THE EFFECT OF GINSENG ON BIOFILM FORMATION BY WILD TYPE AND MUTANT STRAINS OF *PSEUDOMONAS AERUGINOSA*

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by

Premi Shekar

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Director of Thesis

Master's Committee: _____, Chair

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Date

THE EFFECT OF GINSENG ON BIOFILM FORMATION BY WILD TYPE AND MUTANT STRAINS OF *PSEUDOMONAS AERUGINOSA*

Premi Shekar, M. S.
Morehead State University, 2008

Director of Thesis: _____

ABSTRACT

The predominant pathogen in patients with cystic fibrosis is *Pseudomonas aeruginosa*, which results in a chronic lung infection associated with progressive pulmonary insufficiency. Variations in biofilm formation by, *Pseudomonas aeruginosa* (GFP tagged) PAO1 (wild type) and the quorum sensing-deficient mutants PJP1 (Δ *lasI* mutant) and PDO100 (Δ *rhlI* mutant) were examined utilizing three (4.7 μg/mL, 150 μg/mL, 4,800 μg/mL) different concentrations of ginseng for 1, 3, 7 and 10 days, respectively. Scanning electron microscopy and fluorescence microscopy were used to assess the surface view and the biomass of the biofilms, where biomass means the bacterial cells and the extracellular polymeric substance together. It was shown that PAO1 (wild type) showed the least biofilm formation for the highest concentration of ginseng on the first day by visually inspecting the biomass with scanning electron microscope and fluorescence microscope followed by PJP1 (Δ *lasI* mutant) and then PDO100 (Δ *rhlI* mutant). Confocal microscopy was used to obtain the XZY plane view of the biofilms where the biofilm thickness

was measured. The mean thickness of biofilms was greatest in PAO1 (wild type), followed by PJP1 (Δ *lasI* mutant), and then PDO100 (Δ *rhlI* mutant). This could be an indication that when biofilms become thicker, the surface area of the biofilm, which is exposed to the nutrients, could actually decrease. An ANOVA analysis was performed to see if there were significant differences in the effects of all three factors (bacterial strains, ginseng concentration and days of incubation) on biofilm thickness. The result is that all the three factors had a statistically significant effect, independent of one another, on biofilm formation (p value=0.00). The mean thickness of the biofilm was greatest in PAO1 (wild type), followed by PJP1 (Δ *lasI* mutant), and then in PDO100 (Δ *rhlI* mutant). The biofilm thickness decreased with increasing concentration of ginseng and the biofilm thickness increased with increasing number of days of incubation. The R^2 value for this experiment was 83.10% which showed that more variance was accounted for. On the basis of these results, it is suggested that ginseng may have the potential of being a biofilm inhibitor in conjunction with an immunology agent (shown in other studies), thus being a promising natural medicine with other forms of treatment, for CF patients with chronic *P. aeruginosa* lung infection.

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TABLE OF CONTENTS

List of Tables

List of Figures

Introduction

Biofilm formation and persistence is a very important feature of *Pseudomonas aeruginosa.* Quorum sensing has shown to be responsible in the cell-to-cell communication in bacteria during biofilm formation. In cystic fibrosis patients *Pseudomonas aeruginosa* form biofilms which are resistant to most antibiotics and also to muti-drug therapies. Pulmonary infections due to *Pseudomonas aeruginosa* cause major fatalities in cystic fibrosis patients. The need for alternative drug strategies to deal with biofilm formation is very essential. The Chinese herb ginseng has shown to be an immunostimulant, antioxidant, vasodialator etc. It has been demonstrated to enhance the clearance of bacteria from rat lungs and result in a shift of the inflammatory type from a Th2-like to a Th1-line response, (Song *et al.*, 1997). It consists of more then 70 identified substances and some other active substance classified as ginosenosides, (Kharazmi *et al.*, 2004). These ginosenosides are glycosylated steroids. Ginsenocides are chemicals specific to ginseng which are closely related saponins and may therefore possess detergent like quality. The role of ginseng as a biofilm inhibitor is yet to be seen and therefore is of interest.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic human pathogen. It is known for its versatile and ubiquitous nature. It can thrive on plant and animal tissue as well as in soil and marine habitats. *Pseudomonas aeruginosa* is important clinically because of its resistance to most antibiotics. It is a major cause of nosocomial infections. It accounts for about 80 percent of opportunistic infections by *Pseudomonads*. Patients that are immunocompromised, especially those with burns, cancer, cystic fibrosis etc., have a fatality rate of about 50 percent (Baron, 1996). Patients with respirators or with catheters are also at risk. *Pseudomonas* species cause infections such as endocarditis, pneumonia and infections of the urinary tract, central nervous system, eyes, ears, skin, and others.

Pseudomonas aeruginosa can be distinguished in various ways in the laboratory. It is a gram negative, aerobic bacillus. It is a non-fermenter, oxidase positive, beta-hemolytic and can grow at 42° C. It produces a bluish- green pigment called pyocynin on tryptic soy agar plate. It also has a fruity odor.

In the past couple of years, there have been many studies focusing on the virulence factors involved with *Pseudomonas aeruginosa* which fall under the quorum sensing systems that include elastase, rhamnolipid, LasA protease, alkaline protease, exotoxinA, etc. (Davey *et al.*, 2002; van Delden & Iglewski, 1998). Then there are cell associated virulence factors such as flagellum, pilus, and nonpilus adhesion (Figure 1).

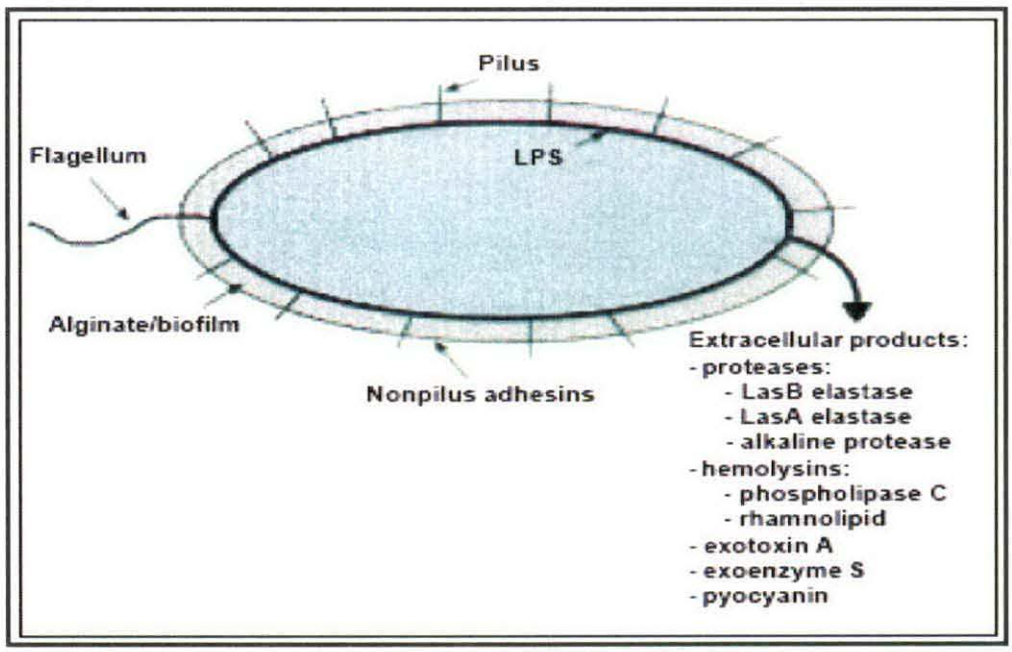


Figure1. Virulence factors of *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* has both cell-associated (flagellum, pilus, nonpilus adhesins, alginate/biofilm, lipopolysaccharide [LPS]) and extracellular virulence factors (proteases, hemolysins, exotoxin A, exoenzyme S, pyocyanin), (Van Delden and Iglewski, 1998).

The primary host defense mechanism against *Pseudomonas aeruginosa* is humoral immunity. Phagocytosis by polymorphonuclear leukocytes as well as antibodies to somatic antigens and exotoxins help in recovery from *Pseudomonas aeruginosa* infection (Baron, 1996). However, in patients with cystic fibrosis this mechanism appears to not clear the infection. Further, formation of *Pseudomonas aeruginosa* biofilms in the airway epithelia of cystic fibrosis patients exacerbates the problem.

Biofilms

One of the most distinctive features of *Pseudomonas aeruginosa* is its ability to produce biofilms. The first documented observation of biofilms was more then 70 years ago by Arthur T. Henrici. He described them as:

> "The slime from rocks, the mucous sheaths of colonial algae, scrapings from the leaves of submerged plants, all show a microbic flora rich in numbers and in diversity of forms. It is quite evident that for the most part the water bacteria are not free floating organisms, but grow upon submerged surfaces; they are of the benthos rather than the plankton"(Henrici, 1932).

