E. coli*, *S. aureus*, and MRSA, respectively. The obtained result indicated that PtI caused enhanced biofilm prevention in *E. coli* and *S. aureus* biofilms. However, the *Ec*-PtI+αA possessed enhancement in MRSA biofilm. Therefore, the PtI enzyme played a vital role in the prevention of all the biofilm formations. The *S. aureus*, *E. coli*, and MRSA biofilm matrixes contain protein components that maintain biofilm integrity [34,48]. The enzyme system of PtI and *Ec*-PtI+αA might be acted initially on bacterial adherence proteins and caused the prevention of biofilm formation. Proteinase and trypsin have frequently been used as efficient biofilm prevention agents that hinder bacterial adherence and biofilm formation in *S. aureus* [48]. The PtI and *Ec*-PtI+αA exhibited significant biofilm inhibition as well as prevention effects, while αA gave the biofilm inhibitions but not prevention. The CV staining data for biofilm prevention was validated by the bacterial colony counting technique in Figure 3. The biofilm after enzyme treatment was mixed with peptone water and cultured on Petri plates. The obtained data are shown in Figure 3. The control and αA-treated biofilm samples showed a very high number of colonies compared to the PtI- and *Ec*-PtI+αA-treated biofilm samples in Figure 3. Thus, the obtained results of CV staining and colony counting corroborated the efficacy of the PtI and *Ec*-PtI+αA for biofilm prevention of *E. coli*, *S. aureus*, and MRSA.

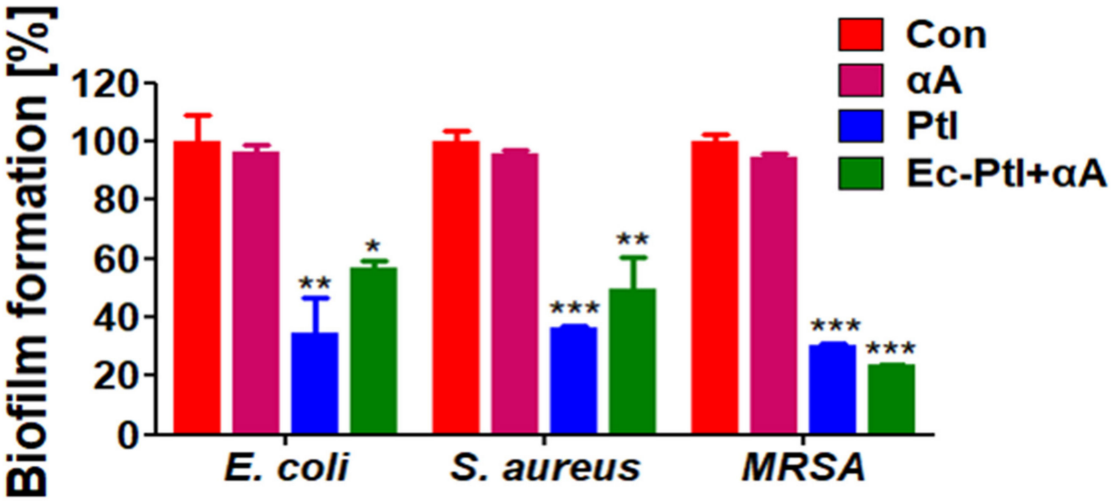

Figure 2. The prevention effect of protease and protease with α-amylase against biofilm formation by *E. coli*, *S. aureus*, and MRSA were performed using quantification of CV staining at 570 nm. All values are expressed as mean ± SEM ($n = 3$) and are significantly different in comparison to controls (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$) by Tukey's multiple comparison test.

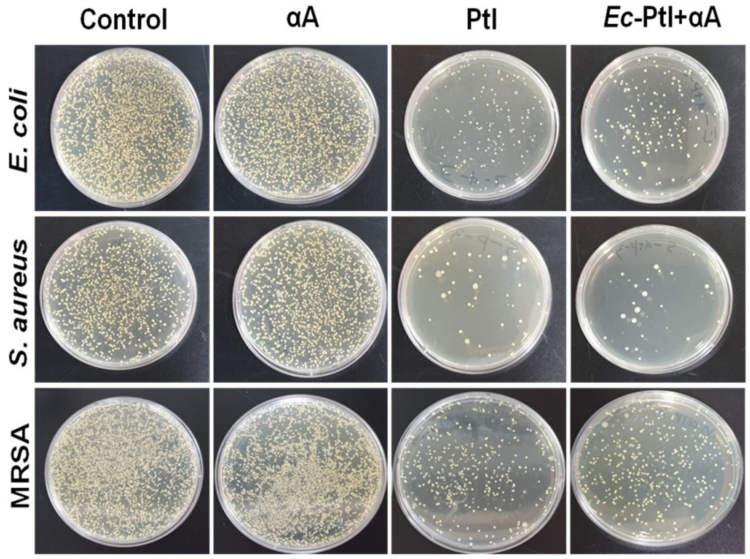

Figure 3. The prevention effect of αA, PtI, and *Ec*-PtI+αA against biofilm formation by *E. coli*, *S. aureus*, and MRSA were performed by colony counting.

