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EFFECTS OF ESCAPIN INTERMEDIATE PRODUCTS (EIP-K) ON

BIOFILMS OF *Pseudomonas aeruginosa*

by

MARWA NABIL ABDELAZIZ AHMED

Under the Direction of Eric Gilbert

ABSTRACT

Escapin is an L-amino acid oxidase that produces antimicrobial metabolites collectively called “Escapin Intermediate Products” (EIP-K). EIP-K and H₂O₂ together were previously shown to be bactericidal towards diverse planktonic bacteria. The present work investigates the ability of EIP-K and H₂O₂ to antagonize bacterial biofilms, using *Pseudomonas aeruginosa* as a model. The project had three aims: 1) determine the most effective concentrations of EIP-K and H₂O₂ necessary to break down existing *P. aeruginosa* biofilms, using a crystal violet assay; 2) examine the ability of EIP-K + H₂O₂ to inhibit biofilm formation, using triphenyl tetrazolium chloride dye; and 3) determine the effect of EIP-K + H₂O₂ on the viability, biomass and structure of biofilms cultivated in flow cells using confocal laser scanning microscopy (CLSM). Results showed that EIP-K + H₂O₂ significantly reduced biofilm biomass relative to controls and that the compounds are effective at nanomolar concentrations.

INDEX WORDS: Biofilm, *Pseudomonas aeruginosa*, Escapin, EIP-K, L-amino acid oxidase, Crystal violet assay, Triphenyl tetrazolium chloride, Confocal laser scanning microscopy

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by

MARWA NABIL ABDELAZIZ AHMED

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2013

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2013

EFFECTS OF ESCAPIN INTERMEDIATE PRODUCTS (EIP-K) ON

BIOFILMS OF *Pseudomonas aeruginosa*

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Office of Graduate Studies

College of Arts and Sciences

Georgia State University

August 2013

DEDICATION

To my parents, I would like to dedicate this thesis because of all the wonderful things they do for me and supporting me all the way.

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1 INTRODUCTION

1.1 Microbial Biofilms

Biofilms are microbial communities encased in a matrix of extracellular polymeric substance (EPS) composed of extracellular DNA, proteins, lipids, and polysaccharides, and they adhere to and grow on biotic and abiotic surfaces [1, 2]. Bacteria form biofilms in response to environmental stress, nutritional starvation, oxygen depletion, or exposure to chemicals including antibiotics. Cells grown in biofilm are greatly different from the planktonic cells of the same organism in terms of gene expression, cellular physiology, and resistance to antibiotics [3-5]. Biofilm formation was shown early in the fossil record (~3.25 billion years ago) and a wide range of organisms in both the *Archaea* and *Bacteria* lineages, including the 'living fossils' in the most deeply dividing branches of the phylogenetic tree were shown to be biofilm forming bacteria [1]. It is evident that biofilm formation is an ancient and essential component of the microbial life cycle, and is a key factor for survival in diverse environments. Recent advances show that biofilms may be structurally complex, dynamic systems that can comprise multispecies and inhabit different ecosystems. Generally, biofilms are formed by different microorganisms as a protected mode of growth that allows cells to survive in hostile environments and also disperse to colonize new niches [1].

1.1.1 Biofilm Formation

Biofilm formation initiates when microorganisms switch their lifestyle from free swimming cells (planktonic cells) to a lifestyle in which cells are firmly adhered to biotic or abiotic surfaces in response to a variety of environmental signals. Cells are irreversibly attached to the substratum

forming monolayer of cells followed by proliferation of cells attached to the surface leading to the formation of microcolonies and extensive network of extracellular polymeric substances. The microcolony matures into a more complex three dimensional structure of biofilm [3, 6, 7]. Cells start to disperse from the biofilm due to nutrient starvation. Dispersal of cells from biofilm is an essential stage of the biofilm life cycle as dispersal enables the bacteria to spread and form new biofilm on other biotic or abiotic surfaces. Biofilm formation involves many regulatory genes and factors that control initial cell-surface interactions, cell-cell communication (quorum sensing), biofilm maturation, and the dispersal of cells from biofilm.

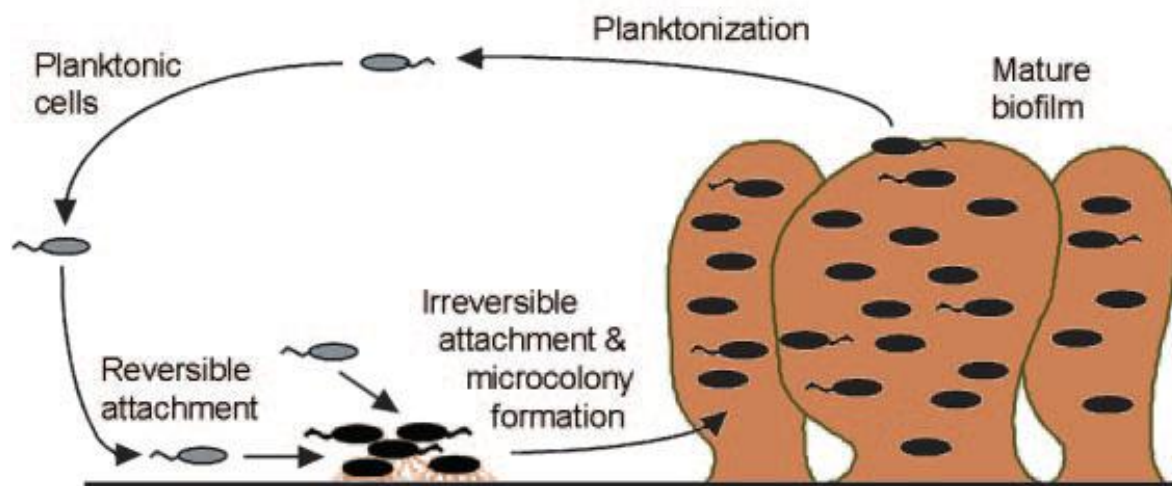


Figure 1. Stages of biofilm development [8]

1.1.2 Significance of Biofilms

Biofilm formation has serious health and environmental impacts. For instance, formation of biofilms on medical devices, such as catheters or implants, often results in chronic infections that are difficult to be targeted by therapeutic drugs [9, 10]. Moreover, nosocomial infections

have been associated with biofilm formation on human surfaces such as teeth, skin, and the urinary tract [11]. However, not all biofilms formed on surfaces are considered harmful. Some of them are beneficial. Cells within biofilm can interact together more collaboratively than individual cells, and this interaction can be exploited for industrial purposes. For example, biofilms can help eliminate petroleum oil from contaminated oceans or marine systems by the hydrocarbon degrading activities of microbial communities [12]. Biofilms are used in microbial fuel cells (MFCs) to generate electricity from a variety of starting materials, including complex organic waste and renewable biomass [13]. Additionally, biofilms grown on skin comprise beneficial species that can prevent colonization of pathogens [9]. Biofilm formation by some bacterial species such as *B. subtilis* prevents infection caused by some plant pathogens, reduces mild steel corrosion, produces novel compounds, and often allows beneficial mutualistic symbiosis [14]. For instance, *Actinobacteria* often grows on ants, allowing the ants to prevent the growth of pathogen fungi in gardens [15]. Thus, biofilms impact human health and environment in many ways; for that reason biofilms are receiving significant attention.

1.1.3 Resistance of Biofilms to Antimicrobial Agents

Biofilms are more resistant to antimicrobial agents such as antibiotics than their planktonic counterparts [2, 16]. Cells grown in biofilm can be up to 1000 fold more resistant to antibacterial agents than planktonic cells and they are protected from even intensive treatment regimens [2, 17-19]. Moreover, biofilms contain persister cells, cells that neither grow nor die in the presence of antimicrobial agents, and thus conferring on them multidrug resistance [20]. This resistance can be due to the thickness of biofilm matrix so the antimicrobials penetrate poorly into the matrix, and the cells are protected from external treatment by reacting or

binding with biocides [21]. Additionally, bacteria living in biofilms adopt an altered metabolic state. This includes increasing extracellular enzymatic activity inside the biofilms which confers on them more resistance to antimicrobials [22]. Extracellular polymeric substances (EPS) may form barriers or make complexes with the antimicrobials, thus preventing or reducing the antimicrobial action. Moreover, biofilms can generate different microenvironments within their layers with altered pH, CO₂ concentrations, oxygen concentrations, cation concentrations, and other variables, which may affect the activity of antimicrobials [23]. For these reasons, biofilms represent an important issue for public health necessitating the development of novel antimicrobial and therapeutic agents.

