Biofilm dispersal effects of Fenton's reagent on *Staphylococcus aureus* biofilm

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Abstract

Staphylococcus aureus biofilms have shown significant resistance against antibiotics. A potential approach to counter this is through inducing biofilm dispersal. Fenton's reagent, a solution which contains the reactive hydroxyl radical (OH•), may be a potential agent of biofilm dispersal. Although Fenton chemistry has proven to have antibacterial effects, the effect of Fenton's reagent itself against a biofilm has not yet been determined. This study aims to evaluate the biofilm dispersal effects of Fenton's reagent on an *S. aureus* biofilm, through the use of the crystal violet assay and microplate reading at OD620. It was found that Fenton's reagent does not induce biofilm dispersal; rather, it induces biofilm formation at 100 µL and has no effect at 200 µL. This may have been due to excess Fe^{2+} ions and due to the reactive hydroxyl radical already being consumed by the time of the reagent's application. It was concluded that Fenton's reagent may induce biofilm formation instead of biofilm dispersal, depending on the ratio between the volume of Fenton's reagent and the biofilm mass.

Keywords: *Fenton's reagent, biofilms, anti-biofilm, biofilm dispersal, Staphylococcus aureus*

Introduction. Planktonic bacteria are capable of assuming an alternative multicellular lifestyle in a form called the biofilm, which are communities of bacteria growing on biotic or abiotic surfaces [1]. Biofilms are composed of bacterial cells and a self-produced extracellular matrix — which is then constituted by extracellular polymeric substances (EPS) such as carbohydrates, proteins, and extracellular DNA that create a highly hydrated structure that protects the biomass from damage [2]. The matrix contributes to the increased resilience of biofilms against antibiotic treatment and has a major role in many chronic infections; thus, it is often theorized that agents that degrade or destroy the biofilm matrix could inhibit biofilm formation or promote dispersal of established biofilm colonies [3]. Biofilm dispersal is especially significant as it detaches the bacterial cells from the biofilm, making them less resistant to antibiotic treatment.

Agents of biofilm dispersal are divided into classes, one of which involves chemicals such as metal chelators and surfactants [4]. Fenton's reagent was shown to be just as effective as a surfactant against the EPS of bound water in sewage sludge [5]. Fenton's reagent is most commonly used to destroy organic compounds that are resistant to other wastewater treatment techniques, and can be synthesized in the lab through the catalytic decomposition of hydrogen peroxide by a ferrous iron to follow the reaction $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$. It has been proposed that this reaction is induced by bactericidal antibiotics, primarily to generate the hydroxyl radical (OH•) in the human body, which becomes part of the immunological response to kill invading bacteria [6]. The hydroxyl radical is also known to oxidize and degrade DNA [7], a known component of biofilm matrices [8,9].

One example of a biofilm with a matrix that has high concentrations of DNA is *Staphylococcus aureus* [10, 11]. *S. aureus* is a Gram-positive bacteria that has become notorious for causing a wide range of diseases, with multi-drug resistant species being recognized as those that often infect medical equipment once in the biofilm phenotype [12]. Thus, a particularly effective way to combat this biofilm is through the use of DNA-degrading substances, be it a nuclease from another organism [13,14], endogenously produced nuclease [15], or the enzyme DNase [16].

Thus the effects of a hypothesized agent of biofilm dispersal that is known to attack and degrade DNA would be most realized in a biofilm which has a matrix in which DNA is a major component. It is hypothesized that Fenton's reagent, which produces the highly reactive hydroxyl radical and has exhibited surfactant-like properties, would be able to induce biofilm dispersal in a DNA-abundant biofilm such as *S. aureus.*

This study aims to evaluate the biofilm dispersal effects of Fenton's reagent on an *S. aureus* biofilm. Specifically, it aims to:

(i) measure optical density of *S. aureus* biofilm samples treated with 100µL and 200 µL of Fenton's reagent through crystal violet assay;

(ii) compare the optical densities of the different treatments.

This study may contribute to the small body of literature covering the use of Fenton's reagent outside of its conventional use, particularly as a potential agent of biofilm dispersal. It may also act as a basis for future studies in the same field.

Methods. Diluted S. aureus biofilm cultures were first prepared and inoculated into a 96-well plate while the Fenton's reagent was prepared. Biofilm

cultures were then treated with Fenton's reagent. The crystal violet assay was used to assess biofilm dispersal, and results were taken as the optical density of each well at OD620.

