Effects of ultrasonic exposure on growth and attachment rates of a capsule-forming bacterium
by Teresa Ann Campbell

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
The initial goal of this project was to study changes in the growth rate of a capsule-forming bacteria (Pseudomonas aeruginosa) with exposure of a culture to low intensity ultrasonic waves. Preliminary experiments had earlier indicated that the possibility existed for increasing the growth rate by "scraping" the capsule layer from the cell. This might reduce the resistance to mass transport, resulting in a higher growth rate. Because the bacterial cell also uses this capsule layer to attach to surfaces, attachment ability was also studied.

Growth rates of cultures were measured by viable cell counts. Cell concentration measurements of sonicated samples were compared to concentration measurements of control cultures exposed to identical environmental conditions with the single exception of the absence of ultrasonic exposure. Although some experimental runs indicated a beneficial effect of ultrasound on the growth rate of the culture, the majority of the data showed that sonication was actually decreasing the growth rate. In addition, these "positive" experimental runs could not be duplicated, even under identical conditions. Varying such parameters as temperature, exposure intensity, exposure duration, or nutrient concentration also did not produce evidence of growth rate enhancement.

Surface attachment of cells after exposure to ultrasound was studied using a specialized flow system. Cell attachment from sonicated cultures was compared to controls at identical environmental conditions. The flow system used in this series of experiments was still in the development phase, so precise quantitative results were not attainable. However, even after taking experimental error into account, results do indicate a marked decrease in attachment after exposure to ultrasound. Additional experimentation was conducted which indicated that this decrease in ability to attach might be passed on to daughter cells, even without additional exposure.

Although precise determination could not be made as to whether or not ultrasonics could be used to decrease the capsule layer of bacteria, some key conclusions can be drawn. First, bacteria can grow and reproduce in a low intensity ultrasonic field, although at a slightly retarded rate. Second, ultrasonic exposure seems to diminish the ability of cells to attach to surfaces, a characteristic which might be passed on to daughter cells.

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by

Teresa Ann Campbell

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APPROVAL

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Date May 28, 1991

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ABSTRACT

The initial goal of this project was to study changes in the growth rate of a capsule-forming bacteria (Pseudomonas aeruginosa) with exposure of a culture to low intensity ultrasonic waves. Preliminary experiments had earlier indicated that the possibility existed for increasing the growth rate by "scraping" the capsule layer from the cell. This might reduce the resistance to mass transport, resulting in a higher growth rate. Because the bacterial cell also uses this capsule layer to attach to surfaces, attachment ability was also studied.

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INTRODUCTION AND BACKGROUND

The first American Institute of Chemical Engineers symposium on ultrasonics was held in Boston, Massachusetts in May of 1950 and was soon followed by another in Columbus, Ohio in December of that same year (1). The Boston symposium was "developed with three specific objectives in mind: 1) to point up the potentialities of sonic and ultrasonic energy, 2) to emphasize known limitations, and 3) to stimulate investigations and reports of further work in this field." Topics discussed in this symposium included application to aerosol collection problems, colloidal effects of ultrasonics, and testing and gaging of materials. The Columbus symposium included topics such as biological effects, power requirements and applications to industrial processing.

As interest in ultrasonics grew, so did the range of applications. By 1971, the term "sonochemical engineering" had been coined to describe the "application of sonic and ultrasonic waves to chemical transport and processing operations" (2). In that year, H. Scott Fogler edited a symposium by the American Institute of Chemical Engineers to report on some of the "unusual phenomena" that had been discovered to be associated with ultrasonic waves. For the

most part, topics in this symposium dealt with mixing, drying, viscosity control, and increases in catalytic reaction rates, all common processes in industrial chemical engineering. The general consensus of this symposium was that ultrasonic energy can greatly affect, often beneficially, typical processes within the field of chemical engineering.

Ultrasonic waves are sound waves above the audible frequency range. The accepted "breaking point" between sonic and ultrasonic waves can range anywhere from a frequency of 16 kHz to a frequency of 20 kHz (1, 2), but since there are no fundamental differences between ultrasonic and sonic energies, the "dividing line" is immaterial.

Sound waves are longitudinal waves, and thus vibration of a particle in the medium is in the direction of wave propagation. The word 'particle' can refer to an actual particle suspended in the medium (most often a liquid), or a fluid particle. The accepted definition of a fluid particle is a volume of fluid in which the pressure, temperature, density, and velocity are considered equal for each molecule.

As sound waves propagate through a medium, they create zones of low pressure and temperature and zones of high pressure and temperature. When passed through liquids or gases, sound waves have produced some interesting effects,

including cavitation, acoustic streaming and interfacial instabilities.

Cavitation is the most studied of these effects, perhaps because it is the most easily observed. Cavitation is the formation, growth, and collapse of tiny bubbles in liquids. When cavitation occurs, the rarefaction portion of the ultrasonic wave is lower in pressure than the vapor pressure of the liquid, causing tiny bubbles to be formed. However, when the high pressure zone propagates through the medium, many of these bubbles will collapse, generally quite violently.