Biofilms are formed in response to certain environmental signals where free-living independent bacteria grow into interdependent aggregate architectural colonies. They can persist in highly complex systems of intercellular interaction and communication to adjust to changing environmental signals. Biofilms can be formed by not only a single microbial species but also by multiple microbial species. They form on biotic as well as abiotic surfaces. Although there are more mixed-species biofilms in most environments, single-species biofilms also exist in a variety of infections and on the surface of medical implants. *Pseudomonas aeruginosa* is one of the most studied single species biofilms.

By analyzing *Pseudomonas aeruginosa* biofilm architecture under a scanning confocal laser microscope of a continuous flow slide culture it was found that the cells were most dense at their attachment surfaces and then became increasingly diffuse at the outer region. They were highly hydrated, open structures containing 73-98% extracellular materials and space (J.R. Lawrence *et al.*, 1991). Acylated homoserine lactone serves as an inducer for the development of these mushroom and pillar like structures which are made up of extracellular polymeric substance forming bacterial clusters (Figure 2). The life cycle of biofilms consists of attachment, proliferation, and quorum sensing. Quorum sensing is a type of cell-to-cell communication which is very important for the formation and growth of biofilms and will be discussed in the next section. Specific genes facilitate the attachment of cells to the surface and also to each other. These genes encode proteins that synthesize cell-to-cell signaling molecules like homeserine lactones, which begin extracellular polymeric substance formation. When bacteria receive nutritional signals and attach to the substratum, they start forming a biofilm by sending signals to other bacteria. For growth and nourishment of the microbial population, the biofilms trap nutrients and stay attached to cell surfaces. As we know, these attached bacteria excrete extracellular polymeric substance forming the structural architecture of the biofilm. The mature biofilms shed bacteria that then enter into a planktonic state (Serralta *et al.*, 2001). These bacteria that are shed do so in order to find other nourishment or to loosen the burden.

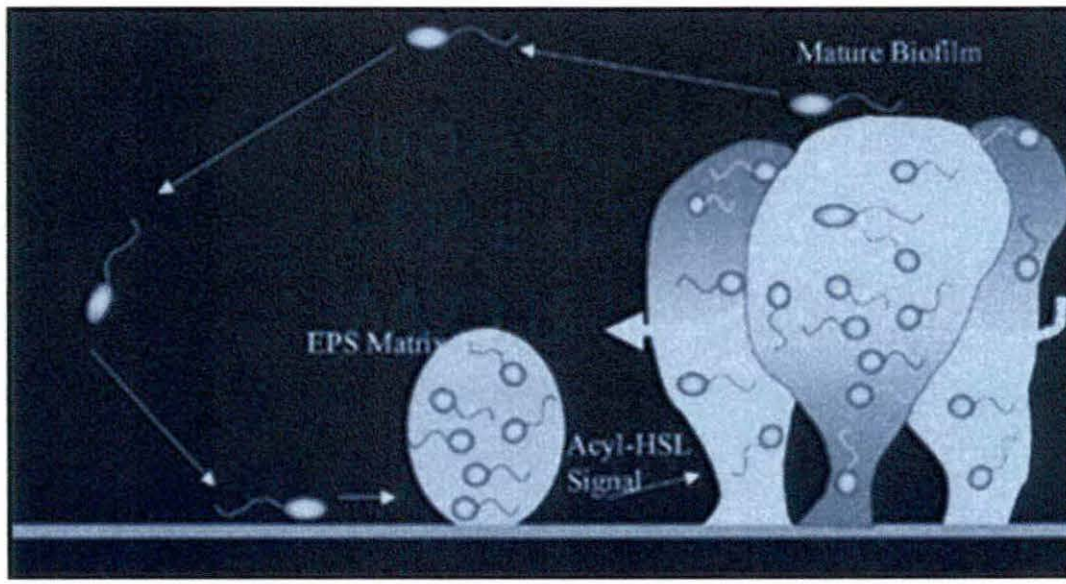


Figure 2. Schematic representation showing development of a *Pseudomonas aeruginosa* biofilm. The white arrow represents a water channel flowing in a void between the three areas that contain bacterial clusters (Serralta, *et al.*, 2001).

The biofilm formation specifically in *Pseudomonas aeruginosa* starts in response to nutritional signals, where they use their flagella for motility and when there is presence of a solid surface they start proliferating forming a monolayer. This monolayer then forms a cluster of cells called a microcolony. This requires the type-IV pili for a mode of surface translocation known as twitching motility. *Pseudomonas aeruginosa* requires twitching motility, type-IV pili and a set of chemotaxis-related genes like the quorum sensing las/rhl dependent genes to form the mushroom like complex architecture (Figure 3; O'Toole, 2000).

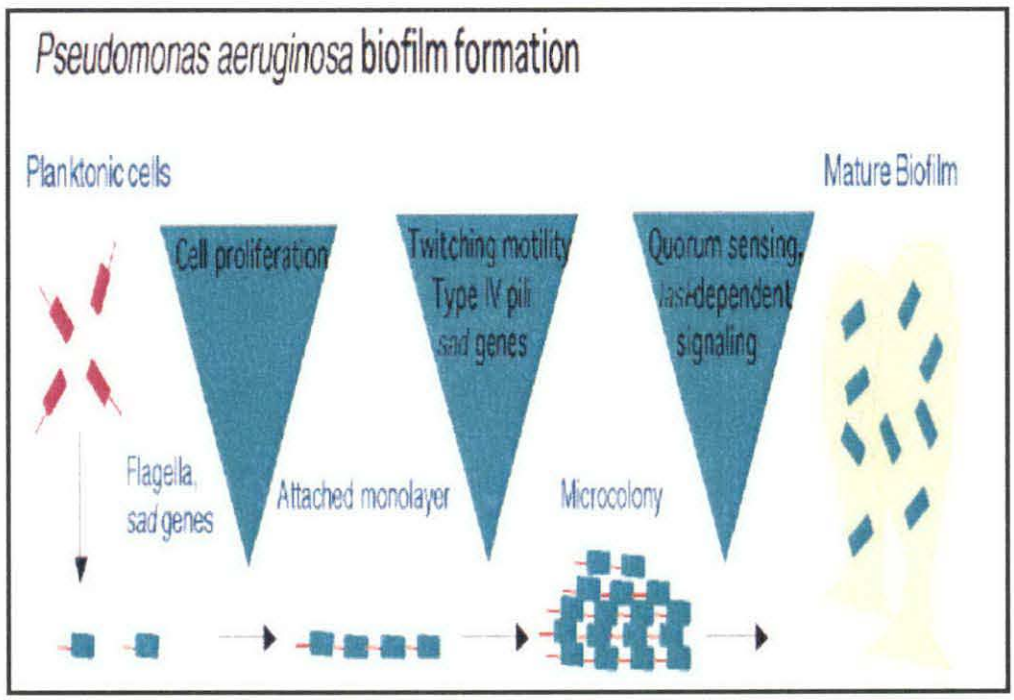


Figure 3. *Pseudomonas aeruginosa* biofilm formation and developmental pathways. Initial response to nutritional signals and in presence of a solid surface. There is formation of monolayer, which forms cluster of microcolonies. It uses type-IV pili for surface translocation known as twitching motility. Chemotaxis-related genes are required to develop the complex architecture and form mushroomlike structure (O'Toole, 2000).

To get a better understanding of the development of biofilm, Figure 4. shows an electron micrograph of *Pseudomonas aeruginosa* grown *in vitro.* Here they are in microcolonies. The cells are in close proximity to each other.

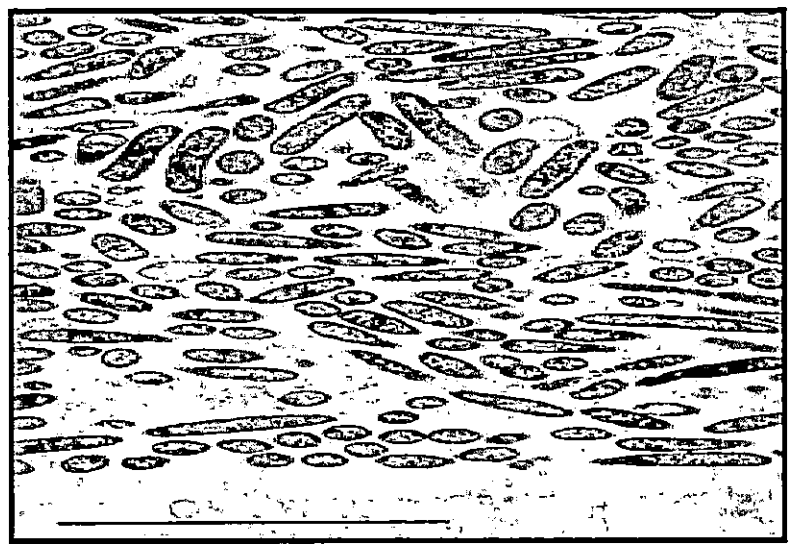
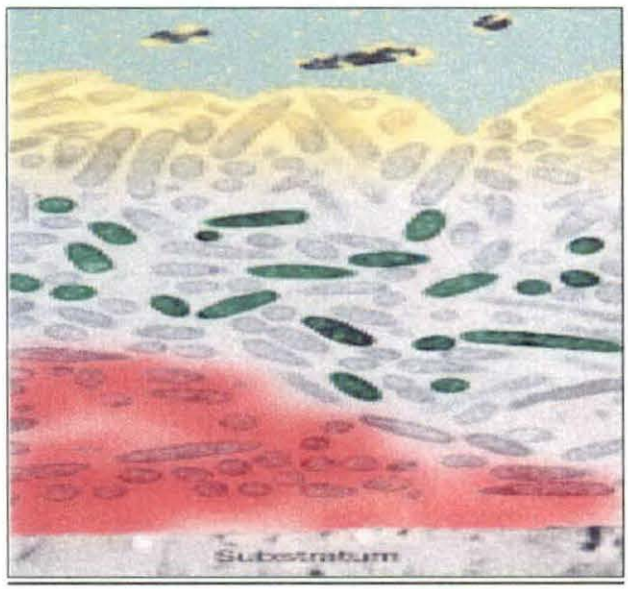