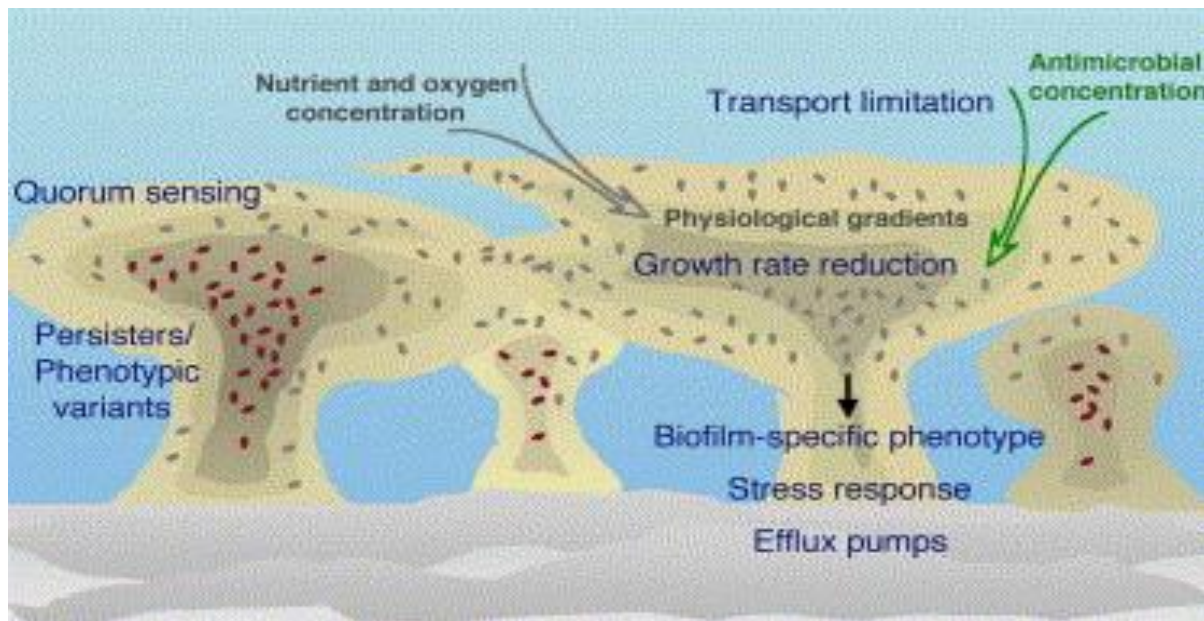


Figure 2. Mechanisms of resistance of *P. aeruginosa* biofilms to antimicrobial agents [24]

1.1.4 *Pseudomonas aeruginosa* Biofilms

Pseudomonas aeruginosa is an opportunistic pathogen that causes chronic infections such as cystic fibrosis [22, 25]. Cystic fibrosis (CF) destroys lung function which makes it a major cause

of morbidity and mortality. In the chronically infected CF lung, *P. aeruginosa* grows as a biofilm and is hard to be eradicated [26, 27]. In addition, extracellular polymeric substances (EPS) produced by *P. aeruginosa* biofilms can inhibit phagocytosis by cells of the immune system.

1.2 Effect of Antimicrobial Agents on Biofilm Matrix and Viability

Recent studies showed that antibiotics alone cannot destroy the biofilm matrix [6] as they have been shown to be effective against biofilm viable mass more than that the biofilm matrix which is mainly responsible for biofilm persistence. Biofilm matrix and viability of cells grown in biofilm are essential for the development of biofilms. Therefore, an effective antimicrobial or biocide should be effective against both biofilm viable mass and matrix [22]. Most biocides show a greater effect on biofilm viability than on matrix. For instance, isopropanol and peractetic acid markedly reduce the biofilm viability of *P. aeruginosa* with a lower effect on biofilm matrix [22]. Hydrogen peroxide has been extensively studied previously for its bactericidal and anti-biofilm activity. Hydrogen peroxide is a powerful antimicrobial agent against both planktonic cells and biofilm of *P. aeruginosa* [6, 22]. Hydrogen peroxide is a powerful anti-biofilm agent because it is active on both biofilm matrix and viable mass, and it can result in a significant eradication of *P. aeruginosa* biofilm at a concentration of 5% after one hour [6, 22]. Also, sodium hypochlorite is effective against both biofilm matrix and viability [22]. Some antimicrobial agents can only affect biofilm matrix and trigger biofilm dispersal. For example, nitric oxide induces dispersal of *P. aeruginosa* biofilm bacteria at low, sublethal concentrations (25 to 500 nM)[28].

1.3 Bactericidal Effect of Escapin

Escapin is an effective inhibitor of many microbes and it is normally produced by sea hares (*Aplysia californica*) as an antipredatory chemical defense[29]. It has both bacteriostatic and bactericidal activities [29]. Escapin uses L-lysine as a substrate to produce α -amino- ϵ -caproic acid, H_2O_2 , and ammonia [29, 30]. α -Amino- ϵ -caproic acid forms an equilibrium mixture of several compounds, which are collectively called escapin intermediate products of L-lysine (EIP-K)[31]. EIP-K reacts with H_2O_2 to produce a mixture of compounds called escapin end products of lysine (EEP-K). EIP-K plus H_2O_2 , but not EIP-K, EEP-K, H_2O_2 , or EEP-K plus H_2O_2 show rapid, powerful, and long lasting bactericidal activity[31]. EIP-K + H_2O_2 together, but neither alone, is a powerful bactericidal agent with a greatest effect against *P. aeruginosa* planktonic cells[32]. EIP-K + H_2O_2 cause long and lasting DNA condensation in bacteria. Therefore, EIP-K + H_2O_2 could be potentially used as antimicrobial agent for biofilm eradication, which can affect either biofilm cells viability or biofilm matrix or can affect biofilm formation.



Figure 3. The ink of *Aplysia californica* containing escapin, a 60 kDa protein [32]

1.4 Objectives

1.4.1 Effect of EIP-K + H₂O₂ on Disrupting Existing Biofilms of P. aeruginosa

A goal of this study was to determine the effect of EIP-K + H₂O₂ on disrupting established biofilms. The rationale for this objective is the bactericidal effect of EIP-K + H₂O₂ against planktonic cells of *P. aeruginosa*.

1.4.2 Effect of EIP-K + H₂O₂ on Killing Cells within Existing Biofilms of P. aeruginosa

A second line of investigation was to determine the effect of EIP-K + H₂O₂ on biofilm viability, biomass and structure. This hypothesis was tested using biofilm cultivation in flow cell model and imaging using CLSM, and plate counts were used to measure the effect of compounds on bacterial cells viability within the biofilm.

1.4.3 Effect of EIP-K + H₂O₂ on Biofilms Formation by P. aeruginosa

This study also hypothesized that EIP-K + H₂O₂ can prevent initial biofilm formation by assuming that both compounds can disrupt quorum sensing, genes, or signals required for biofilm formation. This assumption is based on previous results showing that EIP-K + H₂O₂ caused DNA condensation within cells lasting for at least 70 hr [32]. This hypothesis was tested with biofilms grown in microtiter plates followed by staining with TTC, an indicator of cellular metabolic activity.