Three types of bubbles have been observed, stable resonant, unstable resonant, and collapsing (3). Resonant bubbles are seen to expand and contract with the sound waves in an oscillatory fashion for an indefinite number of cycles. Resonant bubbles are stable if the average size of the bubble is independent of time. Resonant bubbles are unstable if they continue to grow with each successive wave until they reach an unstable size and collapse. Collapsing bubbles are seen to grow very large and collapse in one cycle. The most fascinating aspect of cavitation is the energy generated by the collapse of cavitation bubbles. While measurements are impossible to obtain directly, the pressure may reach twenty-thousand atmospheres and the temperature may reach 10,000^{o}C (4). These regions of high temperature and pressure may be responsible for the

acceleration of chemical reaction rates as well as heat and mass transfer rates.

Acoustic streaming has been less studied, and thus is less well understood. Basically, acoustic streaming refers to an observed pattern of time independent circulations in sonicated fluid, resulting in a "gentle" mixing pattern at all but the highest sonication intensities (2). What exact effects, beneficial or detrimental, acoustic streaming may have on liquid systems are not completely known or understood at this time.

The third major effect of ultrasonics is interfacial instabilities. This effect is due to the fact that particles at the interface of two liquids are subject to oscillations in and out of the two liquids. One result of these oscillations is the emulsification of "immiscible" liquids. While it could be argued that the collapse of cavitation bubbles could also cause emulsification, emulsions of mercury and water have been produced by sonication without the occurrence of cavitation (5).

One of the earliest studies of ultrasonics on biological cell systems was performed by T.F. Hueter (6) as reported in the 1950 Columbus symposium. However, the viability of the cells after ultrasonic irradiation was not a consideration. In most of Hueter's experiments, cells were killed with ultrasonics and then studied. This is typical of the history of ultrasonics and biological cell systems. In

fact, today, ultrasonic exposure is one of the most common methods of cell destruction. In order to study the internal components in cells, the membrane can be disrupted with high intensity ultrasound and then the various constituents can be centrifuged for separation and analysis. Thus, it is known that the effects of ultrasound can produce cell death. However, very little is known about the effects that ultrasound can have at low intensities on living cells.

As previously mentioned, one of the effects of sonication can be an increase in chemical reaction rate. This is illustrated in the work of Kenneth S. Suslick of the University of Illinois at Urbana-Champaign (7). The majority of work in this study dealt with high intensity sound waves and the benefits that cavitation can have on inorganic systems. Although some effects were observed at low intensity, all of the dramatic effects observed came from the fact that as the cavities collapse, the temperature and pressure reach short lived, but extremely high levels. Thus, it only makes sense that many reaction rates will increase as it has been known for quite some time that nearly all chemical reaction rates are temperature dependent. It might seem from these results that since the metabolism of a cell can be thought of as simply a series of chemical reactions, the growth rate could be significantly increased with this higher intensity sonication.

Unfortunately, there are several other aspects to take into consideration before applying these ideas to living systems.

A living cell is a very delicate system, composed of very large and complex organic molecules, a situation not explored by Suslick. According to Heat Systems, one manufacturer of ultrasonic machines, the larger the molecule, the less likely that it will be able to withstand sonication (8). Therefore, it is only reasonable that larger organic molecules and bacterial cells would be more likely to be disrupted by sonication. However, the fact that some inorganic reactions can be accelerated is reason enough to believe that there may be a possibility of accelerating biochemical organic reactions, and thus cell metabolism, under certain controlled conditions.

Perhaps the most important consideration when dealing with living cells and potentially hazardous conditions is the strength and durability of the cell membrane. A diagram of a typical bacterial cell membrane is given in Figure 1. The internal components of the cell are contained in a fluid called the cytoplasm, which is contained within the cell membrane. The cell wall gives structure to the cell and determines the shape. The majority of the molecules in the membrane are phospholipids which are held together by hydrophobic interactions, not actual covalent bonds. Other molecules contained within the membrane