Figure 4. Electron micrograph of a laboratory-grown *Pseudomonas aeruginosa* biofilm. Bacteria live in multicellular clusters with individual cells in close proximity. The biofilm was grown on a plastic substratum (bottom). Bar=5 μm (Stewart and Costerton, 2001).

Biofilms have gained notoriety by evading our immune system, antibiotics and other antimicrobial agents. The main reason they are protected is that it is hard to penetrate them. Biofilms are not only found in the airway of patients with cystic fibrosis, but are also found in medical implants, urinary catheters and artificial joints. It has been estimated that about 10 million people a year in the United States alone are exposed to biofilm infections from *Pseudomonas aeruginosa* and other bacterial species (Madigan *et al.*, 2002). Biofilms have also been a nuisance in the

industrial sector, colonizing pipelines, submerged oil rigs, boats and other flowing systems. The need for biofilm control industrially and medically has lead to intensive research for antibiofilm agents.

The quorum sensing signal molecules of *Pseudomonas aeruginosa* are generated due to the expression of *lasI* and *rhlI*. After examining scanning confocal microscope images of *ΔlasI* mutant, it was determined that the biofilms were thinner than those of the wild-type under the same culture conditions. Extracellular polymeric substance in *Pseudomonas aeruginosa* biofilms could be influenced by the expression of *lasI* and *rhI* (Davies *et al.*, 1998). It has been hypothesized that the biofilm growth could lead to an early general stress response (GSR) which could be a major factor in bacterial antibiotic resistance. This general stress response is mediated by a sigma factor called RpoS which is regulated by quorum sensing (Brown and Barker, 1999). It is also thought that biofilm antibiotic resistance could be influenced by quorum sensing system because extracellular polymeric substance absorbs and/or deactivates biocides (Stewart and Costerton, 2001). In Figure 5, we see how antibiotics could be left ineffective in a biofilm environment. There have been three different hypotheses about how biofilms could render antibiotics useless: delayed penetration of the antimicrobial agent through the biofilm matrix, altered growth rate of biofilm organisms, and other physiological changes due to the biofilm mode of growth (Donlan and Costerton, 2002).



Slow penetration
Antibiotic (yellow0 may fail to penetrate beyond the surface layers of the biofilm

Resistant phenotype
Some of the bacteria may differentiate into a protected phenotypic state (green)

Altered microenvironment
In zones of nutrient depletion or waste product accumulation (red), antibiotic action may be antagonized

Figure 5. Three hypotheses for mechanisms of antibiotic resistance in biofilms. The attachment surface is shown at the bottom and the aqueous phase containing the antibiotic at the top (Stewart and Costerton, 2001).

Shih and Huang (2002) looked at the variable formation of antibiotic resistance by *Pseudomonas aeruginosa* PAO1 (wild type) and the quorum-sensing –deficient mutant, PDO100, JP1 and JP2. They found that the wild type began its maximum accumulation phase of biofilm formation immediately and reached plateau phase after 24 hr while the quorum sensing mutant lagged 36-48 hr before entering maximum –accumulation phase. They also inferred that the wild type biofilms were little affected by kanamycin, even at 100mg/L, whereas PDO100 biofilms were susceptible to the highest concentration of Kanamycin (100 mg/L) but not to lower concentrations (10 and 50 mg/L). In contrast, cells in JP1 and JP2

biofilms were susceptible to all three concentration of kanamycin (Shih and Huang, 2002). Figure 6 demonstrated more biofilm formation in the wild type.

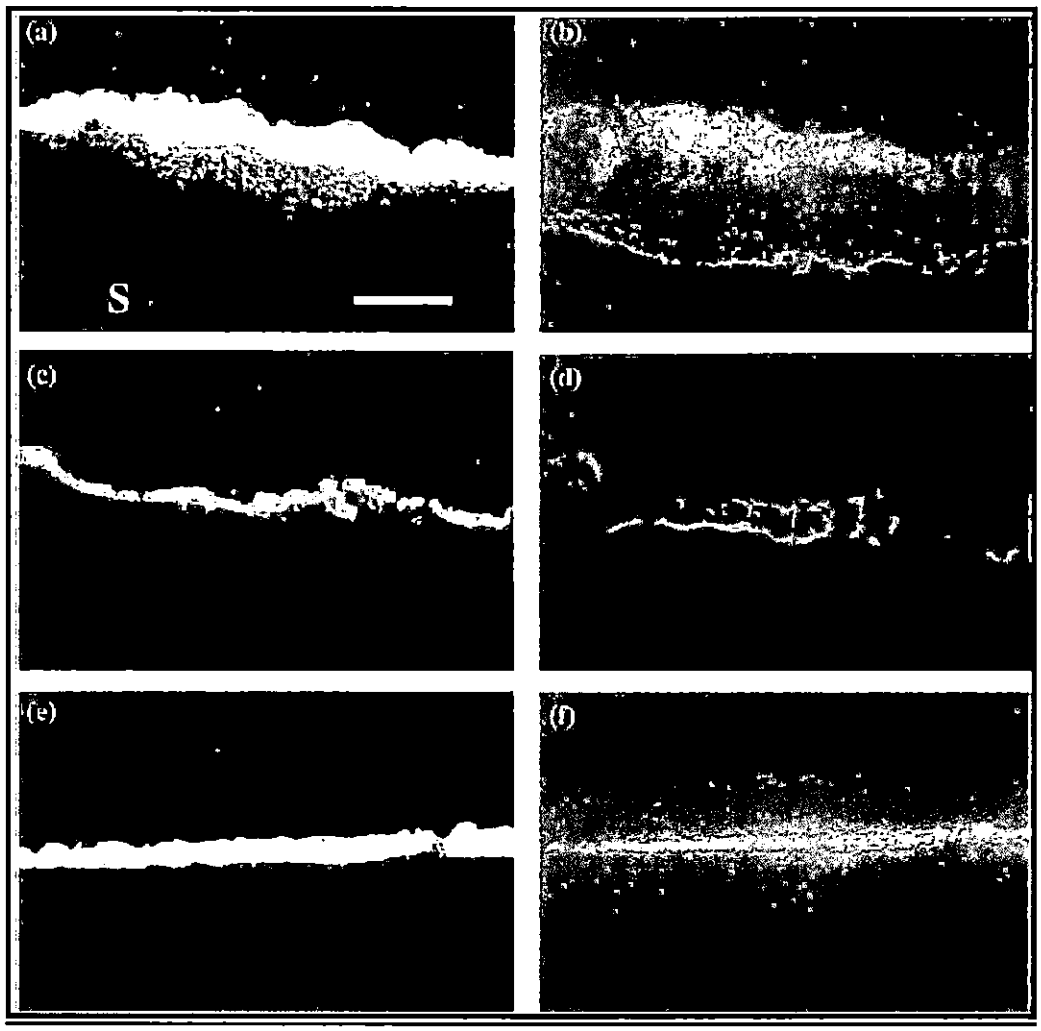


FIG. 6. Epifluorescence microgrpahs of *Pseudomonas aeruginosa* biofilm. Cross-sections stained by ethidium bromide and calcoflour white: (a and b) PAO1; (c and d) PDO100; (e and F) JP1. Substratum; bar=50 um (Shih and Huang, 2002).