2 MATERIALS AND METHODS

2.1 Cultures and Media

Pseudomonas aeruginosa strain PAO1 was used in this study. *Pseudomonas aeruginosa* was stored as frozen stock in 20% glycerol at -80 °C and was cultured on Luria Bertani (LB) agar. Plates were incubated at 37°C overnight (16-18hr). For biofilm formation, the overnight culture was diluted in *Pseudomonas* basal mineral (PBM) media (the amounts are grams per liter of distilled water) (K_2HPO_4 , 12.5g; KH_2PO_4 , 3.8g; $(NH_4)_2SO_4$, 1.0g; $MgSO_4 \cdot 7H_2O$, 0.1g; trace elements solution, 5 ml) with glucose (144 g/liter) as the sole carbon source (pH 7.2) [41] to OD_{600} of 0.01, and then 100 μ l of diluted culture was pipetted into each well in 96-well flat-bottom polystyrene microtiter plate and incubated at 37°C for 24 hr.

2.2 Animals

Sea hares (*Aplysia californica* Cooper 1863) were collected in California by Marinus Scientific (Garden Grove, CA) [32].

2.3 Collection of Ink and Isolation of Escapin

Ink glands were dissected from anesthetized animals and frozen at -80°C until they were used. Purple ink was collected by gently squeezing dissected ink glands in a Petri dish with the blunt end of a scalpel handle. Escapin (ATCC accession no. AY615888) was isolated and purified by using an ÄKTA 100 automated fast protein liquid chromatography system [32].

2.4 Preparation of the Products of Oxidation of L-amino Acids by Escapin.

To produce EIP-K, 55 mM L-lysine or L-arginine monohydrochloride, 1×10^{-3} mg/ml escapin, and 0.13 mg/ml catalase were incubated in deionized water at 30°C on a shaker for up to 24 hours. This solution was filtered using an Amicon Ultra-4 centrifugal filter device (Millipore Corp., Billerica, MA) to remove escapin and catalase and then stored it at -80°C until it was used further [32].

2.5 Biocides Preparation

Hydrogen peroxide solutions were prepared from 30% (w/w) stock solution stored at 4°C and serially diluted in a sterile 50 mM KCl-NaCl solution (pH 7.0). Escapin intermediate products (EIP-K) solutions were prepared from 1 M (w/v) stock solution stored at -80°C and serially diluted in KCl-NaCl solution.

2.6 Assessment of the Effect of EIP-K + H₂O₂ on Biofilm Removal and Viability of *P. aeruginosa*

2.6.1 Crystal Violet (CV) Biofilm Assay

After 24 hr of biofilm growth in microtiter plate, media with planktonic cells were aspirated with a micropipette, and wells were washed three times with 300 µl of KCl-NaCl solution and the plates were left to dry at room temperature for 15 min by placing them face down on tissue. EIP-K and H₂O₂ (1 M, 30% respectively) were then applied to the wells. All six wells in a column receive the same treatment, and each experiment was conducted in at least duplicate. The plates were incubated at 37°C for 30 min. After 30 min, the biocides were discarded and the wells were washed twice with 300 µl of KCl-NaCl solution as described before [6, 22]. After washing, crystal violet staining was performed as described before [3] with some modifications

to assess the effect of EIP-K + H₂O₂ to remove biofilm. These include staining with 150 µl of 0.3% crystal violet for 20 min and afterwards aspirating with a pipette. Excess stain was rinsed off by placing the microtiter plate under running tap water until washings were free of the stain. Excess stain was rinsed off by placing the microtiter plate under running tap water until rinses were free of the stain. Plates were subsequently dried by flipping them on tissue for 15 min. The remaining stain was then solubilized by the addition of 150 µl of 95% ethanol for 30 min standing on bench. Absorbance of the stain was measured using microtiter plate reader at wavelength 590 nm. The efficacy of EIP-K + H₂O₂ (i.e. the percentage reduction in stain) was calculated from the blank (wells containing broth only), control and treated absorbance values in plate using the following equation [6, 22]:

$$\text{Percentage reduction} = [(C-B)-(T-B)/(C-B)]*100$$

where B stands for the average absorbance for blank wells, C stands for the average absorbance for control wells and T stands for the average absorbance for treated wells.

2.6.2 Cultivation of Biofilm in Flow Cells Model and Imaging using Confocal Laser Scanning Microscopy (CLSM)

Biofilms were cultivated in flow cells as described before [19, 33, 34] except with PBM as a growth medium. After growing the biofilms of *P. aeruginosa* PAO1 in the flow cell for 24 hr, the biofilm was rinsed with a sterile 50 mM KCl-NaCl solution (pH 7.0) for 20 min. After rinsing, EIP-K + H₂O₂ was pumped for 30 min through the flow cell, and then the biofilms were rinsed for 10 min. After rinsing, 1 ml of a 1:1,000-diluted LIVE/DEAD® Baclight™ nucleic acid stain (SYTO 9 dye, 3.34 mM; propidium iodide, 20 mM) was pumped into the flow cell. The stain was left

inside the flow cell for 15 min, and then the biofilms were rinsed with KCl-NaCl solution for another 5 min and examined by confocal laser scanning microscope (CLSM). Argon and helium lasers were used with excitation/emission of 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide. Longpass and dual emission filters were used for simultaneous viewing of SYTO 9 and propidium iodide stains. At least four image stacks were collected from each biofilm and at least three independent biofilms were cultivated for each of the tested conditions.

2.6.3 Plate Count

Biofilms grown in flow cells were harvested by pumping the liquid and cells out of flow cells into sterile 1.5 ml microcentrifuge tube. The recovered cells were centrifuged at 8000 rpm for 2 min. Supernatant was discarded and pellet was resuspended in 1 ml of with KCl-NaCl solution. The suspended bacterial culture was serially diluted and plated on LB agar media. Viable cell counts were determined by enumeration of CFU with appropriate dilutions on LB agar media after 24 hours of bacterial growth.

2.7 Assessment of the Effect of EIP-K + H₂O₂ on Biofilm Formation by *P. aeruginosa* using Triphenyl Tetrazolium Chloride (TTC) Dye

After diluting the overnight culture of *P. aeruginosa* with PBM media to give an OD₆₀₀ 0.01, 95µl of diluted culture with 5 µl of EIP-K/H₂O₂ were added to each well of 96-well microtiter plate and then the plates were incubated at 37°C for 24 hr. After 24 hr, media with planktonic cells were aspirated with a pipette and the wells were washed twice with KCl-NaCl solution. After washing, 100 µl of fresh PBM media were added to each well plus 5 µl of TTC (1% v/v) resulting in a final TTC concentration of 0.05%, and then the plates were sealed with parafilm and wrapped in foil to prevent oxidation of TTC dye and incubated for further 24 hr.

After 24 hr, the absorbance of TTC was measured at 540 nm using a microtiter plate reader [18, 26, 35]

2.7 Statistical Analysis

Image stacks collected by CLSM were evaluated by using the digital image analysis program COMSTAT [36], for quantifying features of biofilm structure. Results were analyzed by ANOVA assuming that ($P < 0.05$) is considered significant.

3 RESULTS

3.1 The Effect of EIP-K + H₂O₂ on Disrupting Existing Biofilms of *P. aeruginosa*

Crystal violet assays were used to measure the ability of EIP-K + H₂O₂ to remove *P. aeruginosa* biofilms. To determine the most effective concentrations of each compound for achieving a synergistic interaction, a cross-sectional design was used. First, the H₂O₂ concentration was held constant at 300 µM and a range of EIP-K concentrations were tried in combination with it. The assay indicated that 0.5 mM EIP-K was the most effective concentration of the initial concentrations that were tested (Fig. 4.1). Subsequently, CV assays were carried out with 0.5 mM EIP-K and varying concentrations of H₂O₂ (Fig. 4.2). These experiments indicated that 300 µM H₂O₂ resulted in a small but significant increase in biofilm removal.