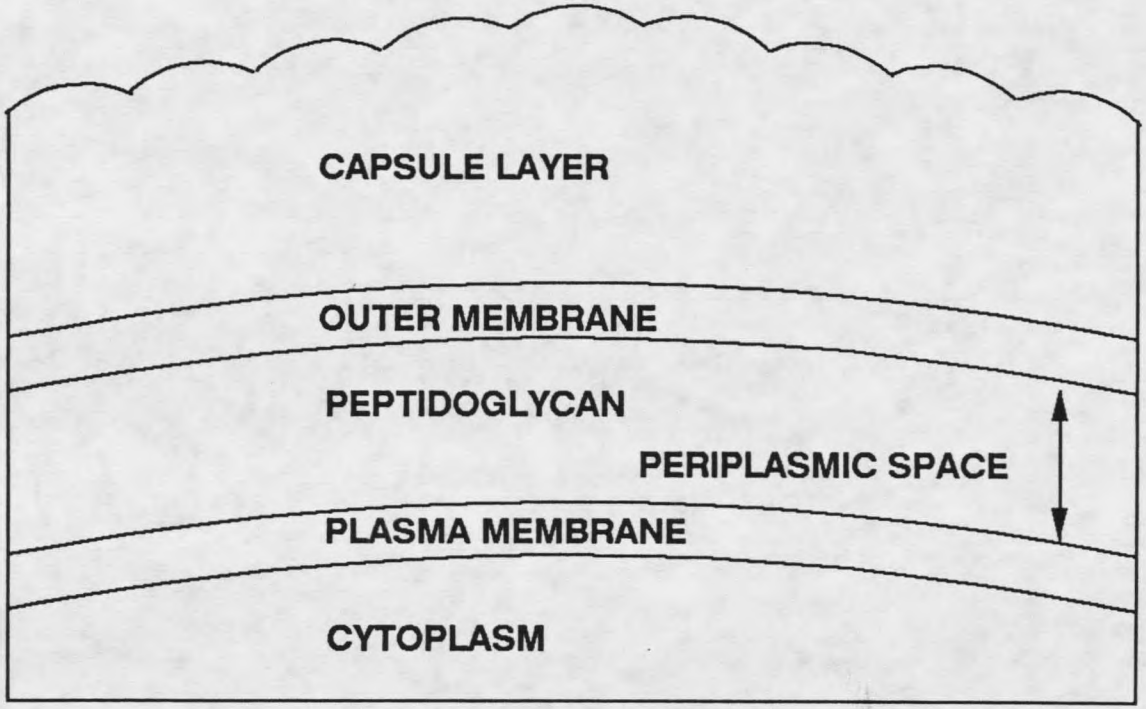

Figure 1. Outer Cellular Structure

include transport proteins, which are responsible for taking in nutrients and disposing of waste products. However, these molecules are not actually bonded to the membrane, but instead are held in place in a sort of a fluid mosaic pattern. It can easily be seen that the membrane of the cell would be one of the weaker parts of the cell and thus likely to be disrupted with ultrasound.

As shown in Figure 1, the exterior of a bacterial cell can be covered with a substance known as a slime or capsule layer. This layer can vary in thickness up to three times the width of the cell itself. The capsule layer is generally composed of lipopolysaccharides and does not have a rigid structure. While this layer serves no known purpose for cell metabolism, it does allow the bacterial cells to adhere to surfaces and to grow together in colonies, thus serving a beneficial purpose by allowing the bacteria to remain stationary. However, it has been proposed that if the capsule layer is thick enough, it may prove to be a hindrance to cell metabolism in that necessary nutrients would have to diffuse through this excessively thick layer in order to get to the cell wall and membrane.

As previously mentioned, sound waves produce vibratory motion in the direction of wave propagation. Due to differences in particle characteristics, the amplitude of vibration is not constant for all particles in the system. These differences can lead to significant shear stresses

between particles and the surrounding media. These differences in amplitude occur at all intensities as do the shear stresses that they produce.

This research project was designed to take into account the properties of the cell as well as the effects of ultrasound. As the effects of ultrasound at a low intensity should be less damaging than those at high intensity, it was speculated that low intensity ultrasound might produce the same vibratory particle movement as high intensity, but result in a gentle scraping of the capsule layer instead of cell destruction. This scraping could either reduce the thickness of the capsule layer, or remove it all together, which would then reduce the resistance to mass transport. This lower resistance should then manifest itself as a higher metabolism which could be observed as an increase in the culture growth rate. By carefully controlling other environmental parameters such as temperature and nutrient availability, any difference between the concentration of a sonicated culture and the concentration of a control culture should be attributable to the effects of the sonication. In this way, growth enhancement due to sonication could be both observable and measurable.

MICROBIOLOGY

The living microbial cell can be thought of as an extremely sophisticated chemical reactor, involving over 1000 reactions in an open operating system (9). Each of these reactions fulfills some purpose of cellular life. Due to the complexity of this system, there is no feasible way to look at the detailed effects of a change in any one parameter on the entire system. One would have to choose between either looking at the effects on the system as a whole, or on each individual reaction system within the cell. Therefore, in the present study, the effects of ultrasound on cells are analyzed for the impacts on cells as whole systems and not for the distinct internal processes of microbial life. Thus, the detailed knowledge of microbiology necessary for the current project is limited and highly specific towards the particular bacterium and its particular growth needs and characteristics.

Pseudomonas Aeruginosa

The bacteria chosen for this study is a strain of Pseudomonas aeruginosa. P. aeruginosa are straight rods, approximately 1 micron in diameter and 1.5 to 2.0 microns in length. They are motile bacteria, the motility being

provided by the one flagellum, or "tail", on one end of each cell. They are very adaptable, and they tend to grow well on all usual laboratory culture media. Like many microorganisms, they require water, oxygen, and a temperature-controlled environment for significant growth to occur.

As mentioned, P. aeruginosa are a rod-shaped bacteria. Since the major thrust of the current project is aimed at enhanced transport through the capsule layer and cell wall, the shape of the cell is very important. The more surface area per unit volume a cell has, the more likely that a transport benefit would be seen. However, it is known that the effects of ultrasound can be violent and that the smaller, more compact cells can generally withstand it much better (10). This would mean that cocci, or spherical bacteria might be best for this study. Unfortunately, these have the least surface area per unit volume of any shape. Thus, the rod-shaped cells are somewhat of a compromise between the two extremes. They provide the added surface area while still retaining a compact shape.