Quorum Sensing

The individual bacterial cells interact with each other by cell to cell communication which requires a quorum of cells. Quorum sensing is a mechanism through which *Pseudomonas aeruginosa* and other microorganisms regulate virulence factor gene expression in a cell density-dependent manner. *Pseudomonas aeruginosa* are able to sense their environment and react accordingly. Cell-to cell signaling was first described in the marine bacteria *Vibrio fischeri* lux system (Fuqua *et al.*, 1996). In gram-positive bacteria, peptide pheromones are used as signals, while in most gram negative bacteria a small molecule called an autoinducer is used (Van Delden and Iglewski, 1998). These autoinducers are homserine lactone-based molecules that differ between one another in length and substitutions on their acyl side chains. The two known quorum sensing systems in *Pseudomonas aeruginosa* are the *las* system and the *rhl* system (Pesci *et al.*, 1997). Each of these systems contains *LasI* or *RhlI* homoserine lactone synthase and regulator protein LasR or RhlR, respectively (Latifi *et al.*, 1996; Pearson *et al.*, 1997). They help in modulation of gene transcription and their cognate activators, N-(3-oxododecanoyl)-L-homoserine lactone (C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) (van Delden *et al.*, 1998; Gambello, 1991).

Both of these systems are interlinked with each other with the *las* system seems to be dominant over the *rhl* system (Figure 7). The *las* quorum sensing

system consists of the transcriptional activator protein *LasR* and the *Pseudomonas* autoinducer PAI-1[*N*-(3-oxododecanoyl)-L-homoserine lactone].

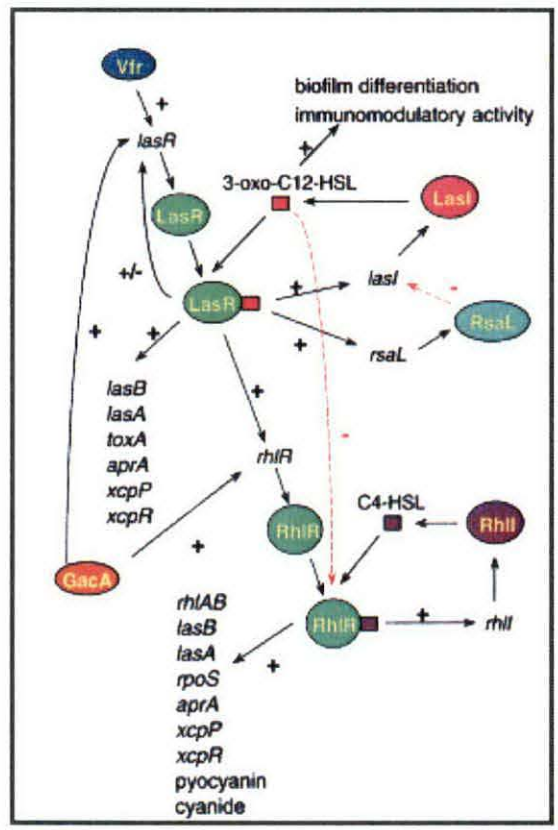


Figure 7. The cell-to-cell signaling circuitry of *Pseudomonas aeruginosa.*
The las cell-to-cell signaling system controls the rhl cell-to-cell signaling system in a hierarchy cascade. The LasR/3-oxo-C12-HSL complex activates the transcription of rhlR, and 3-oxo-C12-HSL blocks the activation of RhlR by C4-HSL.The las system itself is controlled positively by Vfr and GacA, and negatively by RsaL. 3-oxo-C12-HSL is required for biofilm differentiation and has immunomodulatory activity. Both cell-to-cell signaling systems regulate the expression of numerous genes (lasB: LasB elastase, lasA: LasA elastase, toxA: exotoxin A, aprA: alkaline protease, xcpP and xcpR: genes of the xcp secretory pathway, rhlAB: rhamnosyltransferase required for rhamnolipid production, rpoS: stationary phase sigma factor, (Van Delden and Iglewski, 1998).

During high density growth, PAI-1 reaches a threshold concentration and forms a complex with LasR. This complex in then converted to a transcriptional activator which has shown to be involved in transcription of *lasI, lasB, toxA* and *apr*. In fact, *LasR* and PAI-1 regulate the expression of *rhlR* by serving as transcriptional activators of *rhlR*. The *rhl* quorum–sensing system consists of the transcriptional activator protein RhlR and the autoinducer PAI-2 (*N*-butyryl-L-homoserine lactone). The *rhl* quorum sensing has so far shown to be involved in the transcription of *rhlA*, *rhlI*, *lasB*, and *rpoS* (Van Delden and Iglewski, 1998).

Cell-to-cell signaling systems may be responsible in enabling *Pseudomonas aeruginosa* to overcome host defense mechanisms. When extracellular virulence factors are produced by a small number of bacteria, they may be able to produce a host response which would be enough to neutralize these compounds. However, if an entire bacterial population expresses virulent genes at a certain density at a high level then they could overcome the host defense (Figure 8). These factors could lead to increase in bacterial toxins which would then lead to invasion of blood vessels, dissemination, systemic inflammatory-response syndrome, and finally death. Even appropriate antibiotic therapies are often unable to stop this course; therefore, the process must be blocked early, before virulence gene expression can be coordinated (Van Delden and Iglewski, 1998).

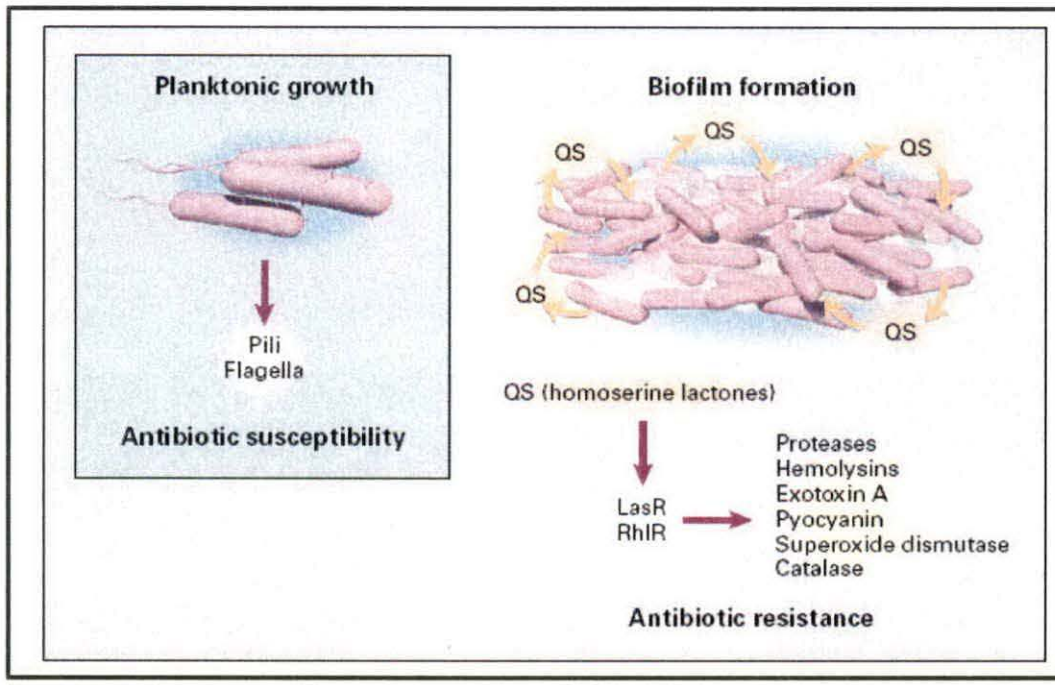


Figure 8. The formation of a biofilm by *Pseudomonas aeruginosa.* In the normal host, inadvertently inhaled bacteria are cleared by the innate defenses of the airway. Under low bacterial density the expression of quorum sensors (QS), which are diffusible homoserine lactones, is negligible and planktonic growth (left-hand panel) predominates. Lactoferrin actively protects against the formation of a biofilm by blocking the primitive motility system of *P. aeruginosa.* Under conditions of high bacterial density (right-hand panel), quorum sensors are secreted by the bacteria and freely diffuse within the bacterial community. Quorum sensors interact with transcriptional activators LasR and RhlR to direct the expression of several factors that facilitate the persistence of bacteria in the lung, such as proteases, hemolysins, exotoxin A, pyocyanin, superoxide dismutase, and catalase, and thus enable the organisms to evade the effects of antibiotics (Rashid *et al.*, 2000).

In another study, the exoenzyme S regulon of *Pseudomonas aeruginosa* was examined. This regulon is comprised of genes for a type III secretion system and for four anti-host effector proteins (ExoS, T, U and Y), which are translocated into host cells. For the first time *RhlR/RhlI* and *RpoS* were linked with the expression of

the anti-host effector *ExoS*, part of the exoenzyme S regulon. The data also suggested that the exoenzyme S regulon may be downregulated during *Pseudomonas aeruginosa* biofilm formation (Hogardt, *et al.*, 2004).