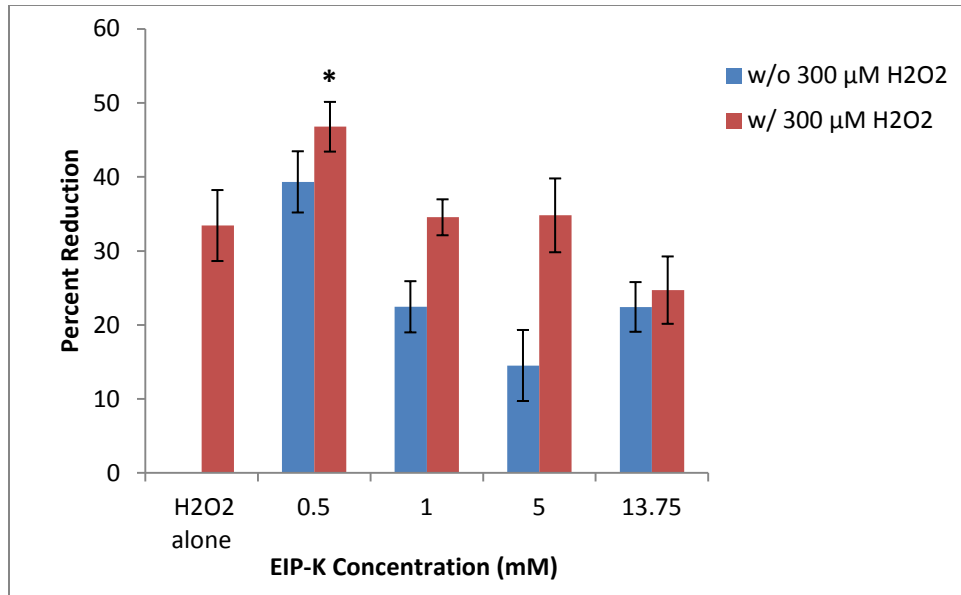


Figure 4.1.Crystal violet assay measuring removal of existing biofilms following treatment with EIP-K, H₂O₂ or EIP-K + 300 μ M H₂O₂. Values are means \pm standard deviation for six replicates from a representative experiment. An asterisk indicates that the effect of EIP-K + H₂O₂ is significantly higher than that of H₂O₂ alone ($P < 0.005$; paired t test).

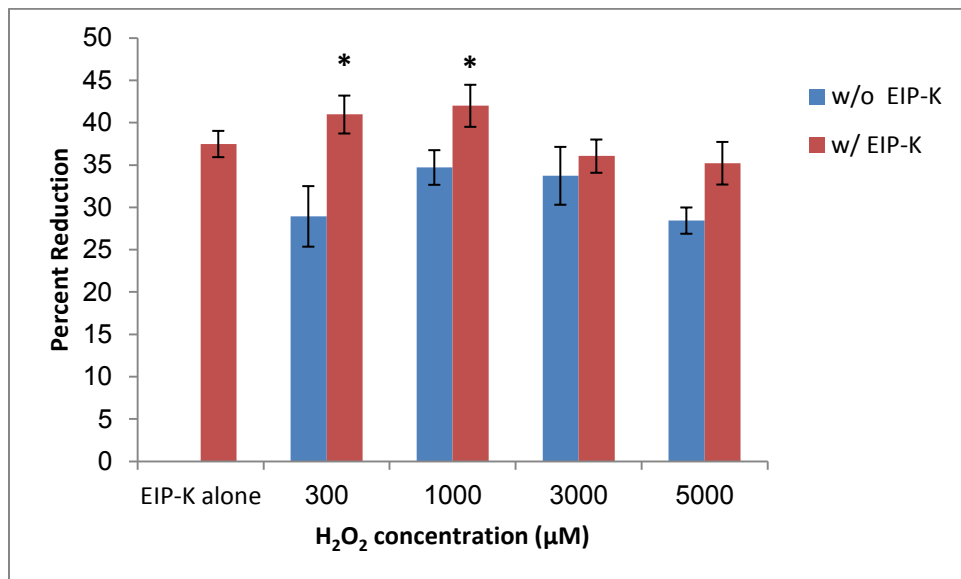


Figure 4.2.Crystal violet assay measuring removal of existing biofilms following treatment with EIP-K, H₂O₂ or 0.5 mM EIP-K + H₂O₂. Values are means \pm standard deviation for six replicates from a representative experiment. An asterisk indicates that the effect of EIP-K + H₂O₂ is significantly higher than that of EIP-K alone ($P < 0.02$; paired t test).

3.2 Flow Cell Assessment of EIP-K + H₂O₂ Activity

Biofilm cultivation in flow cells and imaging using confocal laser scanning microscopy (CLSM) were used to determine the effect of EIP-K + H₂O₂ on both biomass reduction and cell viability of *P. aeruginosa* biofilms. Three concentrations of H₂O₂ ranging from 0.3 nM to 30 nM in combination with 50 μM EIP-K were compared to determine the most effective treatment at this range; these are referred to as the low concentration treatments in the following sections. Additionally, 5 mM EIP-K + 300 μM H₂O₂ was tested and is referred to as the high concentration in the following sections. At each concentration, four conditions were compared: an untreated control, treatment with either EIP-K alone or H₂O₂ alone and treatment with EIP-K and H₂O₂ simultaneously. Representative images for the low and high concentration treatments are shown in Figs. 5.1 and 5.2. 30 nM was found to be the most effective H₂O₂ concentration for reducing biofilm biomass (Fig. 5.3a). 30 nM H₂O₂ resulted in nearly 68% reduction in biomass relative to an untreated control (Fig. 5.3b). The high concentration treatment resulted in approximately a 39% reduction (Fig. 5.3c). Viability staining indicated similar ratios of red and green cells for the low concentration treatment (Fig. 5.4a) In contrast; there was an increase in the number of red cells in biofilms treated with EIP-K + H₂O₂ at high concentration (Fig. 5.4b). Enumeration of cells in biofilms by serial dilution and plate count determined a significant reduction in cell number following treatment with both EIP-K and H₂O₂ at the low concentration compared to the untreated control as well as an increase in cell density following treatment with H₂O₂ only (Fig. 5.5). Plate counts indicated that the high concentration also caused a significant reduction in cell number compared to the untreated control, although to a lower extent than for the low concentration of EIP-K + H₂O₂ (Fig. 5.5).