3.3. Proposed Mechanisms for the Anti-Biofilm Effect

The active PtI and αA acted on the backbone proteins of the EPS of established biofilms (Figure 4), this degraded the EPS backbone resulting in the respective biofilm eradication (Figure 4). In the enzymatic cocktail *Ec*-PtI+αA, PtI with αA acted on both proteins and starch components of EPS and elevated the biofilm eradication in MRSA biofilms (Figure 1).

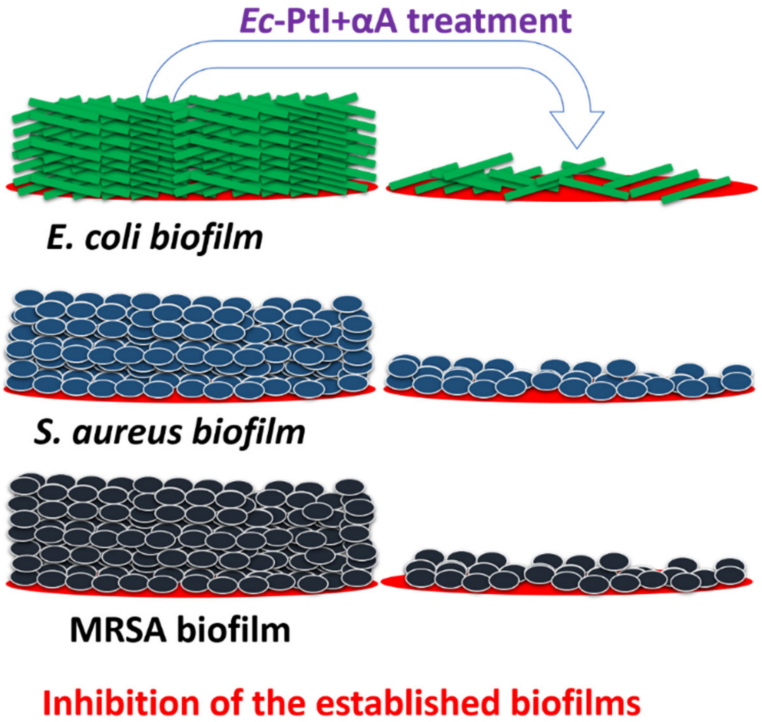

Figure 4. Schematic presentation of the inhibition of established biofilms.

However, in the possible mechanism of the prevention effect, PtI acted on the proteins released from the planktonic cells for biofilm formation (Figure 5). This degradation of the proteins hampered the bacterial adherence processes and ultimately caused the prevention of biofilm formation (Figure 5).

In the interesting observation of the prevention effect study, the individual αA exhibited a negligible biofilm prevention effect. This observation suggests that initially, proteins from EPS play an important role in the build-up of biofilm formation. Biofilm formation includes four main stages: bacteria attachment to surface, microcolony formation, biofilm maturation, and dispersal of bacterial biofilm [15]. The role of protein in the early stage of the bacterial attachment to the surface was investigated by Landini et al., 2010 [49]. Landini et al., 2010 concluded that initially proteins are involved in the bacterial cell attachment and then decipher the very early steps in biofilm formation [49]. The obtained results in our study are in line with this report. Hence, the degradation of the proteins at the early stage of biofilm formation might help the biofilm treatment significantly.