The exact nature of the increased resistance in biofilms is unclear but has been attributed to slow growth, penetration barriers, β-lactamase production, and other factors. *P. aeruginosa* also produces other less well-defined biofilms essential in the colonization of indwelling devices such as catheters. The *las* cell-to-cell signaling system has been shown to be involved in the differentiation of *Pseudomonas aeruginosa* biofilms (Davies *et al.*, 1998). A mutant defective in the production of 3-oxo-C12-HSL formed an abnormal biofilm that, in contrast to the wild type biofilm, was sensitive to low concentrations of the detergent sodium dodecyl sulfate (SDS). Furthermore, the addition of 3-oxo-C12-HSL to the culture media restored production of a differentiated, SDS-resistant biofilm by the mutant. Whether the formation of an undifferentiated biofilm renders this mutant more sensitive to antibiotics is still unknown. Also unclear is whether 3-oxo-C12-HSL is required for the differentiation of alginate biofilms. The link between 3-oxo-C12-HSL and biofilm differentiation highlights the broad range of systems controlled by cell-to-cell signaling in *Pseudomonas aeruginosa* (Davies *et al.*, 1998).

Due to the fact that quorum sensing regulates an array of *Pseudomonas aeruginosa* factors and its deletion attenuates the virulence of these bacteria, quorum sensing may become an ideal target for alternate therapy in these multi

drug resistant bacteria. The mutant was also shown to be aberrant in quorum sensing responses in that production of the quorum-sensing controlled virulence factors elastase and rhamnolipid are severely reduced.

In patients with cystic fibrosis *Pseudomonas aeruginosa* gives rise to a persistent infection. *Pseudomonas aeruginosa* selects for the mucoid producing exopolysaccharide which grow into a biofilm. This protects the bacteria from the patient's immune system by protection against phagocytosis and complement activity as well as external source of antibiotics like aminoglycosides, β-lactam antibiotics, fluoroquinolones and disinfectants. In one study, *Pseudomonas aeruginosa* biofilms were examined in responses to environmental stimuli as a means of studying gene expression and physiology of biofilm bacteria. Bollinger *et al., (2000)* focused on oxidative stress response because the knowledge of antioxidant responses in this organism is firmly grounded genetically and physiologically and that antioxidant enzymes like KatA and KatB (present during the growth phase) are of central importance to the pathogenicity of *Pseudomonas aeruginosa*. The main components of the oxidative stress response are regulated by quorum sensing and it exerts its effects when cell densities are high, a condition found in biofilms that can lead to localized areas of high nutrient demand. They hypothesized that nutrient limitation may also be an important factor to consider in studies aimed at understanding quorum sensing and biofilm biology. By using quorum sensing mutants, they demonstrated that the nutritional status of the cell

must be taken into account when one is evaluating quorum sensing -based gene expression. In the biofilm mode of growth, quorum sensing may also have negative regulatory functions. Quorum sensing based gene regulation models based on studies with planktonic cells need be modified in order to explain biofilm gene expression behavior, as gene expression in biofilms is dynamic (Bollinger, *et al.*, 2000). All these results from different experiments emphasize that quorum sensing is an integral part of biofilm formation.

Cystic Fibrosis

Quorum-sensing systems may play an active role in the *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. Cystic fibrosis is an inherited chronic and autosomal recessive disease that is caused by a mutation in a single gene on chromosome 7. It affects the lungs and digestive systems of about 30,000 children and adults in the United States with about 1,000 new cases diagnosed each year. The incidence in Caucasian populations is approximately 1 in 2,500 live births and a carrier frequency of 1 in 25. The gene involved was identified in 1989 and it codes for a protein called the cystic fibrosis transmembrane conductance regulator (CFTR). The gene, on the long arm of chromosome 7, is 250 kb long, contains 27 exons, and encodes a transmembrane protein of 1,480 amino acids, the CFTR. The protein CFTR is produced in a number of tissues throughout the body, regulating the movement of salt and water in and out of these cells. CFTR functions as a chloride ion channel protein and belongs to a large family of transmembrane

proteins that also includes P glycoprotein. Patients who are homozygous for mutant alleles of the CF gene have severe defects in chloride ion transport and have a characteristically salty sweat. This imbalance can also be detected in the cells lining internal organs which lead to a buildup of sticky dehydrated mucus in male sex ducts, ducts of the pancreas, and the airways of the lungs. These glands behind the ducts continue to secrete, causing them to swell and form cysts (Govan and Deretic, 1996). When the gene coding for this protein is abnormal, it alters the protein. This alteration leads to thick mucus secretion which clogs the bronchial tubes in the lungs and plugs the exit passages from pancreas and intestines, leading to loss of function of these organs. Due to this production of mucous the airway passages are blocked which is inviting to bacterial infections. *Pseudomonas aeruginosa* can cause chronic pulmonary infection. About 80 to 95% of patients with cystic fibrosis have fatalities resulting from respiratory failure brought on by chronic bacterial infection and airway inflammation (Lyczak, *et al.*, 2002).

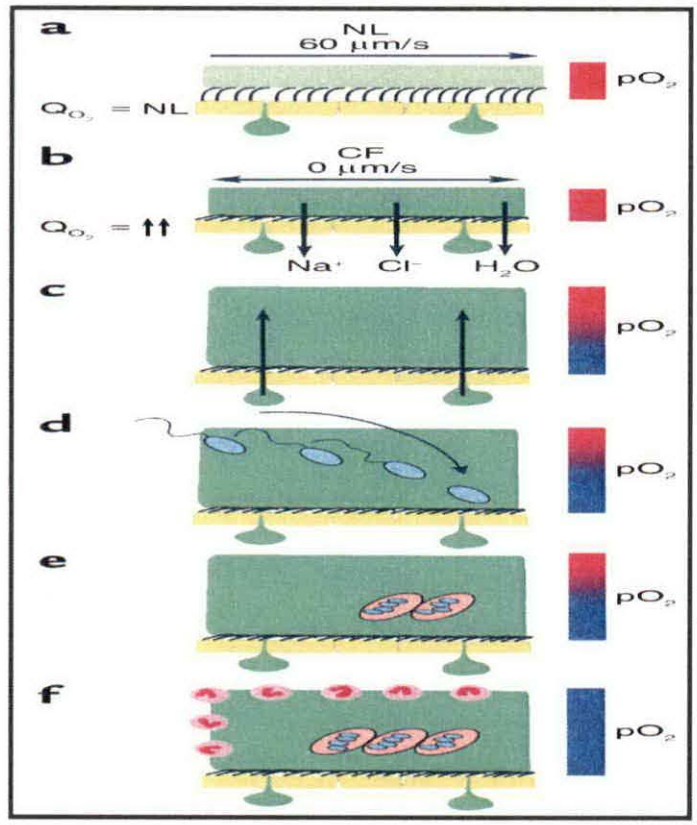


Figure 9. Model of the pathogenic events hypothesized to lead to chronic *P.aeruginosa* infection in airways of CF patients. (a) On normal airway epithelia, a thin mucus layer with low-viscosity periciliary layer (PCL) facilitates efficient mucociliary clearance. A normal rate of epithelial O2 consumption. (b–f) CF airway epithelia. (b) Excessive CF volume depletion removes the PCL, mucus becomes adherent to epithelial surfaces, and mucus transport slows /stops (bidirectional vector). (c) Persistent mucus hypersecretion (denoted as mucus secretory gland/goblet cell units; dark green) with time increases the height of luminal mucus masses/plugs. (d) *P. aeruginosa* bacteria deposited on mucus surfaces penetrate actively and/or passively (due to mucus turbulence) into hypoxic zones within the mucus masses. (e) *P. aeruginosa* adapts to hypoxic niches within mucus masses with increased alginate formation and the creation of macrocolonies. (f) Macrocolonies resist secondary defenses, including neutrophils, setting the stage for chronic infection. The presence of increased macrocolony density and, to a lesser extent neutrophils, render the now mucopurulent mass hypoxic (blue bar). (Worlitzsch, *et al.*, 2002)

Figure 10 shows a scanning electron micrograph of cystic fibrosis respiratory epithelium infected with *Pseudomonas aeruginosa.*

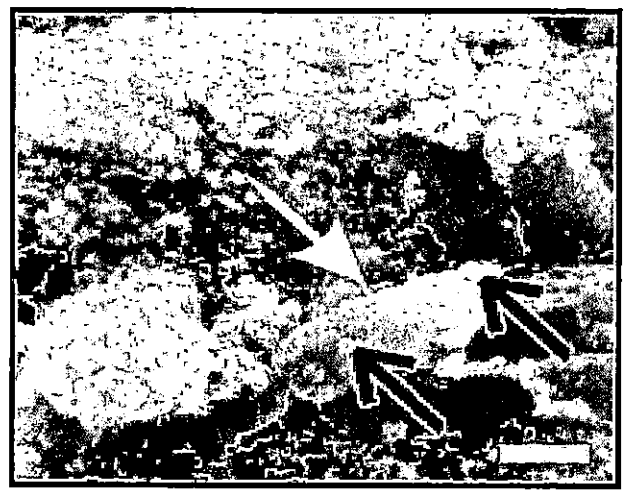


Figure 10. Scanning electron micrograph of mucus coated spheroid derived from CF respiratory epithelium. *P. aeruginosa* (white arrow), were enmeshed in mucus (black arrows) following 2-hour incubation (Worlitzsch, *et al.*, 2002).