Also, P. aeruginosa are Gram-negative. This test distinguishes between the two common types of cell walls. The Gram-negative wall was depicted in Figure 1. In Gram-negative bacteria, there is a second membrane around the cell wall. This has various effects on the cell, one of which is to give the cell added protection, again perhaps

adding to the ability of the cell to withstand ultrasonic exposure.

Finally, P. aeruginosa are known capsule producers. The main thrust of this project is to increase cell growth rates by increasing mass transfer through the slime layer. Thus, this attribute is of prime importance to the current study.

Growth and Metabolism

Microorganisms require a variety of conditions and nutrients in order to grow and reproduce. One important condition is temperature. While a wide range of viable temperatures exists for most microorganisms, there is a relatively narrow band which will result in the maximum growth rate. The optimum temperature is the temperature at which the microorganism will grow the fastest. Figure 2 illustrates how a change in temperature can alter the growth rate (11). P. aeruginosa, the bacteria used for this study, are able to grow in temperatures from 15 - 50 C with optimum growth occurring at 37 - 42 C (12).

The growth of a bacterial culture can be defined as an increase in the number of cells, and the growth rate is the change in cell number per unit time. The growth rate is not constant, but will change with concentration and environmental conditions. A typical growth curve of a batch culture is shown in Figure 3. As shown, the culture will go