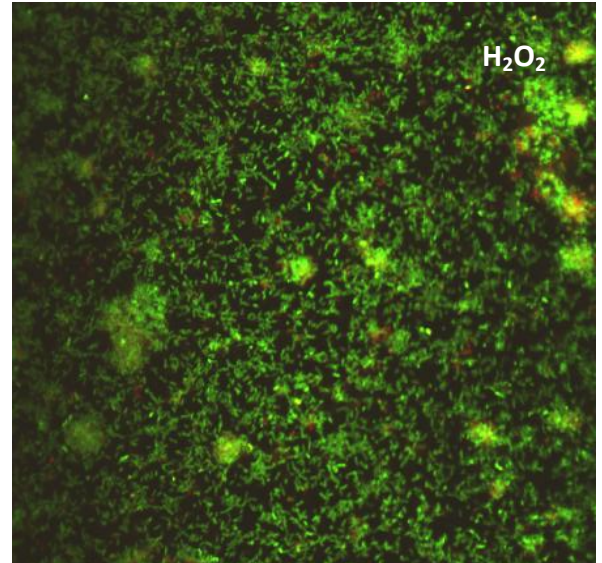
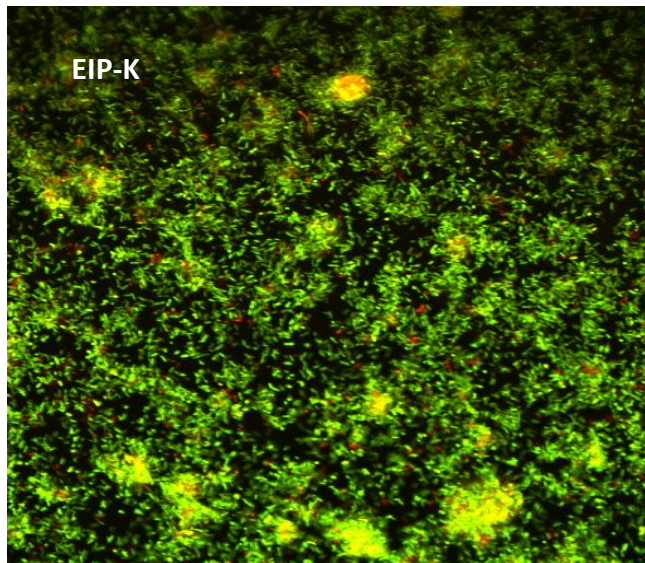
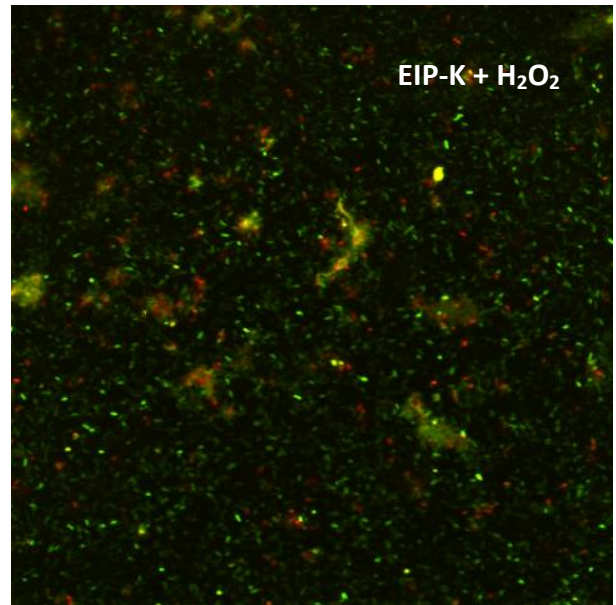
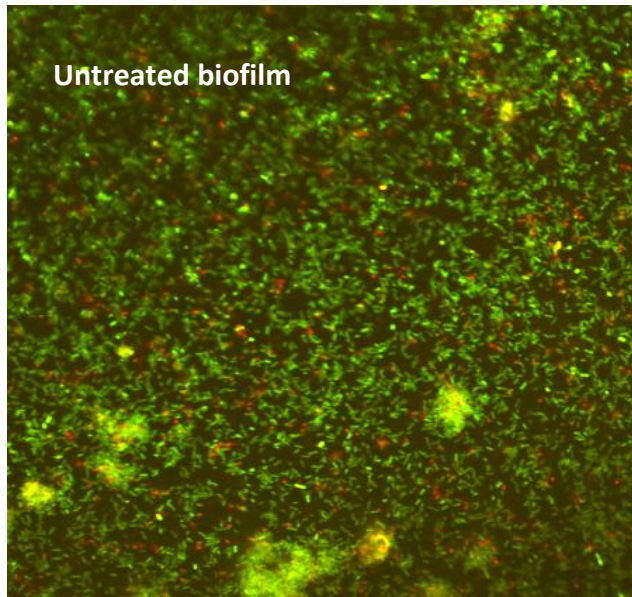


Figure 5.1. Confocal microscopy images of *P. aeruginosa* biofilms grown for 24 h following treatment with 50 μM EIP-K+ 30 nM H₂O₂, EIP-K or H₂O₂ alone for 30 min. Note the increase in black, indicating unoccupied space in the upper right hand panel. Live cells have green or yellow color while dead cells have red color.

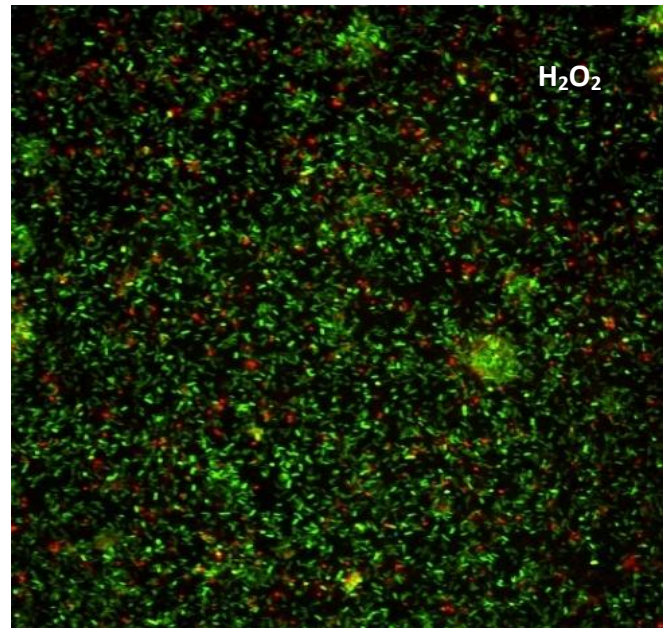
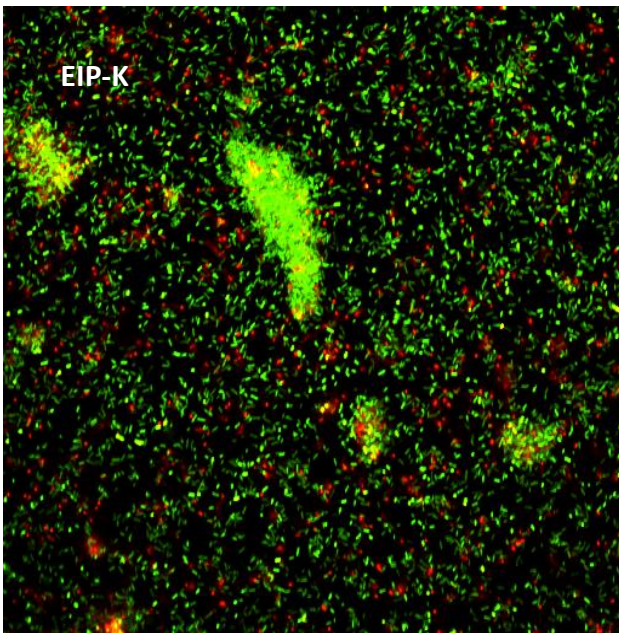
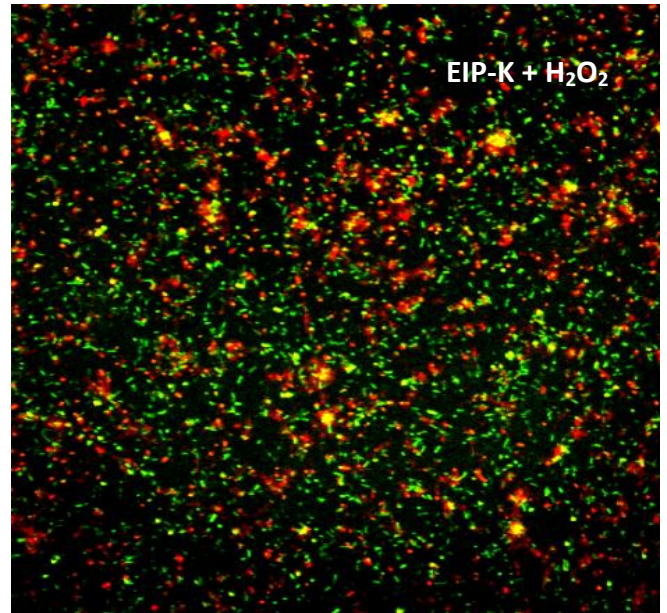
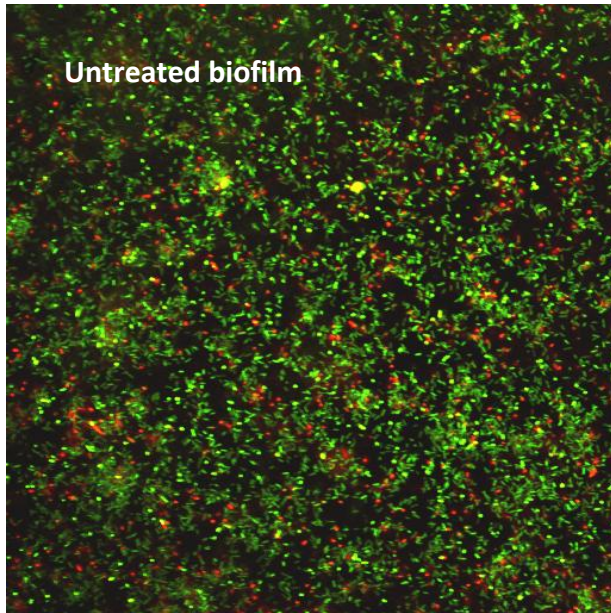
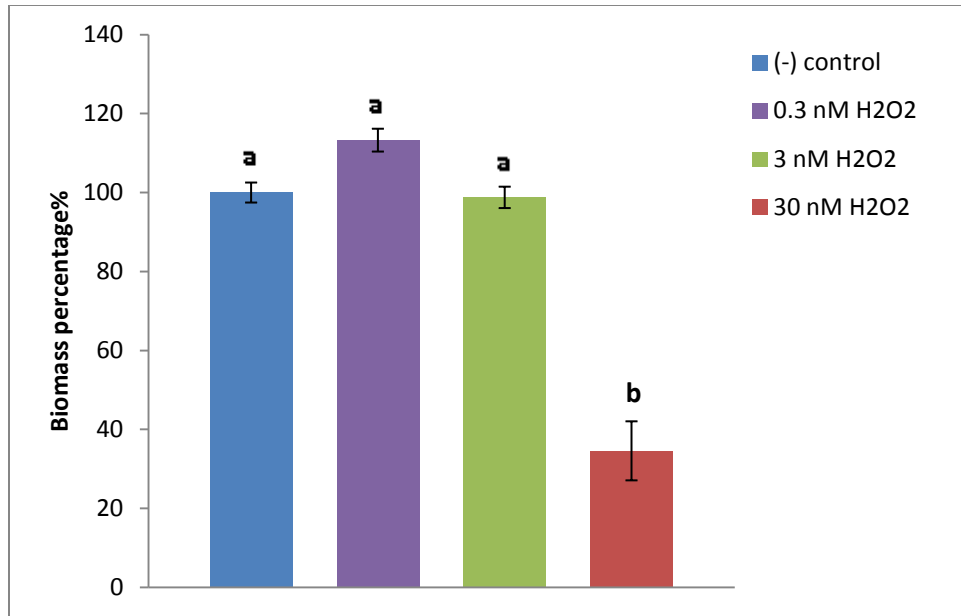
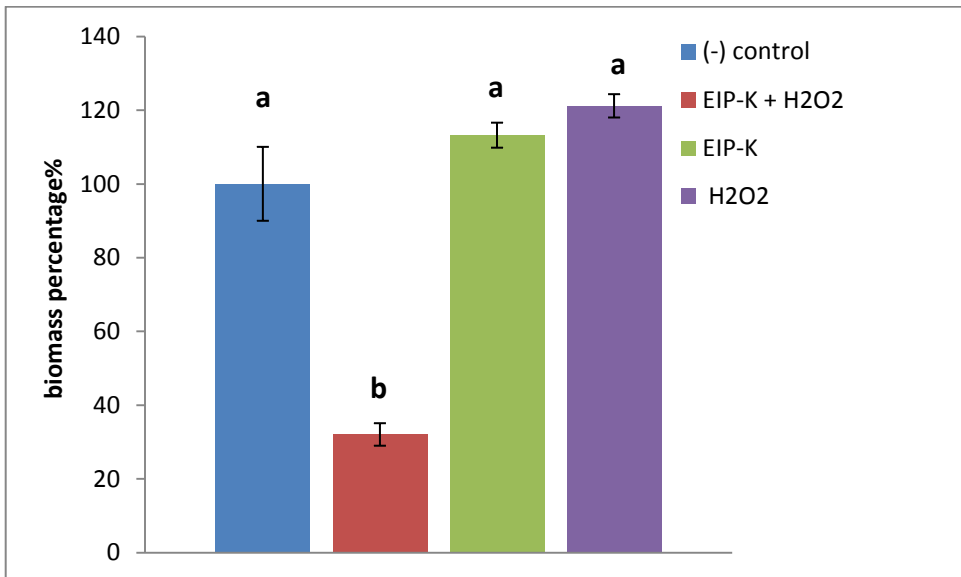