Chemicals and equipment. Crystal violet and concentrated acetic acid for crystal violet assay, and the hydrogen peroxide (H_2O_2) and ferrous sulfate ($FeSO_4$) for Fenton's reagent were sourced from the PSHS-WVC Chemistry SRA. Tryptic soy broth and glucose were purchased from Gregor Mendel Research Laboratories of the University of San Agustin.

Test tubes, culture tubes, and other glassware were sourced from the MEDTECH Laboratories of the University of San Agustin. Sterile microtiter plates, micropipette, micropipette tips used in the study were purchased or rented from Mendel Research Laboratories. The microplate reader used was also located there. A Level 2 Biosafety cabinet was used for procedures involving hazardous biologicals.

Staphylococcus aureus strains. Biofilm-forming cultures of *S. aureus* (ATCC 25923) were purchased from the Microbiology Section of the Clinical Laboratory of the University of San Agustin. At the end of experimentation, biohazardous wastes, test tubes, and any other glassware or equipment exposed to *S. aureus* were first decontaminated with a 10% bleach solution and allowed to sit for 30 minutes and discarded. These were then sterilized for 1 h by autoclave at 121°C under 15 lbs psi of steam pressure.

Media preparation. Tryptic soy broth (TSB) was used throughout the study and was prepared by adding 3.00 g of TSB to 100 mL of distilled water in a media bottle. The media was then mixed and heated on a hot plate with a magnetic stirrer. Once the TSB became clear and yellow-brown in color, the media bottle was removed from heat, covered, and then autoclaved for 1 h at 121°C under 15 lbs psi of steam pressure.

Culture of S. aureus biofilm. First, 140 µL of the *S. aureus* stock culture was diluted with 14 mL of tryptic soy broth supplemented with 1% glucose (1:100) then vortexed [17] in a separate culture tube. The diluted culture was then inoculated into a 96-well plate for crystal violet assay.

Fenton's reagent preparation. The Fe^{2+} catalyst was prepared by dissolving 13.9 g of analytical-grade ferrous sulfate ($FeSO_4$.7H2O) in 100 mL of sterilized deionized water (DI) containing 5 mL of concentrated H_2SO_4 [18]. A volume of 30 mL of analytical grade 30% hydrogen peroxide was added in to the Fe^{2+} catalyst to produce Fenton's reagent. The solution was then left to cool to room temperature in a fume hood.

Biofilm dispersal screening. Biofilm dispersal screening was done with 5 replicates per treatment and 5 replicates for each control set, for a total of 25 samples for validity and accuracy of results, as shown in Figure 1. After vortexing, 100 µL of diluted bacteria was inoculated into 15 wells of a 96-well plate. The microtiter plate was incubated for 44 h at 37°C. The individual wells were treated with the following:

(i) 100 µL distilled water (negative control);

(ii) 100 µL of Fenton's reagent;

(iii) 200 µL of Fenton's reagent.

Blank wells for medium control were supplemented with 200 µL TSB only. The treated and control samples were further incubated for 30 min at 37°C. The contents of the wells were then carefully decanted into a discard container. The wells were washed by adding 200 µL of distilled water to each, then subsequently shaking and blotting the plate to remove excess water, non-adherent cells, and non-adherent components of the biofilm. Once the samples were sufficiently washed, 125 µL of a 0.1% solution of crystal violet in water was added to each well. The plate was incubated at room temperature for an additional 10 minutes before being rinsed, shaken, and blotted again two times. Another set of standard samples for microplate reading was supplemented with 100 µL of acetic acid only. Immediately after blotting and addition of acetic acid, optical density at OD620 was quantified by microplate reader. The plate was left to dry and incubate at room temperature in an inverted position overnight for 20 h. After drying, 125 µL of acetic acid was added to each well to solubilize the crystal violet. Finally, optical density at OD620 was quantified again by microplate reader. The resulting concentration in each well is proportional to the number of cells in the biofilm [15].

Data management and statistical analysis. Qualitative data was in the form of pictures of the stained wells after treatment. Quantitative data was in the form of optical density measured by a microplate reader, where the mean and standard deviation were calculated for each treatment. Raw data was also analyzed using the One-Way ANOVA was used at a confidence interval of 95% to determine whether the means of the three test groups differ significantly from one another. Tukey HSD test, Scheffe Multiple Comparison test, and the Bonferroni and Holm Multiple Comparison test were also conducted as post-hoc analysis. All statistical tests were done using the online statistical calculator using Astatsa's One-way ANOVA with Tukey HSD test, Scheffe Multiple Comparison test, and the Bonferroni and Holm Multiple Comparison test for post-hoc analysis. All statistical tests and results were validated by Ms. Ma. Romy Alexis Consulta, a math and statistics teacher at PSHS-WVC.