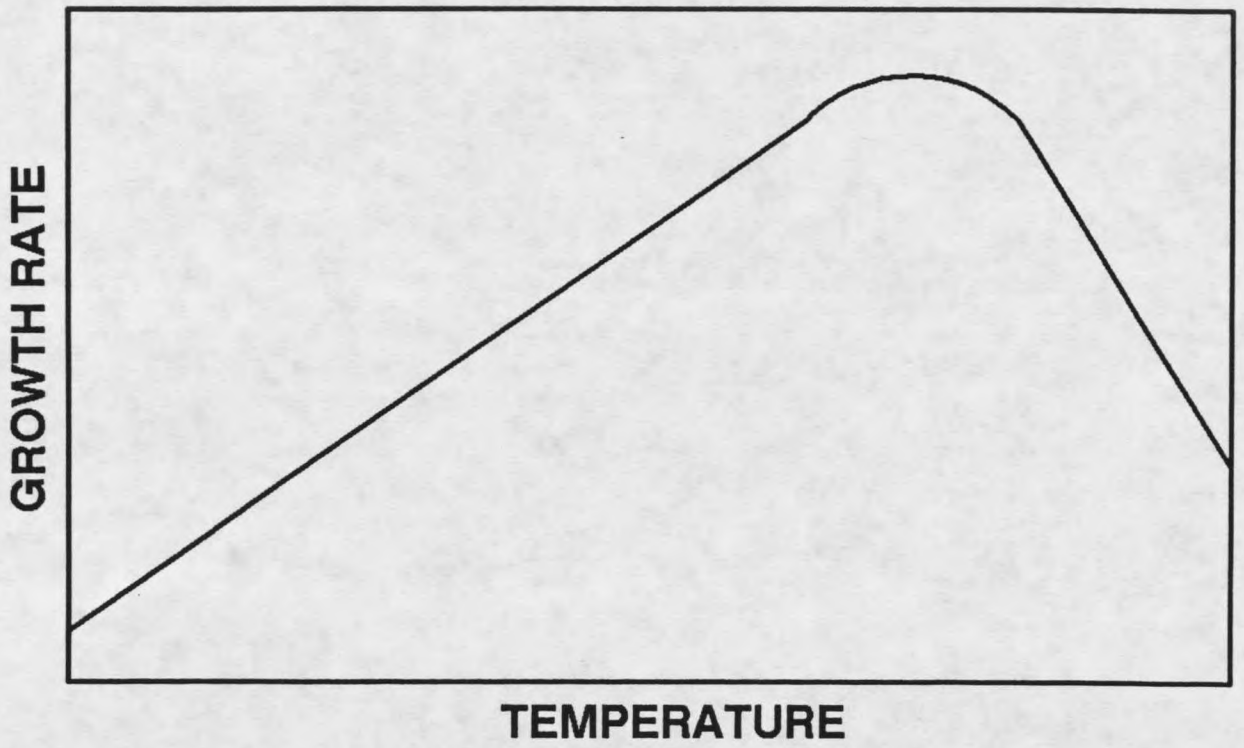

Figure 2. Bacterial Growth Rate versus Temperature

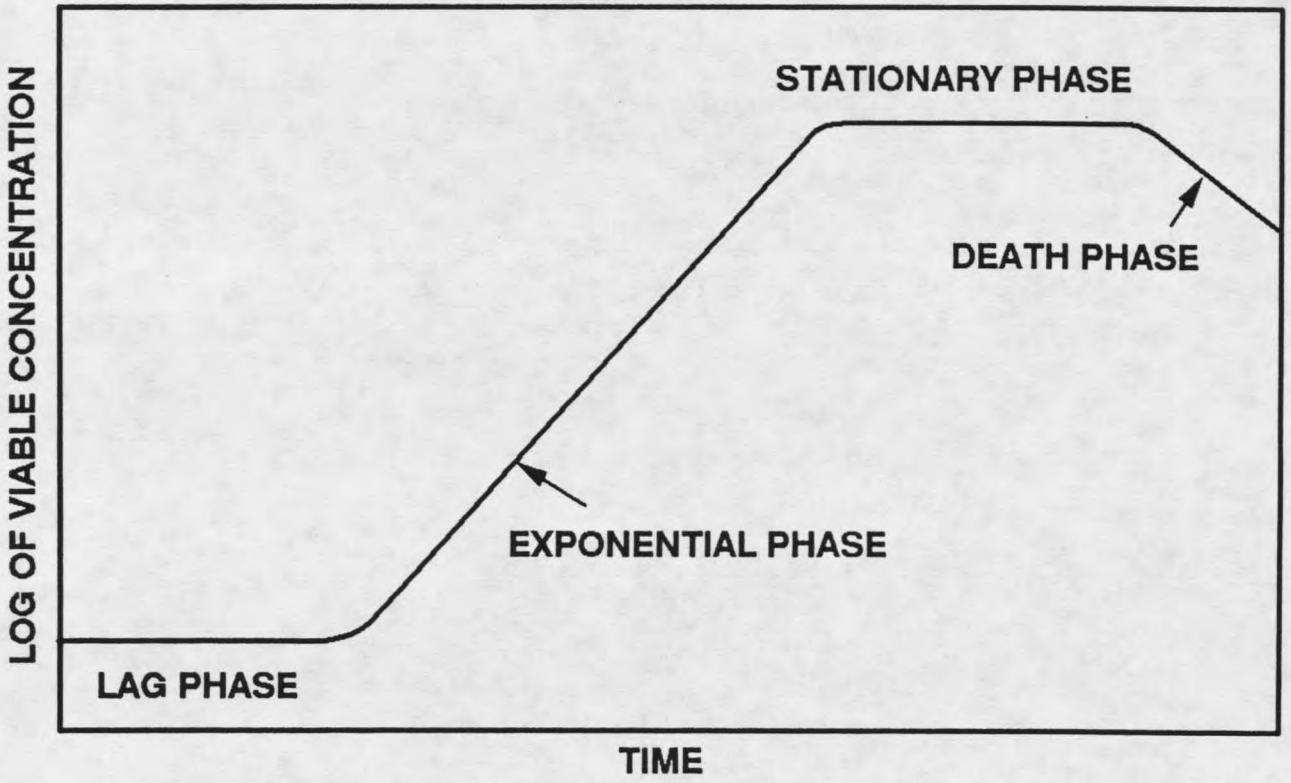

Figure 3. Typical Bacterial Growth Curve (Base 10 Logarithm of the Viable Cell Concentration versus Time

through a lag phase, an exponential phase, a stationary phase, and finally, a death phase. This type of curve is typical of a batch culture that has been freshly inoculated from a parent culture itself in a stationary phase. If the parent culture had been in an exponential phase and then inoculated into a fresh batch of the same media, an exponential phase would have continued. The reason for the lag phase from a stationary parent is due to the condition of the culture in stationary phase, as explained below.

Growth of bacteria in an adequate media is characteristically exponential for a period of time (11). However, it is obvious that this cannot continue forever. The generation time is the time required for the number of viable cells in a culture to double, with twenty minutes being common for many bacterial strains. If exponential growth could continue indefinitely, a typical laboratory culture with a generation time of twenty minutes would produce a population weighing about 4000 times the weight of the earth in just forty-eight hours (13). As the concentration of the culture increases, either a necessary nutrient is depleted or a waste product is accumulated to a level that inhibits further growth, and exponential growth ceases. When this happens, the constituents of the cell that are used for reproduction deplete in concentration within the cell. The bacterial cell is extremely conservative in that it only produces what it actually needs. Therefore, when the environmental conditions are

such that the rate of metabolism of nutrients is decreased, the production of enzymes necessary for synthesis is decreased. When cells in this condition are inoculated into fresh media, a lag time is required to resynthesize enzymes. A lag time also occurs for damaged or injured cells where the time is required to repair the cell. In addition, cells transferred from a rich medium to a poorer one also undergo a lag phase as the balance of the various enzymes must be adjusted to the nutrient concentrations in the new medium.

Attachment

As mentioned previously, P. aeruginosa are motile bacteria, meaning that they have the ability to move themselves through a medium. This gives them the ability to adapt to their environment to the extent that they can move short distances to a more desirable location. Unfortunately, in a liquid medium, a cell in suspension is at the mercy of the currents and any bulk flow that may be occurring. Thus, even though it can propel itself, to some extent, to desirable locations, the cell requires a means of staying there. This is accomplished through attachment. Attachment is the process by which the cells adhere to surfaces, generally by means of either the flagella, the capsule layer, or both. Once attached, the cell can acquire nutrients from the liquid media, allowing for growth,

reproduction and the production of additional capsular lippopolysaccahride. This is the beginning of a "biofilm" and results in "biofouling". Biofouling can result in decreased efficiency and significant corrosion in industrial processes and thus can be a very serious problem.