The CFTR protein, along with being a chloride ion channel and regulator of other ion channels, is also a receptor for epithelial cell internalization of *P. aeruginosa* on the airway surface (Golberg and Pier, 2000). The innate host immunity could come into play in a normal, healthy lung, after epithelial cells uptake *P. aeruginosa* by airway epithelial cells. The binding and internalization events could initiate cellular signaling pathways that lead to apoptosis, which may serve as a mechanism of removing the bacteria-laden epithelial cells from the airway surface such that they can be expectorated and swallowed. Also, these apoptotic bodies derived from these bacteria laden epithelial cells may be

phagocytosed by dendritic cells (Rubertelli, *et al.*, 1997) leading to presentation of bacterial antigens to T cells (Figure 11).

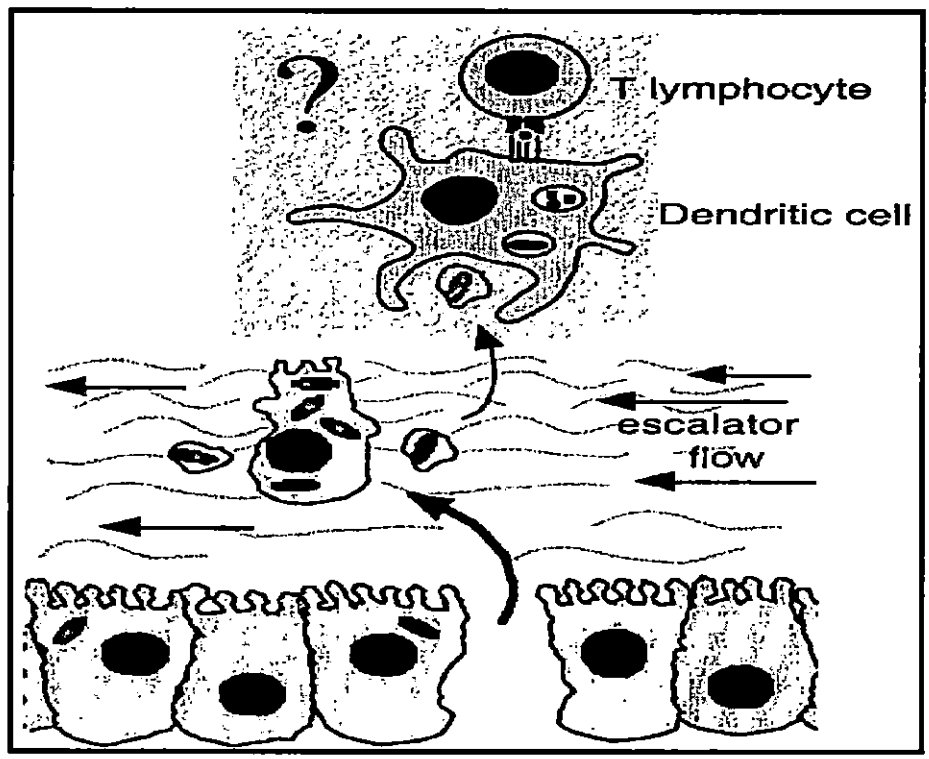


Figure 11. CFTR-dependent internalization of *Pseudomonas aeruginosa* causes apoptosis and dequamation of bacteria-laden epithelial cells. In the normal airway, theses apoptotic cells and the bacteria they contain are probably removed via the mucociliary escalator. In the CF airway, this clearance mechanism does not function normally due to inefficient internalization of bacteria stemming from a lack of CFTR protein. It is possible that apoptotic bodies derived from desquamated epithelial cells are later phagocytosed by dendritic cells for subsequent proceeding and presentation of bacterial antigen to T lymphocytes (Lyczak, *et al.*, 2002).

Early isolates of *Pseudomonas aeruginosa* appear much like environmental isolates in their phenotype (non-mucoid); later isolates are more resistant to antibiotics and frequently mucoid. With time, they change into alginate-producing

mucoid biofilms. Alginate is an exopolysaccharide which is secreted by mucoid *Pseudomonas aeruginosa*, giving rise to their appearance. Alginate contains O-acetylated linear polymers of D-mannuronate and L-guluronate residues (Evans & Linker, 1973). These alginate producing strains are responsible for poor lung function in cystic fibrosis patients. Alginate production has also shown to slow host immune clearance in animals which could lead to tissue damage and survival in the lung (Boucher, *et al.*, 1997; Song *et al.*, 2003).There are three main proposed ideas for the pathogenic roles of alginate, in the form of a mucoid bacterial coating or as a free substance, and they are (i) the mucoid capsule-like material serves as a direct barrier against phagocytic cells and effective opsonization; (ii) alginate may function as an immunomodulatory molecule; and (iii) alginate production may play a role in biofilm-related phenomena, including contribution to adhesion and antibiotic resistance (Govan and Deretic, 1996).

One of the molecular mechanisms for the constitutive expression of the exopolysaccharide alginate has been discovered and involves the alternative sigma factor, AlgU. Alginate is produced in copious amounts by transcriptional activation of the regulatory protein AlgR and its subsequent upregulation of the 12 alginate biosynthetic genes (*algD* through *algA*). AlgR directly represses the Rhl quorum-sensing circuit in a biofilm-specific manner (Morici, *et al.*, 2007).

There are distinct genetic strains of *Pseudomonas aeruginosa* that colonize the upper and lower airway of cystic fibrosis patients (Burns *et al.*, 2001). Once

Pseudomonas aeruginosa biofilms are established, they are almost impossible to treat, with a combination of several classes of antibiotics, including aminoglycosides, β -lactams, and tetracyclines. These strains can produce β-lactamase in order to produce a host immune response to the action of β-lactam antibiotics. Due to *Pseudomonas aeruginosa's* intrinsic resistance, cystic fibrosis patients have a recurring infection which can cause destruction of lung tissue. Patients with cystic fibrosis do mount a significant humoral response to *Pseudomonas aeruginosa* antigens, and there can be significant antibody response to surface polysaccharides and exoproducts. This initial antibody response to surface proteins may actually be an indicator of chronic *Pseudomonas aeruginosa* infection (Burns, *et al.,* 2001). The antibiotic resistant phenotypic variants of *Pseudomonas aeruginosa* that have the ability to form biofilms arise at high frequency both *in vitro* and in the lungs of CF patients. A regulatory protein called phenotype variant regulator (PvrR) that controls the conversion between antibiotic-resistant and antibiotic-susceptible forms has been identified. It directs gene expression in response to specific environmental conditions. The overexpression of this single locus resulted in antibiotic-susceptible strains with a decreased propensity to form biofilms. The finding of this single locus helped in understanding that there is a correlation between increased ability to form biofilms and multidrug resistance. Compounds that affect PvrR function could have an important role in the treatment of CF infections. The activation of regulatory

elements like PvrR could prevent biofilm formation and render bacteria more susceptible to antimicrobial agents (Drenkerd and Asubel, 2002).

To understand the importance of quorum sensing in chronic *Pseudomonas aeruginosa* lung infection, an *in vivo* study was done to see the pathogenic effects of the wild-type *Pseudomonas aeruginosa* PAO1 and its double mutant, PAO1 *lasI rhlI*, in which the signal generating parts of the quorum sensing systems that were defective were compared. A rat model of *P. aeruginosa* lung infection was used in the present study. The results of this study showed that during the early stages of infection, the PAO1 double mutant induced a stronger serum antibody response compared to its wild-type counterpart. On days 14 and 28 post-infection, significantly milder lung pathology, a reduced number of lung bacteria, but increased lung interferon γ production were detected in the group infected with the PAO1 double mutant when compared with the PAO1-infected group (Hong Wu *et al.*, 2001).

Even after testing positive for *Pseudomonas aeruginosa,* some individuals show no clinical or bacteriologic response to appropriate microbial therapy. Therefore chloramphenicol and polymyxin B sulfate therapy could be prescribed by some experienced clinicians. These drugs are not generally used by the rest of the medical community but they do occasionally induce clinical responses. The failure to eradicate susceptible bacteria from the lungs of patients with cystic fibrosis, who already have a large bacterial burden (108 to 109 colonyforming units per

milliliter), can result not only in selection for and persistence of multidrug-resistant organisms but also in infection with exotic opportunists, such as *Achromobacter xylosoxidans, Stenotrophomonas maltophilia,* and *Burkholderia cepacia* complex (Prince, 2002).