Results and Discussion. Biofilm dispersal of untreated *S. aureus* biofilms and *S. aureus* biofilms treated with 100 µL or 200 µL of Fenton's reagent was observed in a biofilm dispersal assay using crystal violet staining in a 96-well (see Figure 1). Crystal violet stain is not visibly present in the blank samples (7A-7E). Faint crystal violet stain is visible in the negative control samples (1A-1E). Relatively dense crystal violet binding is evident in the samples treated samples. Samples treated with 100 µL of Fenton's reagent (3A-3E) show increased binding of crystal violet, as compared to samples treated with 200 µL of Fenton's reagent (5A-5F). Coloration due to crystal violet binding in wells indicates presence of bacterial biofilm.

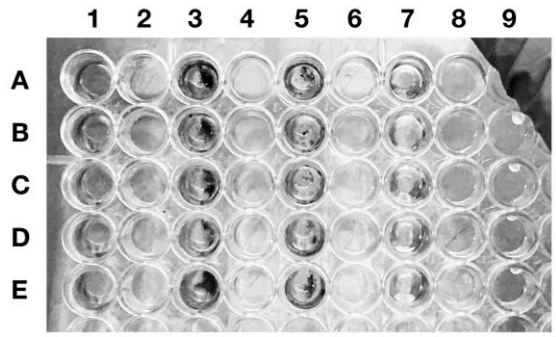

Figure 1. Photograph of crystal violet-stained wells in a 96-well plate. Negative control samples supplemented with 100 µL distilled water (1A-1E); Fenton's reagent-treated samples at 100 µL (3A-3E); Fenton's reagent- treated samples at 200 µL (5A-5F); blanks with only TSB (7A-7E); standards with only acetic acid (9A-9E).

Results were then validated through microplate reading. OD620 of the individual wells were acquired, where sample wells having an optical density greater than the mean optical density + 2 SD of the blank sample wells with only TSB were considered to have been able to grow a biofilm. All samples treated with 100 µL of Fenton's reagent, 200 µL of Fenton's reagent, or 100 µL of distilled water tested positive for biofilm formation.

After 22 hours of drying at room temperature, optical density of *S. aureus* biofilm samples treated with 100 µL of Fenton's reagent, 200 µL of Fenton's reagent, or supplemented with 100 µL of distilled water (control) were measured at OD620 using a microplate reader. Five replicates were used for each sample set. The control samples had a mean optical density of 0.466 ± 0.09. Samples treated with 100 µL of Fenton's reagent had a mean optical density of 0.744 ± 0.21, while samples treated with 200 µL of Fenton's reagent had a mean optical density of 0.441 ± 0.10. There was a significant difference between the mean optical densities of the control samples and samples treated with 100 µL of Fenton's reagent; this indicates that there was increased biofilm biomass in the samples treated with 100 µL of Fenton's reagent as compared to the control, implying biofilm formation. A significant difference was also found between the mean optical densities of the samples treated with 100 µL of Fenton's reagent and samples treated with 200 µL of Fenton's reagent. No significant difference was found between the mean optical densities of the control samples and samples treated with 200 µL of Fenton's reagent.

Table 1. Summary of optical density measured at OD620 expressed in mean±SD.

Control	100 µL of Fenton's reagent	200 µL of Fenton's reagent	TSB
0.4660 ±0.0862	0.7436 ±0.2082	0.4414 ±0.0965	0.1274 ±0.0601

It was found that Fenton's reagent did not induce biofilm dispersal at either treatment, as illustrated in Figure 2. Treatment with 100 µL of Fenton's reagent significantly induced the formation of more biofilm, while treatment with 200 µL did not significantly affect the biofilm. It is notable that treatment with 100 µL was almost double that of the control, with a mean optical density of 0.744 ± 0.21 compared to 0.466 ± 0.09.