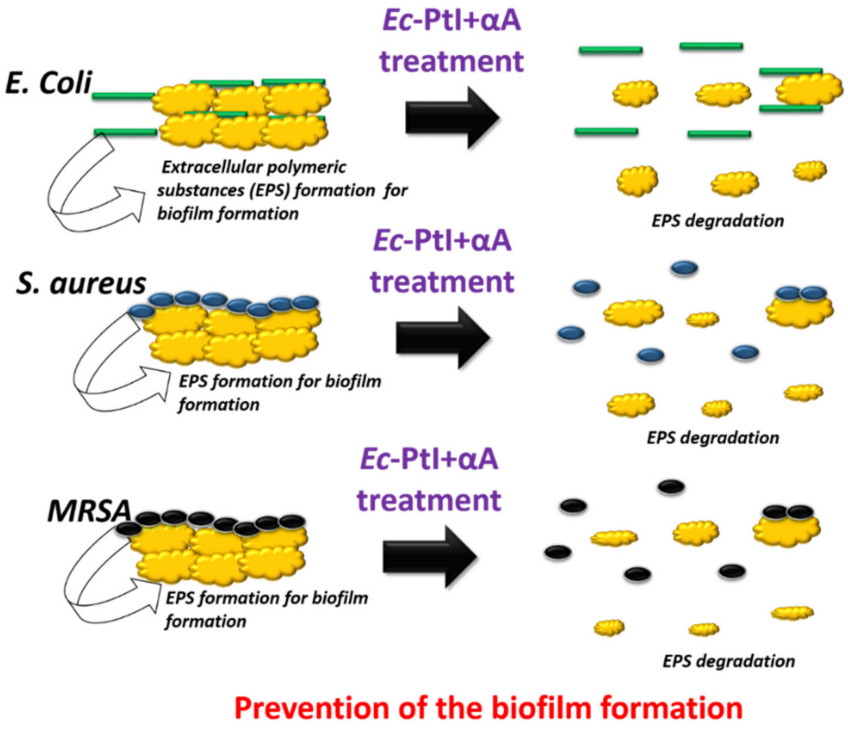

Figure 5. Schematic presentation of the prevention of the biofilm formation.

After a close inspection of all the obtained results, it is very much clear that the different biofilms (*E. coli*, *S. aureus*, and MRSA) give a different response to αA, PtI, and *Ec*-PtI+αA enzyme systems. This is mainly due to the different scaffold and EPS composition of different bacteria [11]. Therefore, it is important to understand the EPS compositions in detail. The detailed composition of EPS of the *E. coli* biofilm was explained in [50]. The major proteins of the *E. coli* biofilm EPS are adhesins, these adhesins are transported to the extracellular environment by auto-transporter adhesins and explore the adhesin potential of *E. coli* [50]. Along with the expression of adhesins by *E. coli*, formation of biofilm matrix is vital for biofilm maturation. This biofilm matrix is an extremely complex, three dimensional background, and is fundamentally composed of water (97%), proteins, exopolysaccharide polymers, lipids/phospholipids, nucleic acids, metabolites, and absorbed nutrients [17,50–53]. The *S. aureus* biofilm EPS have mature *S. aureus* cells, primary oligosaccharide (polymer of *N*-acetyl-β-(1-6)-glucosamine), polysaccharide intercellular adhesin, eDNA, teichoic acids, secrete and lysis-derived proteins, accumulation-associated protein (Aap), and surfactant-like peptides (at the end of biofilm cycle for detachment) [54–59]. Thus, EPS of both the bacteria have a complex and three dimensional structure, and hence it is hard to quantify, as it varies greatly from species to species [14]. The adhesion extracellular proteins play a key role in complex EPS of both the biofilms. Therefore, breakdown of extracellularly secreted adhesins by PtI might be a key factor for the obtained anti-biofilm results for *E. coli* and *S. aureus*. The *E. coli* and *S. aureus* anti-biofilm effect observed in

the presence of αA is less than that for PtI, and it is suggested that breakdown of the proteins than polysaccharides from EPS elevates the anti-biofilm effect. However, the combination of enzymes *Ec*-PtI+αA helped to encounter MRSA biofilm EPS, and hence degrading polysaccharides and proteins at the same time elevated the anti-biofilm effect in MRSA compared with the individual αA and PtI.

Earlier PtI was reported for the extraction of the hemicellulose from wheat germs [60]. The bovine pancreas is a rich source of many therapeutic enzymes [61]. The major function of the bovine pancreatic acinar cell is the synthesis, storage, and secretion of several digestive enzymes, such as proteases, amylase, lipase, elastase, and ribonucleases, to catalyze the food constituent hydrolysis into absorbable forms [30]. This is mostly applied in human therapeutic applications [28,29,31,62]. In line with that, our study exploits the PtI from the bovine pancreas for the anti-biofilm properties. Our study showed the PtI encompasses excellent anti-biofilm properties against both "drug-resistant MRSA", and non-drug resistant pathogenic *E. coli* and *S. aureus*, biofilms. As the pancreatic enzyme is a well-known source of therapeutic enzymes, PtI as a potent anti-biofilm enzyme might be valuable in future studies.