Figure 5.2. Confocal microscopy of *P. aeruginosa* biofilms grown for 24 hr following treatment with 5 mM EIP-K + 300 μ M H₂O₂, EIP-K and H₂O₂ alone for 30 min. Live cells have green or yellow color while dead cells have red color.

(a)



(b)



(c)

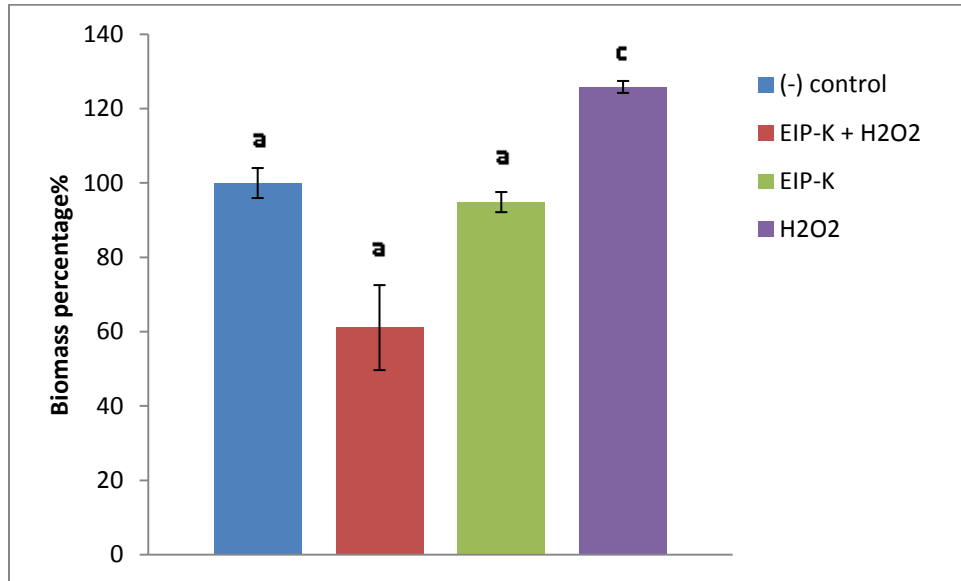
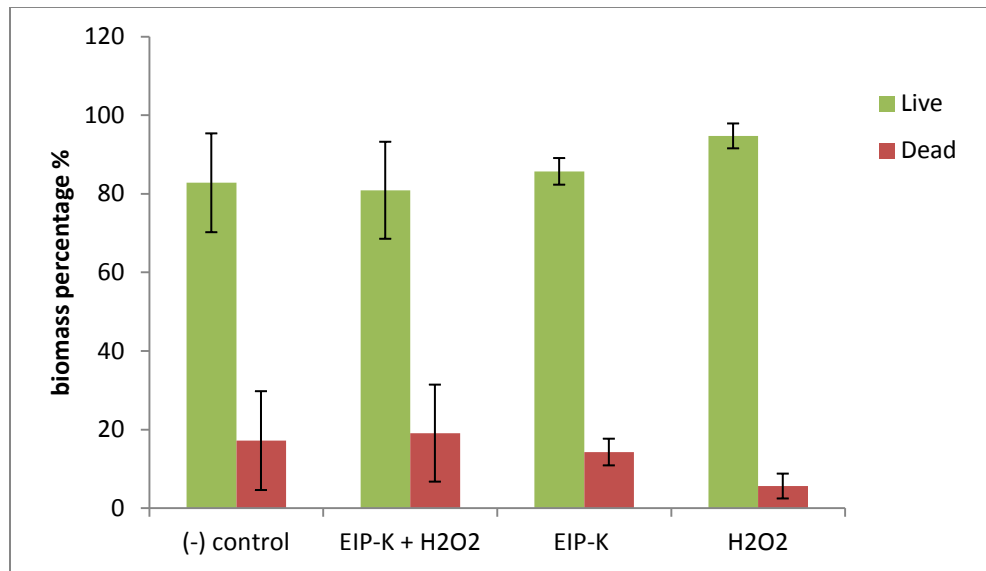


Figure 5.3.Effect of EIP-K + H₂O₂ on *P. aeruginosa* biofilm removal for biofilms grown in flow cells for 24 hr.(a) biofilm treatment with 50 μM EIP-K+ 30, 0.3 and 3 nM H₂O₂.(b) biofilm treatment with 50 μM EIP-K+ 30 nM H₂O₂, EIP-K and H₂O₂ alone.(c) biofilm treatment with 5 mM EIP-K + 300 μM H₂O₂, EIP-K and H₂O₂ alone. Experiment was carried out in quadruplicate. Values are means ± standard error of means for 16-20 replicates from a representative experiment. Columns labeled with different letters are significantly different from one another (p<0.02; paired t test).