Ginseng

Ginseng is a medicinal herb that has been used as a traditional Chinese medicine for more then 4,000 years to treat various diseases (Figure 12). It consists of more then 70 identified substances and some other active substance classified as ginosenosides, (Kharazmi, *et al.*, 2004). These ginosenosides are glycosylated steroids. The three most commonly used ginseng are Asian or Korean ginseng (*Panax ginseng* C.A. Meyer [Araliaceae]), American ginseng (*Panax quinquefolius* L.), and Siberian ginseng, "eleuthero" (*Eleutherococcus senticosus* Maxim. [Araliaceae]; (Block and Mead, 2003). *Panax ginseng* is the most and best studied species. In *Panax ginseng*, 36 different ginsenosides and many minor constituents (essential oils, phytosterols, amino acids, peptides, vitamins, and minerals) have been extracted and isolated from the root, stem, and leaves (Wilkie, 1994). Today, in the western world, this herb has been incorporated in many studies as it has substantiated its capability to treat different diseases. There are more then 300 scientific papers that have been published on ginseng. Ginseng has been shown to be an immunostimulant, antioxidant, vasodialator etc. It has been demonstrated that ginseng can enhance the clearance of bacteria from rat lungs, reduce lung abscesses

and result in a shift of the inflammatory type from a Th2-like to a Th1-like response (Song, *et al.*, 1997). It has also been demonstrated that ginseng modulates interleukin-12 production to induce this Th1 response which results in a better protection against this infection (Larsen, *et al.*, 2004).

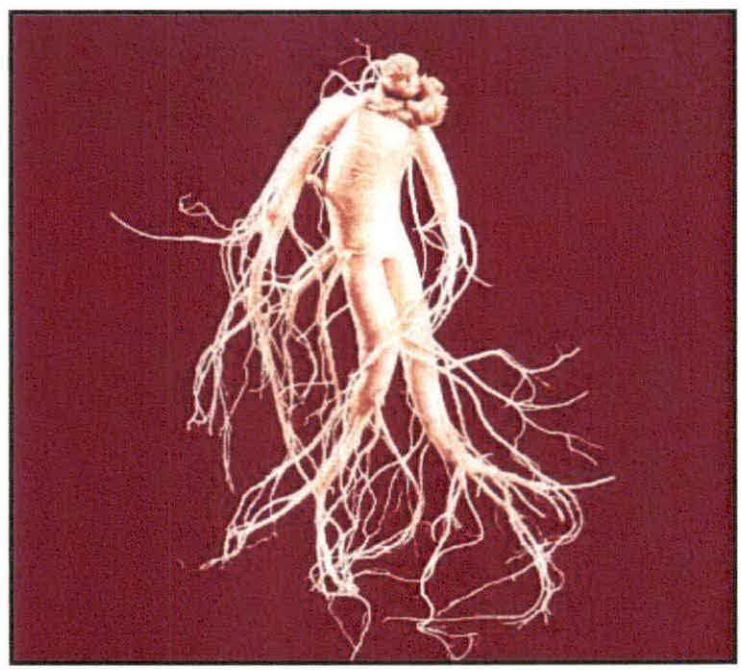


Figure 12. Fresh *Panax ginseng* C.A Meyer (Taik-Koo Yun, 2001).

The disruption of biofilm formation is one of the most important weapons in trying to eradicate *Pseudomonas aeruginosa* infection from the lungs of cystic fibrosis patient. Because of the multi drug resistant nature of these bacteria, alternate therapies have been explored. Use of ginseng has shown evidence of bacterial clearance *in vivo*. By analyzing the effect of ginseng during biofilm formation, we can get an insight into whether it could have a role as a biofilm

inhibitor as it has already shown to clear bacterial load by initiating a Th-1 like response. Using the quorum sensing mutant PDO100 and PJP-1, it can be seen if ginseng has an effect on the quorum sensing. Because quorum sensing was shown previously to be involved in biofilm differentiation, these findings have important implications for the design of biofilm prevention and eradication strategies.

Objective

To study the *in vitro* effect of ginseng on biofilm formation by three strains of *Pseudomonas aeruginosa,* PAO1 (wild type), PJP1 (Δ *lasI* mutant) and PDO100 (Δ *rhlI* mutant).

Statement: *Pseudomonas aeruginosa* when exposed to ginseng exhibit inhibition in biofilm formation.

Significance

Patients with cystic fibrosis are predominantly infected with the pathogen *Pseudomonas aeruginosa*, which results in a chronic lung infection associated with progressive pulmonary insufficiency. The Chinese herbal medicine ginseng has been shown to significantly improve bacterial clearance from the lungs of a rat model with a chronic *Pseudomonas aeruginosa* pneumonia mimicking that of a patient with CF. Two weeks after the challenge with *P. aeruginosa*, the ginseng-treated group showed less severe lung pathology, lower lung abscess incidence, and fewer mast cell numbers in the lung foci. The findings indicated that ginseng treatment of experimental *P. aeruginosa* pneumonia in rats promoted a cellular

response resembling a TH1-like response (Song, *et al.*, 1997). Using ginseng *in vivo* has shown to have an immunological response. However, there haven't been *in vitro* studies done to show the effect of ginseng on the bacteria without an immune response factor. This experiment will analyze if there is inhibition of bacterial biofilm *in vitro*. If there is inhibition of the bacterial biofilm with ginseng then several classes of antibiotics, including aminoglycosides, β -lactams, and tetracyclines would be able to be more effective in clearing the infection because it is easier to fend off the bacteria when there is minimal biofilm formation. The use of quorum sensing mutants PJP1 (Δ *lasI* mutant) and PDO100 (Δ *rhlI* mutant) in comparison with PAO1 (wild type) will show the relationship between biofilm formation and quorum sensing. It will reveal if there is a significant difference between the strains when different concentration of ginseng is used. It will also reveal if ginseng is able to target the quorum sensing genes.

The use of ginseng in this study could show ginseng as an inhibitor of biofilm formation along with being an immunologically important agent as shown in other studies. Ginseng may have the potential to be a promising natural medicine, in conjunction with other forms of treatment, for CF patients with chronic *P. aeruginosa* lung infection.

Materials and Methods

Strains and growth conditions

Pseudomonas aeruginosa strains PAO1 (wild type), PAO-JP1 (Δ *lasI)* and PDO-100(Δ *rhlI*) all with GFP were used in this study (University of Washington, Seattle, Microbiology Dept.). The growth medium for this consisted of tryptic soy broth (TSB). All the strains were grown at 37° C for 1, 3, 7 and 10 days utilizing three different concentrations of ginseng: 4.7 μg/mL, 150 μg/mL and 4800 μg/mL.

The ginseng (Figure 13) was bought from a local Walmart. It was manufactured by Nature's Bounty, Inc. It contains 7% of ginsenocides. This ginseng was a root extract. The other ingredients listed include maltodextrin, gelatin, cellulose (plant origin), silica, vegetable magnesium stearate. Ginsenocides are chemicals specific to ginseng which are closely related saponins. A stock solution of ginseng was made by weighing out 4.8 mg of ginseng powder which was removed from the gelatin capsule. This 4.8 mg of ginseng was then dissolved in 100 mL of distilled water. This was then sterilized by vacuum sterilization using Nalgene disposable filter unit which was gamma irradiated and had 20 μm-diameter pores. The ginseng concentration of 150 μg/mL was calculated from a previous study (Song, *et al.*, 2002) where 150mg/kg of body weight (rat model) of ginseng was injected subcutaneously. The ginseng used in Song, *et al.*, (2002) study was bought from Denmark, however it was also the root extract of *Panax* Korean ginseng. To see the effect of ginseng at lower and higher concentrations

then 150 µg/mL, a ± five fold concentration was used. After that the different concentrations of ginseng were made by a doubling dilution (Table 1).

Table 1. Doubling dilutions for different concentrations of ginseng. The concentrations in bold were the three concentrations used in all the experiments.

Tube #	1	2	3	4	5	6	7	8	9	10	11
Dilution	1/1	½	1/3	¼	1/5 /	1/6	1/ 7	1/8	1/9	1/1 0	1/1 1
Concentration s µg/ml	**480 0**	240 0	120 0	60 0	30 0	**15 0**	75	37. 5	18.7 5	9.3 7	**4.7**

Figure13. Korean Ginseng (Spring Valley).

The three different strains of *Pseudomonas aeruginosa* were grown on tryptic soy agar plates at 37° C for 24 hours. As shown in Figure 14 PAO1 expresses pyocyanin, which is the bluish green pigment within 24 hours of incubation. Pyocynin has been shown to be positively regulated by the *rhl* system Ochsner and Reiser, 1995) All three strains were mucoidy on the plate.