3.4. Anti-Bacterial Assessment of the Enzymes αA, PtI, and Ec-PtI+αA

After assessing the biofilm inhibition and prevention activities, it is very important to test the anti-bacterial effect exerted by αA, PtI, and *Ec*-PtI+αA on *E. coli*, *S. aureus*, and MRSA. Hence, the αA, PtI, and *Ec*-PtI+αA was tested for anti-bacterial studies (Figure 6). The obtained data in Figure 6 conveyed that αA, PtI, and *Ec*-PtI+αA do not inhibit the bacterial growth significantly in *E. coli*, *S. aureus*, and MRSA. However, a very low 5–7% inhibition was observed in the PtI- and *Ec*-PtI+αA-treated samples. This might be due to the enzyme PtI. There are several proteases which have anti-microbial properties [63,64]. The mechanism of the proteinase inhibited growth of the *Fusarium solani* and *Staphylococcus aureus* and was explained as the alteration of cell plasma membrane by protease activity [63]. A similar mechanism of membrane alteration might have occurred with PtI-mediated very low anti-microbial activity. The overall results validated the not significant anti-bacterial nature of the studied enzymes αA, PtI, and *Ec*-PtI+αA. Hence, our study recommends, the individual enzymes PtI and *Ec*-PtI+αA are mainly used to combat the most daunting task of biofilm dispersal. After the dispersal of the bacteria, it can easily be killed by either antibiotics or nano-formulations. Futhermore, it may be further possible to make a combination of these enzymes with the anti-microbial compounds to achieve both the goal of dispersal and killing of the bacteria at the same time.

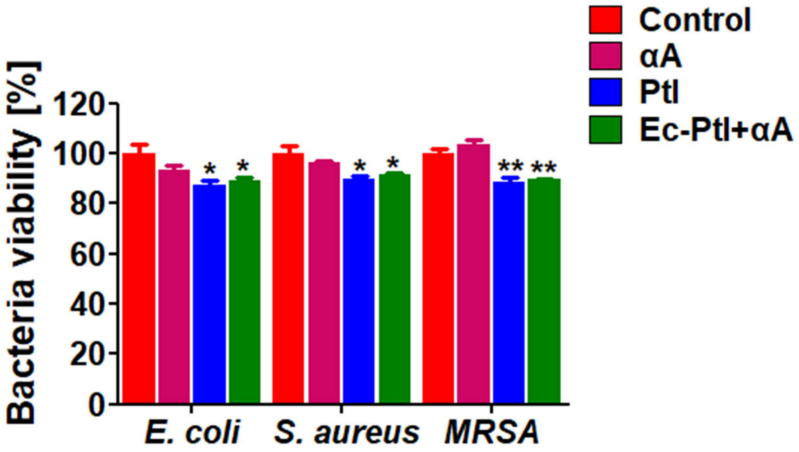

Figure 6. Anti-bacteria effect treated with protease and co-treatment with α-amylase against *E. coli*, *S. aureus*, and MRSA, respectively. All data were expressed as the mean ± SEM (n = 3) * $p < 0.05$ and ** $p < 0.01$ vs. control.

4. Conclusions

In summary, the enzyme PtI and its combinations with αA were assessed for anti-biofilm activities (inhibition of established biofilms and prevention effect on biofilm formation) against three major biofilms of *E. coli*, *S. aureus*, and MRSA. The PtI showed excellent anti-biofilm activities both in biofilm inhibition and prevention against the *E. coli*, *S. aureus*, and MRSA biofilms. This study marks the importance of PtI for future anti-biofilm treatments. In the future, PtI can be coupled with potent anti-microbial masteries to enhance biofilm treatment. Thus, PtI and its combination with αA can be used as an excellent treatment approach for biofilm dispersal. These enzymatic assessments might be extremely helpful in the development of the future treatment of biofilm infection.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4360/12/12/3032/s1. Figure S1. Inhibition effect of PtI and *Ec*-PtI+αA against biofilm was performed by crystal violet (CV) staining. Figure S2. Inhibition effect of protease and protease with α- amylase against *E. coli*, *S. aureus*, MRSA in biofilm were performed by colony counting. Figure S3. Prevention effect of PtI, αA, and *Ec*-PtI+αA against biofilm was performed by crystal violet (CV) staining.

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