LITERATURE SURVEY

To date, nearly all of the work relating ultrasonics to biological systems has dealt with the destructive effects of cavitation. In cavitation, tiny bubbles are formed by the rarefaction portion of the wave where the pressure is low and are collapsed by the high pressure crests. The violence of the collapse causes extremely detrimental effects on biological systems, such as cell lysis, and thus cavitation was avoided whenever possible for the current project.

Cavitation is enhanced by several factors, including dissolved gases and particles in solution (10). However, it has also been found that these factors which enhance cavitation also tend to reduce the severity of effects if cavitation does occur (10). Therefore, for this study, if some cavitation did occur due to dissolved gases and the presence of the bacteria themselves, there is some reason to believe that the cells would be able to survive anyway, provided the intensity of exposure was not too strong.

There have been many misconceptions regarding ultrasonics in the past. One of these is that ultrasonics could cause DNA depolymerization (14). This was found to be incorrect by several investigators (14-19). The reason for

wrong conclusions in the literature was due to errors in experimental interpretation. Sound waves are a form of energy and will dissipate through a medium in the form of heat. Heat can and will cause depolymerization, so the damage observed was due to the temperature increase and not the sonication itself. One opponent of damage due to ultrasonics stated that "neither pulsed nor continuous wave application of ultrasonics cause chromosome damage" (20).

It was also thought that enzymes could be inactivated by ultrasonics (21). However, it has since been found that the observed deactivation was again due to the temperature increase and not the sonication itself (12-25).

There are also other effects of sonication that could have beneficial results on microbial systems. One such effect is micro-streaming. When the bubbles of cavitation form in liquid systems, they do not always collapse immediately but will often build in size until they reach a critical size and then collapse. When this occurs, the fluid is seen to eddy about the bubbles and suspended particles are seen to rotate about an equilibrium axis without damage to the particles themselves (26). This could indicate that if cavitation did occur, but at a low intensity, the bacterial cells could withstand the treatment.

Another effect of sonication on biological cells is that in some instances, the cell walls of bacteria have appeared

thinner (10), indicating a significant effect on the outer surface of the cell. One investigator even suggested that "it may be possible ... to strip a polysaccharide outer coating of a bacteria cell wall, with very low intensities and without injury to the cell itself." (10).

Lastly, it has been shown that shear stress may increase mass transfer in systems of biological cells. The hemoglobin in blood is normally found only within the red blood cells and not in the plasma. In fact, hemolysis, or the release of hemoglobin into the blood system can be very dangerous to human beings. However, hemoglobin has been found in solutions of blood after exposure to shear stresses without breaking of the cell walls (27), indicating a transfer through the cell membrane. Admittedly, the external structure of mammalian cells is much thinner and much less rigid than bacterial cells, but the fact that mass transfer was accomplished without cell destruction shows some indication that mass transfer may be enhanced by ultrasound without destruction to cell membranes.

All of this information indicates that very little is actually known about the effects of low intensity ultrasonic energy on living systems. However, if ultrasonic energy could be applied at a level which would not kill bacterial cells, but would still create vibratory motion of the medium, it could be possible to enhance growth through sonication by increasing the metabolism of the culture.

RESEARCH OBJECTIVES

This research was undertaken to explore the possibilities of enhancing bacterial growth through the ultrasonic removal of the cell capsule layer. Due to the exploratory nature of this project, other objectives were developed as additional information was obtained. These included inhibiting attachment of cells to surfaces by the removal of the capsule layer.

Initially, the primary goal of this work was to show a increase in the growth rate of the bacteria after exposure to ultrasound. Based on initial results, other parameters, such as temperature and nutrient concentration, were to be evaluated for their effect on growth enhancement.

The primary goal at the end of this work was to show a decrease in the attachment of bacteria after exposure to ultrasound, which could indicate a partial removal of the capsule layer. These results can be used to identify a suitable course for future research.

EXPERIMENTAL APPARATUS

The Sonicator

All sonication in this study was done using a Heat Systems Ultrasonic Sonicator with a one-half inch titanium horn. While the frequency of this machine is not variable, being set on 20 kHz, there is some room for exposure regulation by controlling the output intensity, the percent duty cycle, and the cycle time.

The output control regulates how much of the power of the system is actually put out through the horn, thus controlling the sonication intensity. Settings are from zero to ten, which proportionally represent intensities from zero to 450 watts through the half-inch horn tip. Control from zero to one was not judged as precise. Since the amplitude of vibration and cavitation effects are directly related to the intensity, this control was always set relatively low, usually on one, or roughly 50 watts.

The machine is also designed so that sonication can occur for intermittent cycles. This is done using the cycle time and the percent duty cycle. The cycle time indicates how long the complete cycle of sonication and silence will

last. The choices on this system are one, two, or five seconds, or continuous. "Continuous" is the setting used if cycles are not desired. The percent duty cycle determines the fraction of the total cycle time during which sonication will occur. The range for this is from ten to ninety percent. Thus, a cycle time of five seconds and a percent duty cycle of ten percent would result in sonication for 0.5 seconds and silence for 4.5 seconds.

To ensure even exposure of all of the bacterial cells to the sonication, a continuous flow cell was used. The majority of the effects of ultrasound are felt within approximately one-quarter of an inch from the probe itself, making intimate contact with the probe by all the cells very important. By using the flow cell, as shown in Figure 4, this can be accomplished. For these experiments, flow entered the chamber of the cell using the bottom port and exited the cell using the higher side port, ensuring that all liquid, and thus all cells, came into close contact with the probe, giving the most even exposure possible.