(a)



(b)

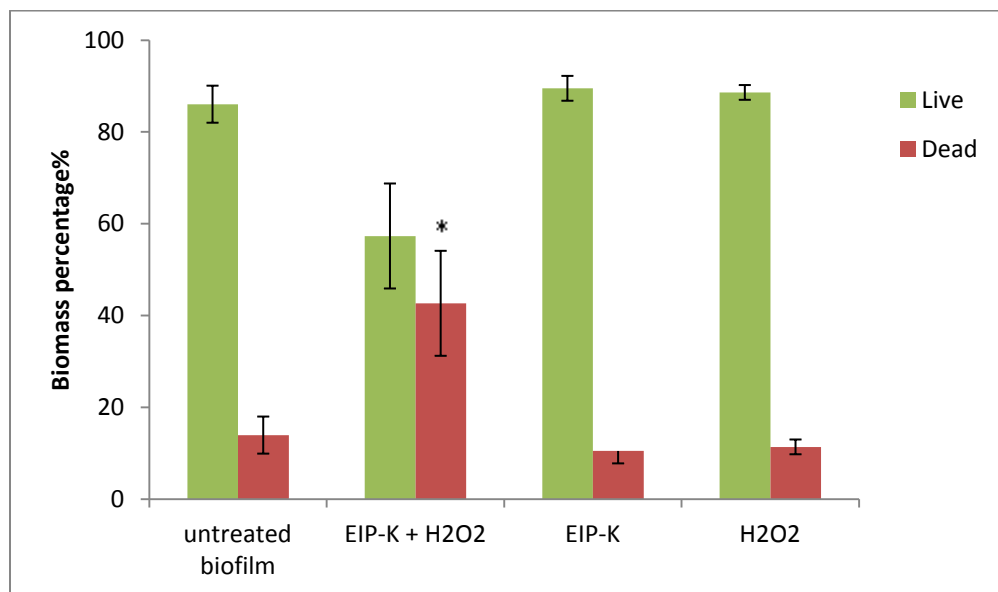


Figure 5.4. Effect of EIP-K + H₂O₂ on *P. aeruginosa* biofilm cells viability after 30 min. of treatment. (a) Biofilm treatment with 50 μM EIP-K + 30 nM H₂O₂, EIP-K and H₂O₂ alone. (b) Biofilm treatment with 5 mM EIP-K + 300 μM H₂O₂, EIP-K and H₂O₂ alone. Values are means ± standard error of means for 16-20 replicates from a representative experiment.

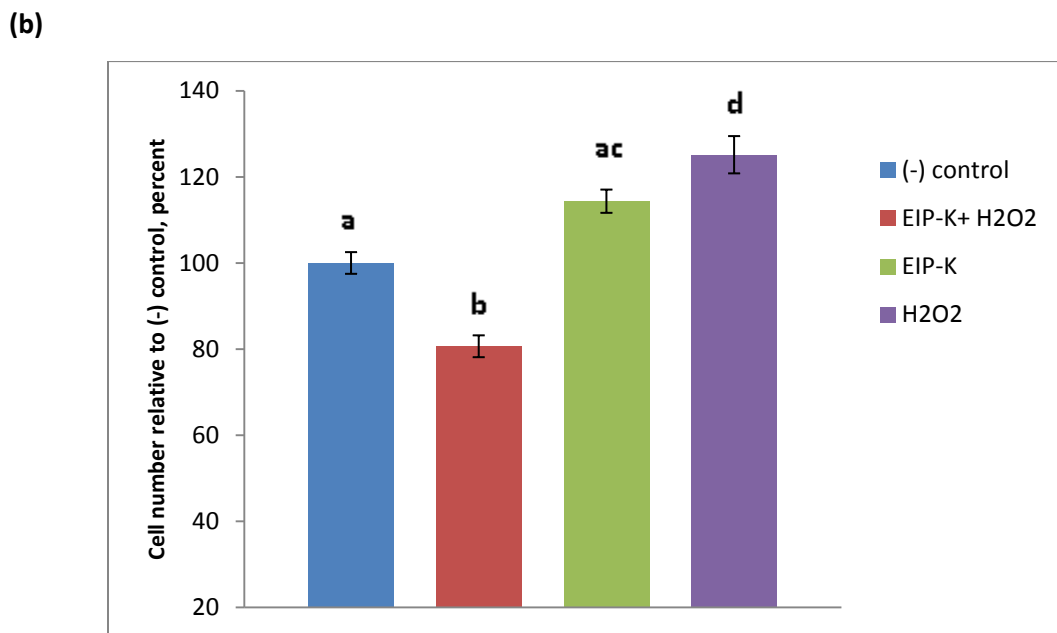
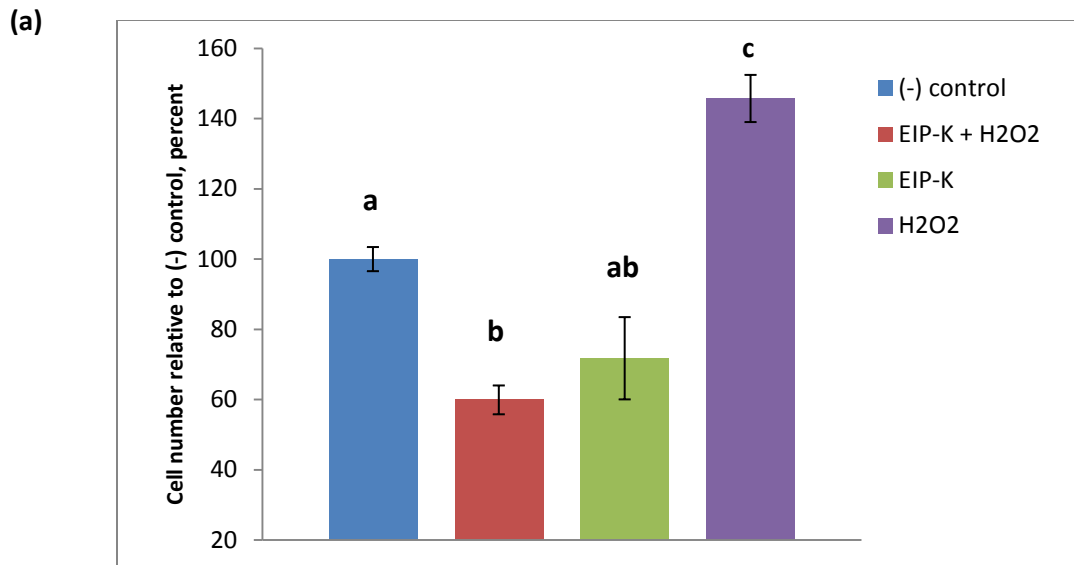


Figure 5.5. Percent of cells in *P. aeruginosa* biofilm enumerated by serial dilution and plate count. (a) Biofilm treatment with 50 μ M EIP-K + 30 nM H₂O₂, EIP-K and H₂O₂ alone. (b) Biofilm treatment with 5 mM EIP-K + 300 μ M H₂O₂, EIP-K and H₂O₂ alone. Values are means \pm standard deviation of means for 3 replicates from a representative experiment. Columns labeled with different letters are significantly different from one another ($p < 0.05$; paired t test).