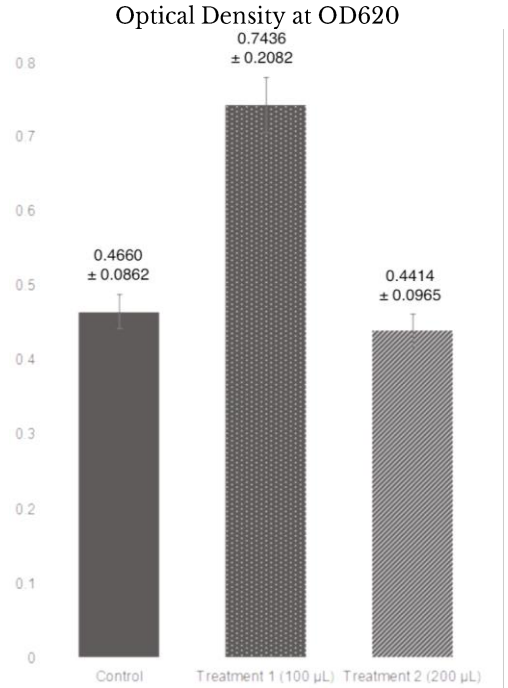

Figure 2. Average optical density measured at OD620 after incubation of 22 h.

As the results show, Fenton's reagent does not induce biofilm dispersal in spite of its components having the expected ability to degrade DNA, a major component of of the *S. aureus* biofilm matrix. Fenton's reagent is composed of a mixture of ferrous iron and hydrogen peroxide, which has the products of Fe^{3+} ions, hydroxyl anions (OH^-), and hydroxyl radicals. Hydroxyl radicals, as a reactive oxygen species, have been reported to cause strand breaks and base modifications in DNA [7], and is known to be highly reactive to many biological molecules such as proteins and lipids [19]. However, they are also reactive in general, such that the hydroxyl radical may still react further with hydrogen peroxide or the Fe^{3+} ions to produce hydroperoxyl (HO_2•) or superoxide anion (O_2•$^-$) radicals [20], neither of which are very reactive with biological molecules. It is possible that the hydroxyl radicals in the Fenton's reagent prepared already reacted to form hydroperoxyl and superoxide radicals, as this reaction is very quick and has a rate constant of 3.3×10^7 $M^{-1}s^{-1}$ at pH 4 [20]. Another factor that may have caused this is the cooling down time of the Fenton's reagent. The prepared Fenton's reagent was allowed to cool down for ten minutes, as the reaction caused by the addition of hydrogen peroxide to ferrous sulfate is volatile and exothermic. This was done so that the *S. aureus* biofilms would not be affected by the high temperature of the Fenton reaction.

While the effect of Fenton's reagent on biofilm dispersal has not yet been documented, other studies have reported about the ability of Fenton's reagent to cause bacterial inhibition. However, it is important to note that instead of separately preparing Fenton's

reagent, these studies induced a Fenton reaction in the bacteria's environment itself in order to stimulate the production of hydroxyl radicals. This is usually done through the addition of hydrogen peroxide to an iron-supplemented medium in the test apparatus itself, and in such a study it was found that hydrogen peroxide was able to cause the bacterial inhibition of *S. aureus* more effectively when it was added to cultures that had been grown in an iron-supplemented medium rather than when it was added to cultures that had iron added to them exogenously after they had matured [21]. Addition of hydroxyl radical scavengers were then able to reverse the bacterial inhibition, which implies that it is the hydroxyl radical that is the main instigator for antibacterial activity. If so, then the fast production of hydroxyl radicals and their subsequent rapid reactions with surrounding molecules must be an integral part of the ability of the Fenton's reaction to induce antibacterial activity and anti-biofilm activity as well. This seems to be validated by the results of other studies, as hydroxyl radicals have been reported to be effective against both planktonic bacteria [22] and against biofilms [23].

It was also hypothesized that the Fenton's reagent would be able to cause biofilm dispersal because of DNA-degrading properties. However, these properties are derived from the DNA-degrading properties of the hydroxyl radicals that are formed as a result of the Fenton reaction, as they are able to induce oxidative damage [24] and strand breaks to DNA, both of which are dependent on the concentration of hydroxyl radicals [7]. Thus, there is also a possibility that the concentration of hydroxyl radicals was not lethal enough to degrade a significant enough amount of DNA and induce biofilm dispersal.