The Flow System

The experimental setup for these experiments is shown in Figure 5. A constant temperature water bath was used to

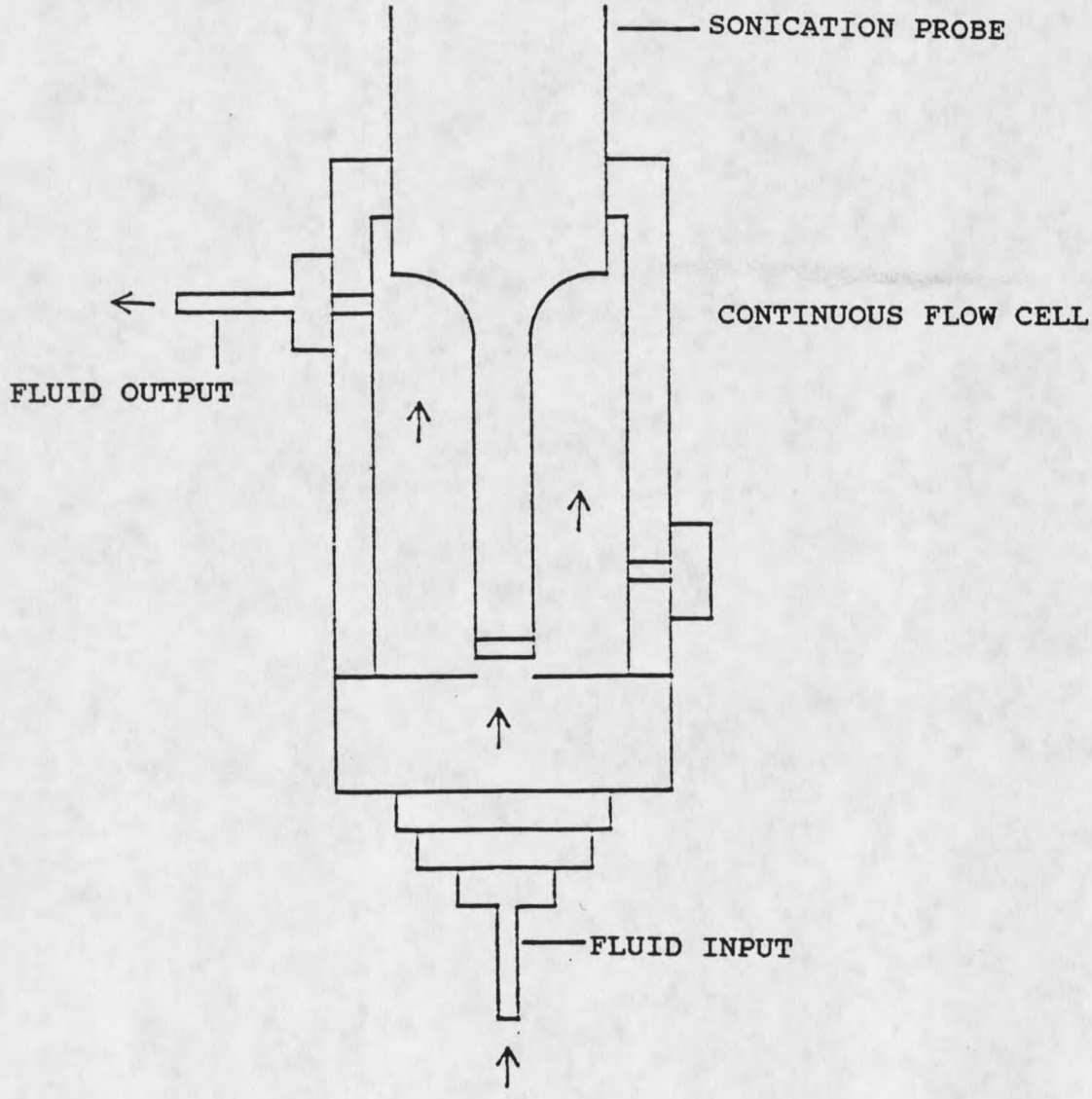

Figure 4. Sonication Probe with Continuous Flow Cell

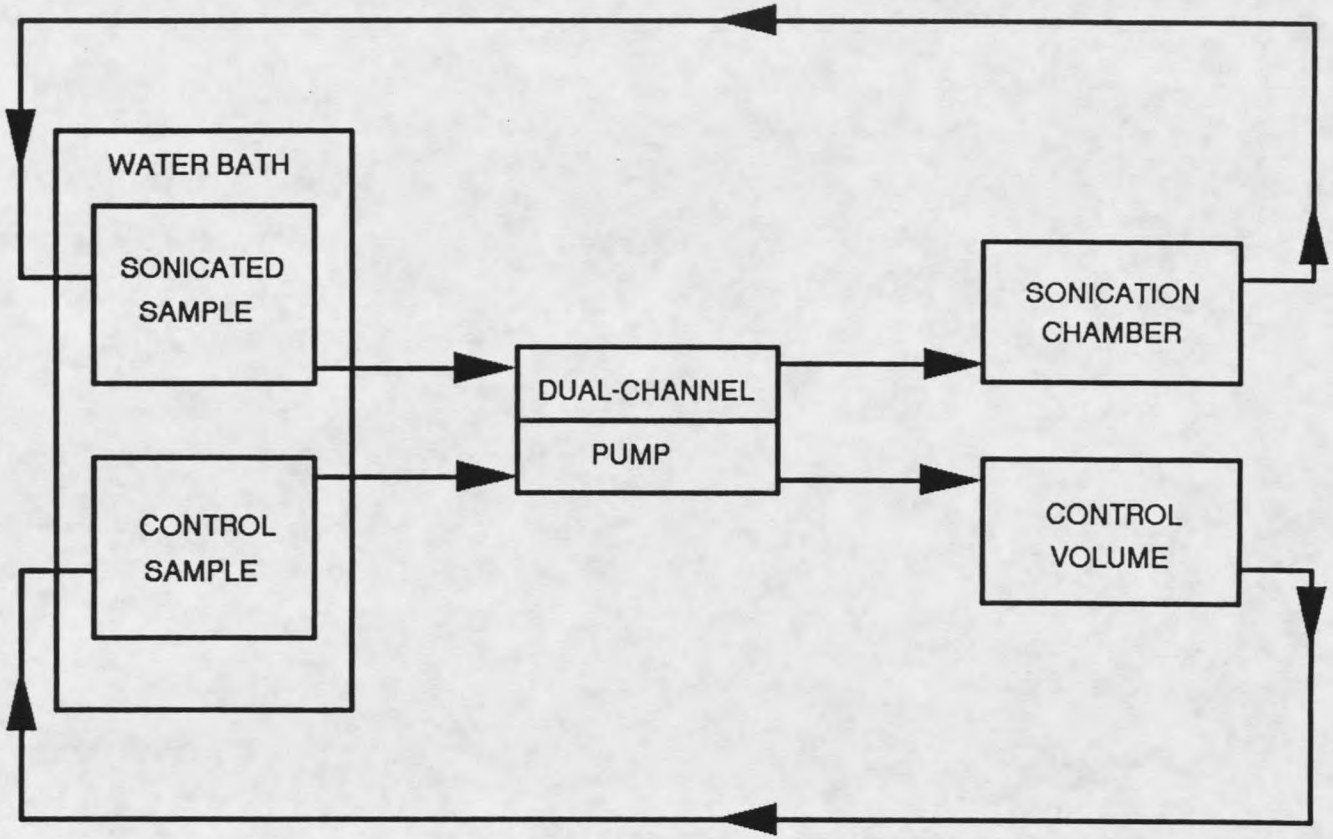

Figure 5. Experimental Apparatus

maintain and regulate temperature for the system. A Masterflex (trademark) peristaltic pump model N-06241-20 was used to force flow through the sonication cell and to provide mixing in the system. The 1 to 100 rpm model was chosen because it was thought that the 6 to 600 rpm model might cause excessive shear stresses in the liquid within the pump head. Since the basis of the experiments was to alter the growth characteristics of the cells through shear stress provided by the sonication, additional stresses were not desired, and the 1 to 100 rpm model was chosen.

For the sonicated sample, fluid was drawn out of the flask, through the pump, through the sonication cell, and back to the flask. The flow line for the control sample was identical with the exception of the sonication cell. Since there was not a sonication cell for the control line, the cell was replaced by a control volume, a stoppered test tube that had a volume as close to the volume of the sonication cell as possible. Volume was still a small amount less than the sonication cell, so the additional volume needed to give both lines the same total volume was obtained by using additional tubing. Since the flow configuration for both lines was nearly identical, any differences in growth would have to be due to the sonication itself.