3.3 The Effect of EIP-K + H₂O₂ on Biofilms Formation by *P. aeruginosa* using TTC Dye

Triphenyl tetrazolium chloride (TTC) dye was used to measure the effect of EIP-K + H₂O₂ on preventing biofilm formation by measuring the metabolic activity of cells following treatment with EIP-K + H₂O₂. Each experiment was performed in duplicate with six replicates being tested per treatment. Different concentration of both EIP-K and H₂O₂ were investigated. The effect of EIP-K + H₂O₂ decreased with increasing concentration. EIP-K + H₂O₂ showed a significant effect on preventing biofilm formation compared to negative control (P<0.007) but it is not significant compared to the corresponding EIP-K and H₂O₂ alone. As the concentration of H₂O₂ increased, biofilm formation was stimulated until 5 mM, a concentration which completely prevented biofilm formation.

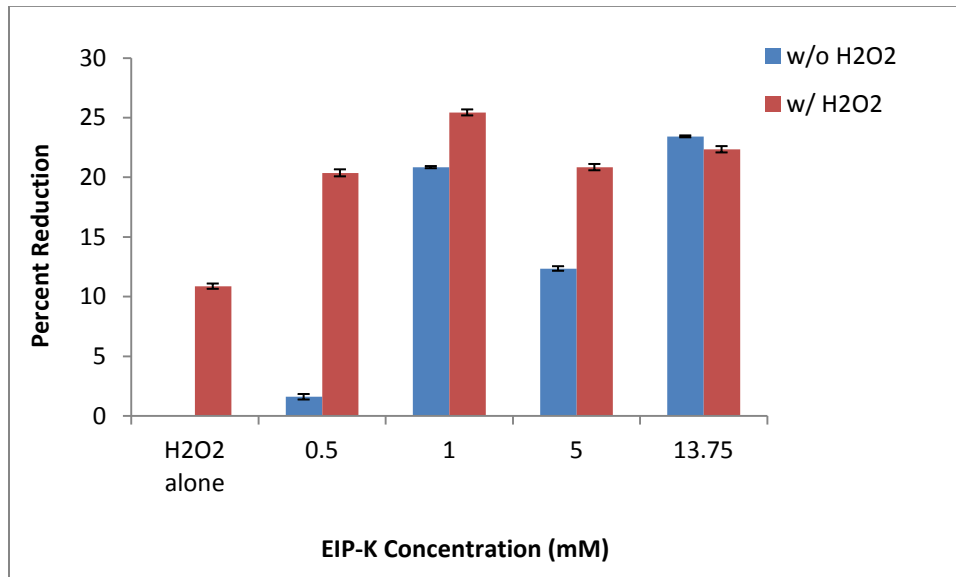


Figure 6.1.Effect of EIP-K, H₂O₂ or EIP-K + 300 μM H₂O₂ on *P. aeruginosa* cells viability during biofilm formation after 30 min of treatment. Values are means ± standard deviation for six replicates from a representative experiment.

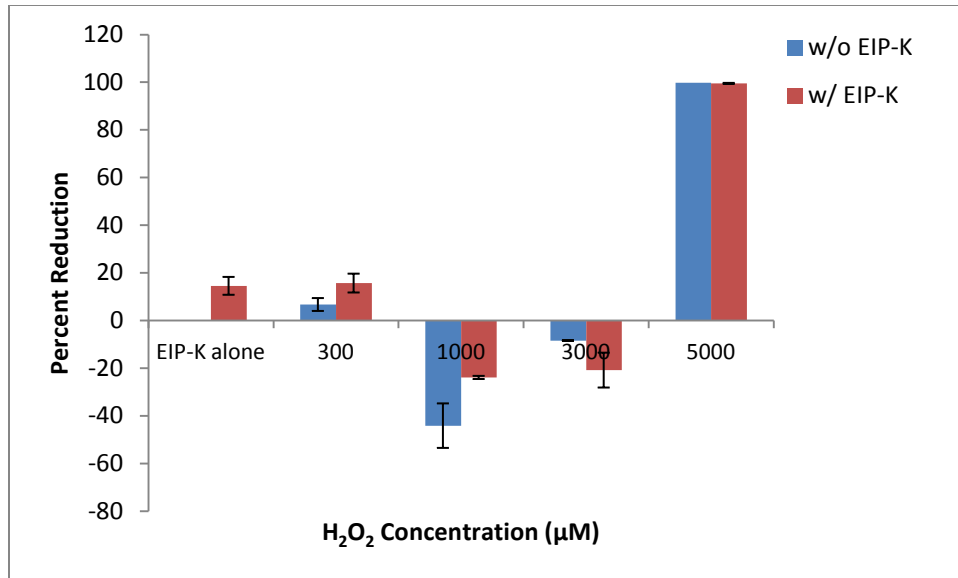


Figure 6.2. Effect of EIP-K, H₂O₂ or 13.75 mM EIP-K + H₂O₂ on *P. aeruginosa* cells viability during biofilm formation after 30 min of treatment. Values are means ± standard deviation for six replicates from a representative experiment.

4 DISCUSSION

EIP-K and H₂O₂ showed promise as an antimicrobial agent for targeting *P. aeruginosa* biofilms. A crystal violet staining technique was used as a screen to identify the range of concentrations where EIP-K and H₂O₂ would work together most effectively. The crystal violet assay results showed that EIP-K + H₂O₂ had a significant effect on biofilm removal of *P. aeruginosa* compared to the negative control, EIP-K or H₂O₂ alone. EIP-K + H₂O₂ was more effective at low concentrations than at high concentrations, suggesting the occurrence of an Eagle effect previously reported for EIP-K + H₂O₂ with *E. coli* [31, 32] and for some antibiotics such as penicillin against streptococci and *Staphylococcus aureus* [37]. As a result, flow cell experiments were carried out to address the effect of EIP-K + H₂O₂ at low concentrations.

Flow cell analysis is a technique that facilitates non-destructive imaging of biofilms and is effective for measuring changes in biofilm parameters in response to antimicrobial agents. The flow cell data presented herein indicated that treatment with EIP-K + H₂O₂ significantly reduced the biomass of *P. aeruginosa* from established biofilms. This result was supported by image analysis data as well as enumeration by serial dilution and plate count. LIVE/DEAD staining revealed no significant differences in the proportion of viable cells among treatments and suggested that EIP-K + H₂O₂ acted by stimulating cellular detachment. Several factors have been reported to promote *P. aeruginosa* detachment, including enzymatic disruption of the surrounding EPS matrix, oxygen radical-dependent killing of bacteria[38], prophage-mediated bacterial death that enhances dispersal of cells from biofilm [25] or the release of amyloid fibers linking cells in the biofilm together, a process regulated by d-amino acids [39]. EIP-K + H₂O₂ may act by stimulating one or more of these mechanisms. If the detachment seen in the flow cell experiments can be replicated in vivo, EIP-K + H₂O₂ could potentially be a useful as part of therapies to control *P. aeruginosa* infections. The effectiveness of EIP-K + H₂O₂ could possibly be enhanced by working in combination with antimicrobial agents shown elsewhere to be effective against *P. aeruginosa*.

Another way that EIP-K + H₂O₂ could be an effective antimicrobial treatment is by preventing the formation of mature biofilms. Using a TTC assay, it was observed that EIP-K + H₂O₂ reduced biofilm formation by *P. aeruginosa*. These data suggest that EIP-K + H₂O₂ inhibited the growth of bacteria that attached to the substratum, leading to reduced biomass relative to controls following 24 h of growth. Generally, the collected data indicate that the potential of EIP-K +

H_2O_2 to prevent biofilm formation was lower than that to remove biofilm. The reduced effect may be due to the neutralization of H_2O_2 by bacterial cells over the course of the experiment. Overall, EIP-K + H_2O_2 promoted biofilm detachment at nanomolar concentration, similarly to the activity of d-amino acids [40] and nitric oxide [28]. Further investigation into the mechanism of action of EIP-K+ H_2O_2 is warranted.

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