Understanding the Effects of Sonication for Planktonic Bacterial Removal

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Abstract

Low frequency ultrasound (LFU) is interrogated for its ability to decrease the viability of planktonic *Pseudomonas aeruginosa*. The presented work herein details a novel approach to quantify the bactericidal effect of LFU as a function of intensity and exposure time. The results of this study indicate that planktonic *Pseudomonas* experiences an exponential rate of diminished viability with exposure time. Higher LFU intensities increase cell death. Additionally, smaller volumes of the planktonic bacteria are more prone to sonication than larger volumes. Future studies will focus on the response of the biofilm phenotype to insonation.

Introduction

Biofilms are the most common form of microbial growth, and a major problem in many engineered systems. For example, biofilms can foul water filtration systems [1], increasing energy demands. They also can coat pipelines and ship hulls [2], increasing the hydraulic drag. Most biofilm removal approaches are ineffective, expensive, or not “green” [3].

The formation of a biofilm begins with adherence of planktonic bacteria to a surface. The formation of a monolayer and production of a matrix then begins until the biofilm forms a microcolony[2,4]. When the biofilm is mature enough, it releases planktonic bacteria into its environment.

This study addresses the foundations of biofilm formation -- planktonic bacteria. There are several ways to remove planktonic bacteria from substrates. There are chemical approaches such as antibiotics and the combination of antimicrobials and ultrasound. This study, however, will focus exclusively on planktonic bacteria removal with ultrasound.

It has been shown in several papers that continuous low frequency ultrasound (LFU) is the most effective towards decreasing biofilm viability [5]. For instance, continuous ultrasound for 6 hours led *E. coli* to have no reproductive ability and resulted in 97% killing in the first two hours (70kHz) [6]. It seems once the ultrasound stops, the bacteria are quick to repair any broken parts of their armor.

Pulse waveform was found to be just as effective as continuous ultrasound at the same power density [7]. However, pulse waveform worked best for *in vivo* experiments (no skin damage - related to the average intensity of ultrasound. Can adjust in duty cycle) (1:3 or 1:6 duty cycle) [8]. It is also of interest to note that the Synergistic antimicrobial effect of pulsed wave ultrasound is related to the temporal peak intensity, not the temporal average intensity .

In this study, planktonic *Pseudomonas aeruginosa,* the building blocks of a biofilm, are sonicated with different intensities of ultrasound and are then characterized by cell viability. These methods are described in the next section.

Methods

The effects of sonication on suspended planktonic *Pseudomonas Aeruginosa* bacteria were investigated with the following techniques / procedures.

Inoculating bacteria

A stock solution of *Pseudomonas Aeruginosa* was prepared by removing a loop of bacteria from the main dish which operated at -80˚C . The loop was used to inoculate the bacteria which was placed in an incubator at 37˚C for 12 hours. This strain had no prior resistance to any antibiotics.

Suspended Liquid Cultures

To begin the liquid culture, lysogeny broth (LB) was poured a quarter of the way up in a test tube and set aside near an open flame for sterilization purposes. A small sample of the inoculated bacteria was then moved from the agar plate and thoroughly mixed into the liquid broth. The test tubes were then placed in an incubator moving at 160 RPM and operating at 37˚C. The liquid suspension was left to grow for 20 hours.

Optical Density and Washing

The optical density of the bacteria in the lysogeny broth was measured in a spectrophotometer at 600 nm. The bacteria were transferred to 1 mL centrifuge tubes that had been previously sterilized in the Autoclave and centrifuged. The bacteria were then “washed” as the broth was taken out, making sure not to disrupt the bacteria pellet, and transferred to 1 mL of PBS buffer. The bacteria were then diluted to an optical density (OD) of 0.1 at 600 nm to allow for the sonication to affect as many of the bacteria as possible.

Sonication

1 mL of the suspended bacteria in PBS was transferred to new centrifuge tubes and insonated at different intensities and times with both probe and bath sonicators.

Probe sonication was conducted using a Qsonica Q125, ⅛” diameter probe which operates at 20 kHz with a power rating of 125 Watts. Due to the heating of the bacteria during sonication, a 125 mL beaker was filled with ice and the 1 mL centrifuge tube was placed in the ice held down with play-doh. The probe was then lowered half way into the liquid bacteria. It is important to note that the probe did not come into contact with any part of the centrifuge tube, only the PBS buffer and mixed bacteria. Bacteria were sonicated for 1, 2, and 5 minutes at 25 and 50 % intensity. After sonication, the tubes were quickly capped and immediately diluted and plated. An example of the probe sonication set up can be seen in the following figure.