Another factor to consider is that the Fe^{2+} ions were in excess, which was purposefully done because no explicit protocol for the preparation of Fenton's reagent has been found by the researchers and the preparation seemed to vary from study to study. These excess Fe^{2+} ions may have been used by the biofilm for further maturation and growth, as several studies report that iron regulates the biofilm formation of *Staphylococcus* species [25]. More significantly, the ferrous sulfate in the Fenton's reagent may have been a primary factor in the biofilm formation caused by the treatment, as ferrous sulfate has been shown to aid in the formation of an *S. aureus* biofilm [26].

Should both situations be true, that the hydroxyl radicals were either already consumed by the time of its application or were at volumes too low to be effective, and that the excess Fe^{2+} ions were used as nutrients, then it would be natural that biofilm formation would occur instead of biofilm dispersal as was hypothesized. This can be seen in the results for the samples treated with 100 µL of Fenton's reagent, where there is a notable increase compared to the control. Both factors may also be exacerbated by the age of the cells used to culture the biofilm, as it may have an effect on the rate at which the biofilm forms or disperses.

There is no clear reason as to why the results for the samples treated with 200 µL of Fenton's reagent match with that of the control. It may be possible that the *S. aureus* biofilm had, similar to the samples treated with 100 µL, also went through biofilm formation but then subsequently went through biofilm dispersal as well as a result of the increased concentration of hydroxyl radicals. However, there is no proof that would support this as the concentration of hydroxyl radicals was not monitored nor calculated.

Conclusion. Fenton's reagent induces biofilm formation in *S. aureus* biofilm at 100 µL but not at 200 µL. It is therefore concluded that Fenton's reagent may induce biofilm formation rather than biofilm dispersal, depending on the ratio between the volume of Fenton's reagent and the biofilm mass. However, it is unclear whether there is a direct, indirect, or no correlation between volume of Fenton's reagent and biofilm biomass.

Recommendations. It is recommended that that a standard protocol for the preparation of Fenton's reagent be made before it is studied further. The properties and effects of Fenton's reagent be studied more in depth, and it that should be subjected to chemical analysis regarding the concentration of hydroxyl radicals and presence of iron ions or other products that may have contributed to the reactivity of the reagent. For the evaluation of biofilm growth and dispersal, it is recommended that different methods should be used aside from the quantification of optical density through microplate reading and that these results be visualized using SEM imaging. It is also recommended that the liquid that is washed off during the crystal violet assay should be tested and analyzed under the microplate reader as well, as this would quantify the planktonic cells that were dispersed. Different volumes of Fenton's reagent must also be tested to see if there is a trend that can be observed in regards to its effects.

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References

[1] Landini P, Antoniani D, Burgess JG, Nijland R. 2010. Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. Appl Microbiol Biotechnol. 86: 813-823. DOI: 10.1007/s00253-010-2468-8.

[2] Kostakioti M, Hadjifrangiskou M, Hultgren SJ. 2013. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. Cold Spring Harb Perspect. 3: a010306.

[3] Kaplan JB. 2009. Therapeutic potential of biofilm-dispersing enzymes. Int J Artif Organs. 32(9): 545-554. DOI: 10.1177/039139880903200903.

[4] Xavier JB, Picioreanu C, Rani SA, van Loosdrecht MCM, Stewart PS. 2005. Biofilm-control strategies based on enzymic disruption of the extracellular polymeric substance matrix — a modelling study. Microbiology. 151(12): 3817-3832: DOI: 10.1099/mic.0.28165-0.

[5] Xing Y, Wang ZQ, Hong C, Yang Q, Feng LH, Jia MM, Li YF. 2017. Mechanism and parameter optimization of Fenton's reagent integrated with surfactant pretreatment to improve sludge dewaterability. PLoS One. 12(1): e0169523. DOI: 10.1371/journal.pone.0169523.

[6] Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell. 130: 797-810. DOI: 10.1016/j.cell.2007.06.049.

[7] Su M, Yang Y, Yang G. 2006. Quantitative measurement of hydroxyl radical induced DNA double-strand breaks and the effect of *N*-acetyl-L-cysteine. FEBS Letters. 580: 4136-4142.

[8] Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. 2002. Extracellular DNA required for bacterial biofilm formation. Science. 295(5559): 1487.

[9] Fuxman Bass J, Russo DM, Gabelloni ML, Geffner JR, Giordano M, Catalano M, Zorreguieta A, Trevani AS. 2010. Extracellular DNA: a major proinflammatory component of Pseudomonas aeruginosa biofilms. J Immunol. 184:6386-6395. DOI: 10.4049/jimmunol.0901640.