Wide-mouth 500-ml Erlenmeyer flasks with specially designed stoppers were used for these experiments. The stoppers and entry/exit tubes were designed to maximize

mixing of both the liquid and gas portions in the flasks. This is shown in Figure 6. The fluid was drawn from the bottom of the flask on one side and returned near the top of the liquid level on the other. As for the gas line, pure oxygen was put in near the gas/liquid interface and allowed to escape near the top of the flask. As the gas outlet was the portal in the system open to the surroundings, a short piece of tubing was attached with the open end facing down to prevent air-borne contamination from entering the system.

The flasks could contain a maximum of about 550 ml, including both the gas and liquid phases. This gave an operating volume in the flasks of about 350 ml liquid and 200 ml gas. The sonication cell contained 60.5 ml of fluid, while the entire flow loop contained approximately 100 ml of liquid when size 17 (0.322 cm^2 cross sectional area) silicon tubing was used. The silicon tubing was chosen because it is compatible with biological systems and because it has a relatively long "tubing life" within the pump head (825 hours at 100 rpm). A high flow rate with a low flow loop volume was desired, so size 17 tubing was chosen. Size 16 tubing has a maximum flow rate of 167 ml/min at 100 rpm (as opposed to about 250 ml/min with size 17) while size 18 would have resulted in an increase in flow loop volume of approximately 30 percent. Thus, the size 17 tubing was judged to provide the best compromise for operation of the present system.