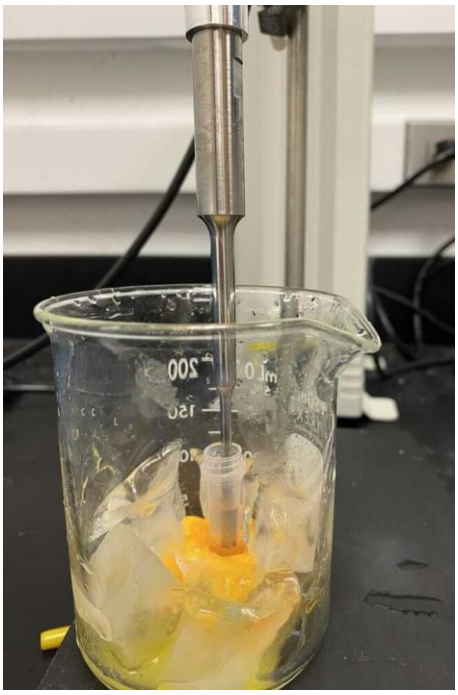

Figure 1. Probe Sonication assembly.

Bath sonication was executed using a Fisher Scientific FS30 Ultrasonic Cleaner. The bath operated at a fixed 40 kHz and produced 130 W of power. The bath was filled to the max limit with DI water. Ice was placed to keep the water temperature from getting above room temperature. A rack was then placed in the water bath to hold the centrifuge tubes. Tubes were placed in the bath for 15 and 30 minutes.

Dilutions

Ten centrifuge tubes were sterilized under a HibriLinker. An agar plate was then split into sections containing a “control”, “Negative control”, “10^{-1}”, “10^{-2}”, etc. The control plate was composed of the bacteria in the diluted PBS buffer. The negative control was composed of only PBS to ensure that there were no bacteria present. Each centrifuge tube received 900 µL of PBS buffer. 100 µL of the sonicated bacteria were placed in the 10^{-1} tube. The contents were thoroughly mixed. 100 µL of the 10^{-1} tube were placed in with 900 µL of PBS buffer in the 10^{-2} tube. This process was

repeated until a dilution of 10^{-9} was reached. Three, evenly spaced, 10 µL drops from each dilution centrifuge tube were then placed on the agar plate which had been previously split into sections depending on the dilution. The plated bacteria were grown in the stagnant incubator for 24 hours at 37˚C and in the ventilation hood to compare.

Colony Counting and Spectrophotometer

The colonies found on the plate have units of colonies / dilution factor. The colonies were counted and multiplied by their dilution factor and then changed to units of mL. The spectrophotometer was used in this initial viability count and was very accurate with the colony counting method, however we deemed it inaccurate for further testing because the spectrophotometer does not take into account whether the bacteria are dead or alive when measuring absorbance. It was used, however, on new liquid suspensions we made before sonication to make sure they were consistent in absorbance to what we used for the other experiments. An example of the colonies on an agar plate are depicted below.

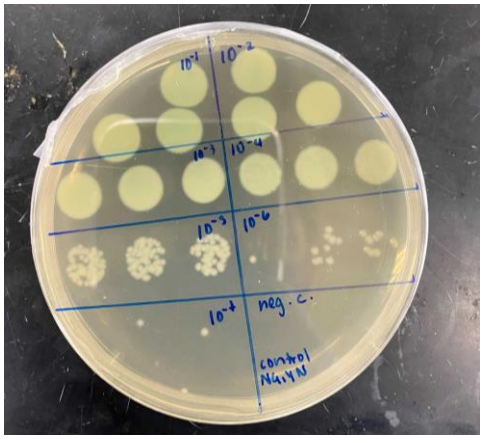

Figure 2. Diluted colony growth for the control.

Results

The results from probe sonication are given in Figure 3. The results from bath sonication are given in Figure 4. Size-effect is explored in Figure 5.