[10] Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L, Tsang LH, Smeltzer MS, Horswill AR, Bayles KW. 2009. Modulation of eDNA Release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS ONE. 4(6): e5822. DOI: 10.1371/journal.pone.0005822.

[11] Dengler V, Foulston L, De Francesco AS, Losick R. 2015. An electrostatic net model for the role of extracellular DNA in biofilm formation by *Staphylococcus aureus*. J Bacteriol. 197(24): 3779–3787. DOI: 10.1128/JB.00726-15.

[12] Chung PY, Toh YS. 2014. Anti-biofilm agents: recent breakthrough against multi-drug resistant *Staphylococcus aureus*. Pathog Dis. 70: 231-239. DOI: 10.1111/2049-632X.12141.

[13] Kiedrowski MR, Kavanaugh JS, Malone CL, Mootz JM, Voyich JM, Smeltzer MS, Bayles KW, Horswill AR. 2011. Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*. PLoS ONE. 6(11): e26714. DOI:10.1371/journal.pone.0026714.

[14] Shields RC, Mokhtar N, Ford M, Hall MJ, Burgess JG, El Badawey MR, Jakubovics NS. 2013. Efficacy of a marine bacterial nuclease against biofilm forming microorganisms isolated from chronic rhinosinusitis. PLoS ONE. 8(2): e55339. DOI: 10.1371/journal.pone.0055339.

[15] Nijland R, Hall MJ, Burgess JG. 2010. Dispersal of biofilms by secreted, matrix degrading bacterial DNase. PLoS ONE. 5(12): e15668. DOI: 10.1371/journal.pone.0015668.

[16] Izano EA, Amarante MA, Kher WB, Kaplan JB. 2008. Differential roles of poly-n-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Appl Environ Microbiol. 74(2): 470-476. DOI: 10.1128/AEM.02073-07.

[17] Stepanovic S, Vukovic D, Hola V, Bonaventura GD, Djukic S, Cirkovic I, Ruzicka F. 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 115: 891-899.

[18] Selvakumar A, Tuccillo ME, Muthukrishnan S, Ray AB. 2009. Use of Fenton's reagent as a disinfectant. U.S. Environmental Protection Agency Papers. 152: 9p. DOI: 10.1002/rem.20208.

[19] Dizdaroglu M, Jaruga P. 2012. Mechanisms of free radical-induced damage to DNA. Free Rad Res. 46(4):382–419. DOI: 10.3109/10715762.2011.653969.

[20] Metz KM, Mangham AN, Bierman MJ, Jin S, Hamers RJ, Pedersen JA. 2009. Engineered nanomaterial transformation under oxidative environmental conditions: development of an in vitro biomimetic assay. Environ Sci Technol. 43(5): 1598–1604.

[21] Repine JE, Fox RB, Berger EM. 1981. Hydrogen peroxide kills *Staphylococcus aureus* by reacting with staphylococcal iron to form hydroxyl radical. J Biol Chem. 256(14): 7994-7096.

[22] Ikai H, Nakamura K, Shirato M, Kanno T, Iwasawa A, Sasaki K, Niwano Y, Kohno M. 2010. Photolysis of hydrogen peroxide, an effective disinfection system via hydroxyl radical formation. Antimicrob Agents Chemother. 54(12): 5086-5091. DOI: 10.1128/AAC.00751-10.

[23] Choi H, Lee DG. 2012. Antimicrobial peptide pleurocidin synergizes with antibiotics through hydroxyl radical formation and membrane damage, and exerts antibiofilm activity. Biochimica et Biophysica Acta. 1820: 1831-1838. DOI: 10.1016/j.bbagen.2012.08.012.

[24] Kohen R, Nyska A. 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol. 30(6): 620-650. DOI: 10.1080/0192623029016672-4.

[25] Lyte M, Freestone PP, Neal CP, Olson BA, Haigh RD, Bayston R, Williams PH. 2003. Stimulation of *Staphylococcus epidermidis* growth and biofilm formation by catecholamine inotropes. Lancet. 361(9352): 130-135. DOI: 10.1016/S0140-6736(03)12231-3.

[26] Lin MH, Shu JC, Huang HY, Cheng YC. 2012. Involvement of iron in biofilm formation by *Staphylococcus aureus*. PLoS ONE. 7(3): e34388. DOI: 10.1371/journal.pone.0034388.