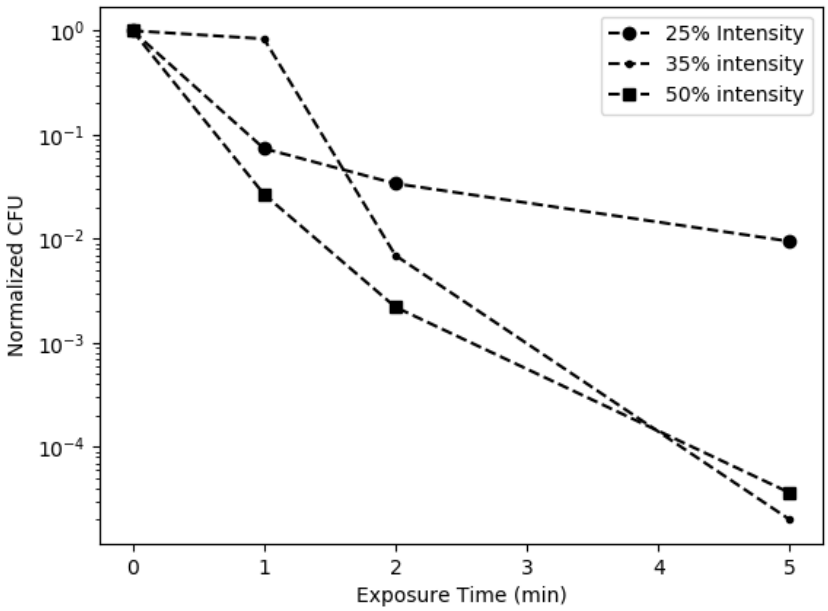

Figure 3. Normalized CFU-exposure profiles for 25%, 35%, and 50% sonication.

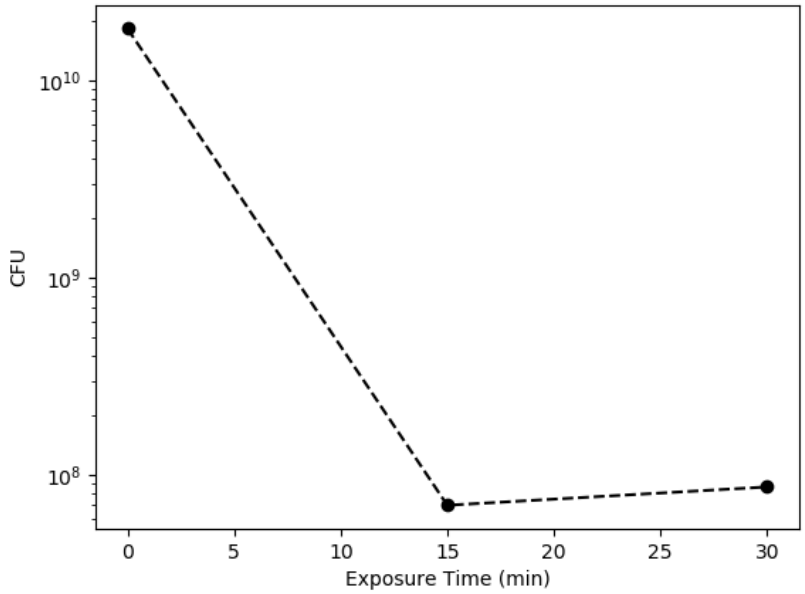

Figure 4. CFU-exposure profile for bath sonication. CFU measured in Colonies/mL.

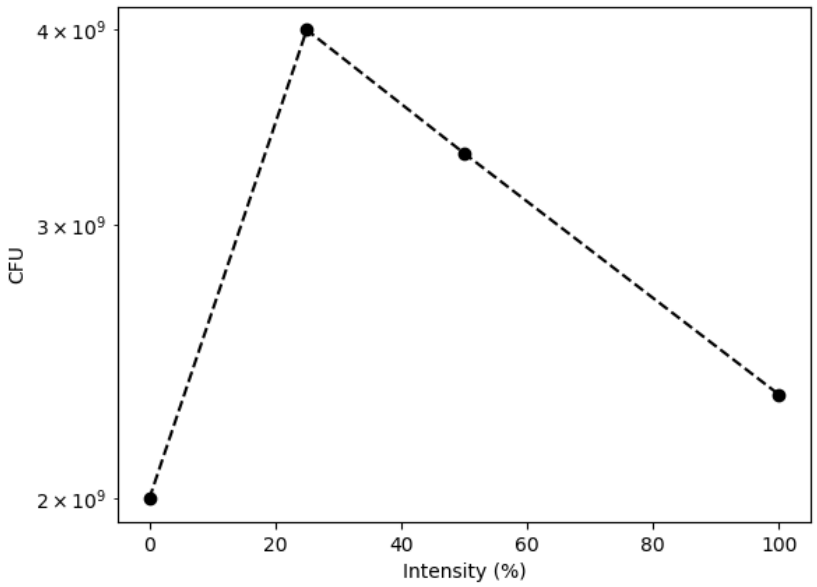

Figure 5. Cell viability of bacteria that were exposed to several intensities over a 5-minute interval in a 10 mL flask (a 10% increase in volume to the 1 mL centrifuge tube).

Discussion

Probe Sonication

Probe sonication induced the highest rate of cell death. The 50% and 25% intensity were measured in the same batch while the 35% intensity results were measured differently. This led to the normalization of the CFU (colony forming units).

The 25% and 50% intensity CFU-exposure profiles show a nearly exponential decrease in viability with respect to exposure time. The intermediate intensity shows a net decrease in viability; however, it is not exponential. This discrepancy might be explained by the fact that the 35% CFU-exposure profiles were produced from separate cultures, sonication setup, and counting assay. Given this variability, comparisons should be limited to the lowest and highest intensities.

In the 25% and 50% results, bacteria were killed the fastest in the first minute of sonication. By minute 2 and 5, the killing decreased by at least two orders of magnitude. The 35% intensity results are much more variable, given that the largest decrease in viability was between the second and fifth minutes. This decrease in the first half of sonication could be explained by the volume of bacteria in each sample. After the LFU kills much of the bacteria in the first minute of sonication, it has a lot less bacteria to bump into. Furthermore, we hypothesize that as the LFU moves through the PBS media and hits the walls of the centrifuge tube, it loses a significant

amount of its intensity. When it hits the bacteria having a reduced intensity, it may hinder the bacteria's growth rather than kill it.

Past research has shown that the volumes in which the bacteria are sonicated are very important to the decrease in cell viability [5] . Indeed, Figure 5 shows the failure of the sonications ability to affect the cell viability. We hypothesize that in larger volumes, declumping of the bacteria gives rise to higher cell counts. This declumping could be occurring during minutes two through five. In the smaller volumes as seen in Figure 3, however, there are not as many colonies to declump. Therefore, the killing rate of the ultrasound dominates its declumping rate.

Bath Sonication

In the first 15 minutes of bath sonication at 40 kHz and produced 130 W of power, the cell viability decreased by over two orders of magnitude. At 30 minutes, however, there was an increase in cell viability. This implies that the bacteria were actually growing during the sonication. This can be explained by systematic error that was overlooked during the experiments.
Bath sonication is done in a closed system -- where the top is covered and there is little room for equilibration with the ambient lab temperature. While sonicating using a bath, the water around the bacteria will heat enough to create an incubator-like environment. One misstep of our procedure that we did not account for was that we did not add enough ice to the bath to simulate a constant, cold, temperature. Consequently, we hypothesize that the bacteria were able to grow in the warmer temperatures and withstand the LFU.

Future Work

Based on the present study's treatment of planktonic bacteria, future work should interrogate biofilm properties including mechanical, biochemical, and topographical.

Quantifying mechanical properties of sonicated biofilms includes measuring the viscosity of biofilms subjected to various LFU regimens using shear rheometry and characterizing the nanomechanical properties of biofilms subjected to various LFU regimens in an AFM.

Quantifying the viability of sonicated biofilms includes growing and sonicating biofilms from a pure Pseudomonas culture. Synthetic biofilms along with naturally grown biofilms should be considered and compared with the planktonic bacteria. This will give the scientific community further insight on the relationship between these different states of bacteria. Cell viability could be measured in two ways. A live cell staining technique I recently developed could be implemented along with culture counting used in my past research.

Qualitatively characterizing the morphology of the EPS of sonicated biofilms includes imaging the biofilms in their native state with an eSEM. The eSEM enables a precise

characterization of the morphology because samples do not need to be fixed or sputter coated. This characterization could enable modal analysis with finite element methods.

Conclusions

The study herein details a methodology for measuring the viability of sonicated planktonic *Pseudomonas aeruginosa*. This methodology includes sample preparation, insonation, dilution, and colony counting. Results indicate that:

(1) Probe sonication decreases cell viability at a higher rate than bath sonication.
(2) Higher intensities of LFU decrease cell viability.
(3) Longer time intervals in the probe sonication kills more planktonic bacteria.
(4) Smaller volumes of bacteria subjected to LFU demonstrate lower viability than larger volumes.
(5) Larger volumes induce the declumping mechanism, a confirmation of a trend previously identified in the open literature [5].

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