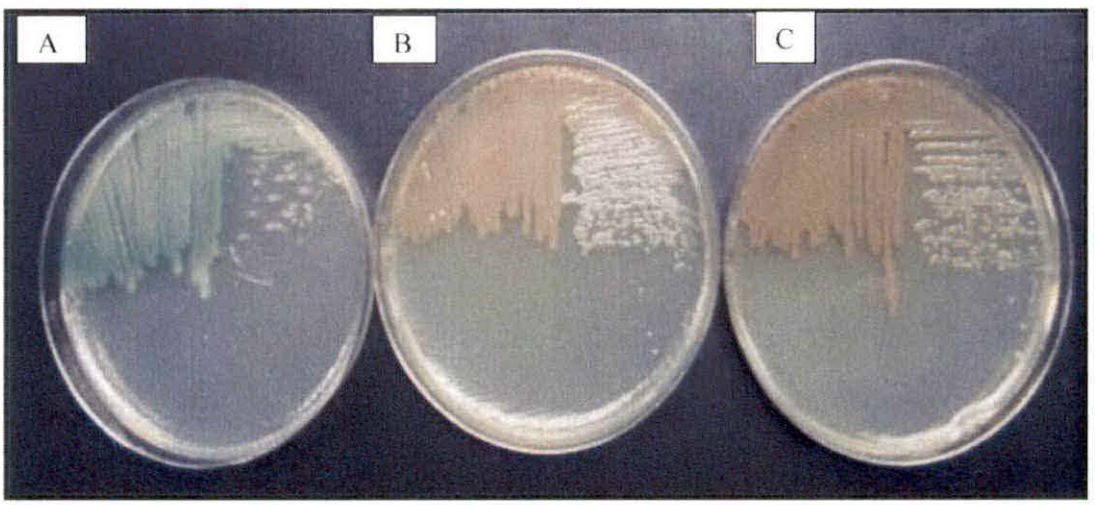


Figure 14. *Pseudomonas aeruginosa* strains on TSA. (A) PAO1 (wild type) (B) PDO-100(Δ *rhlI*) and (C) PJP1 (Δ *lasI*), all with GFP after 24 hours incubation on TSA plates.

Isolated colonies from the TSA plate cultures were then inoculated in 4- mL TSB tubes and incubated in a shaking incubator (Figure 15). It took 6-7 hours for all the strains to reach an optical density at 595nm (OD_{595}) of 0.4 - 0.6. At this optical density bacteria are in a log phase growth i.e., they grow exponentially or logarithmically where the rate of increase in cell number is a multiplicative function of cell number.

The bacteria at log phase growth are shown in Figure 15.

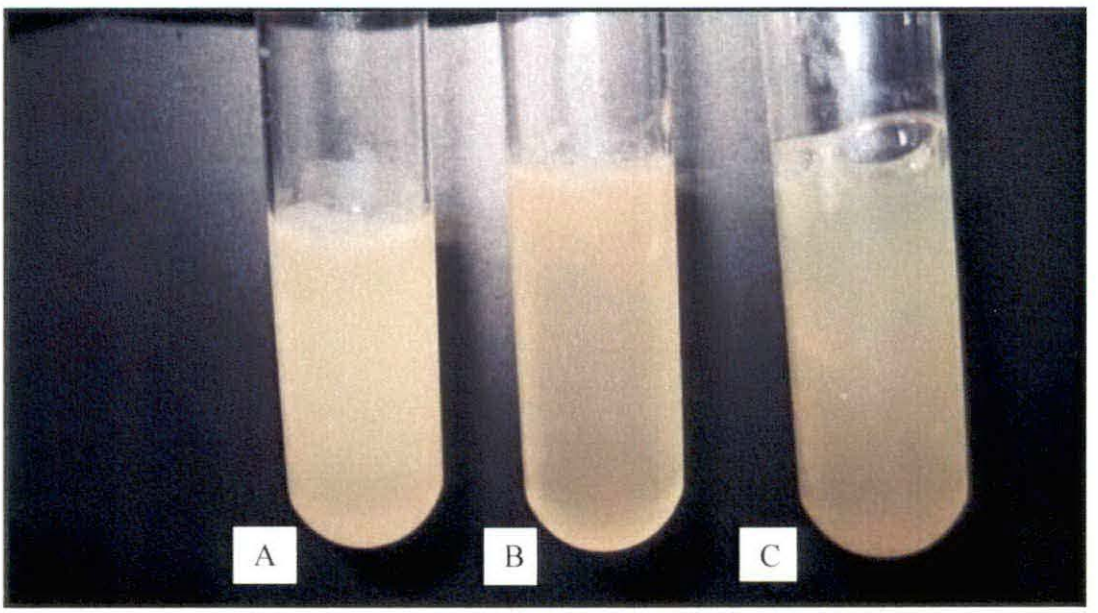


Figure 15. *Pseudomonas aeruginosa* strains in TSB. (A) PAO-JP1 (Δ *lasI*), (B) PDO-100(Δ *rhlI*) and (C) PAO1all with GFP after incubation on TSB.

Scanning electron microscopy

Scanning electron microscopy (SEM) is used to study three dimensional features of individual cells and even whole organisms. SEM (Figure 16) has been not only used in the field of biology but also in the electronics industry. The lenses of the SEM are used to generate a demagnified, focused spot of electrons that is scanned over an electrically conductive specimen. When these electrons strike the specimen, they give rise to a variety of signals which also include low energy secondary electrons from the uppermost layers of the specimen. Some of the

secondary electrons are collected, processed and eventually translated as a series of pixels on a cathode ray tube or monitor. For each point where the electron beam strikes the specimen it generates secondary electrons. A corresponding pixel is displayed on the viewing monitor. The brightness of the pixel depends on the number of secondary electrons generated from the specimen surface. The three dimensional appearance of the SEM images is due to differences in contrast between various structural features of the specimen when they are displayed on the viewing monitor. Contrast arises when different parts of the specimen generate differing amounts of secondary electrons when the electron beam strikes them. There are also backscattering electron which could be used to generate an image. Secondary electrons are used to generate an image based on topographic contrast, whereas backscattering electrons are not normally used to study topographies. Instead, contrast is based on detecting areas of different atomic numbered elements. However, in biological sciences most of the elements present in specimen are of relatively low atomic number and yield few backscattered electrons. Therefore, here secondary electrons are used.

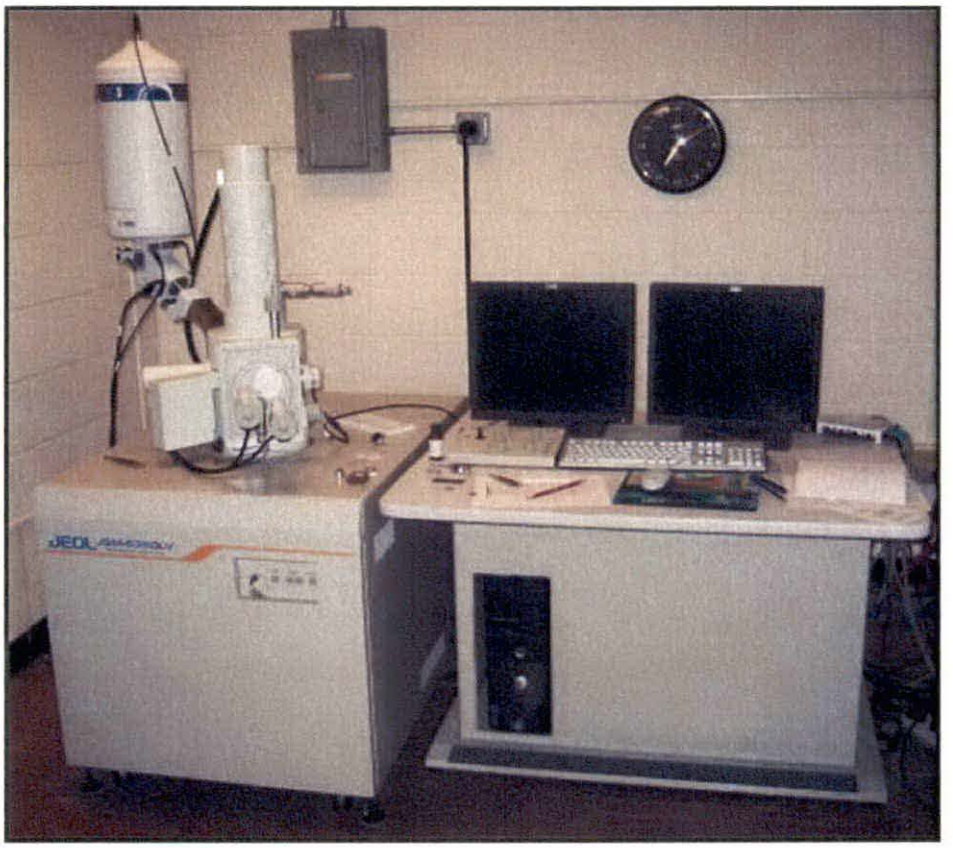


Figure 16. Scanning electron microscope (Jeol, ISM-6380LV).

In this experiment, the biofilms were first cultured in 24-well plates (Figure 17). Nitrocellulose membrane was used as a substratum. Pieces of nitrocellulose membrane (~1.5 cm- 1.5 cm) were cut to fit inside each of the wells. One mL of TSB was dispensed into each well. Then 200 μL of three different concentrations of ginseng was added to the wells. After that, 100 μL of PAO1 (wild type), PAO-JP1 (Δ *lasI)* and PDO100 (Δ *rhlI*) exhibiting an optical density at 595nm (OD_{595}) of 0.4 - 0.6 was dispensed into the wells. There were also control wells for each strain with TSB. This plate was then incubated at 37°C for 1, 3, 7

and 10 days, respectively, and then the nitrocellulose membranes were fixed to view under the SEM.

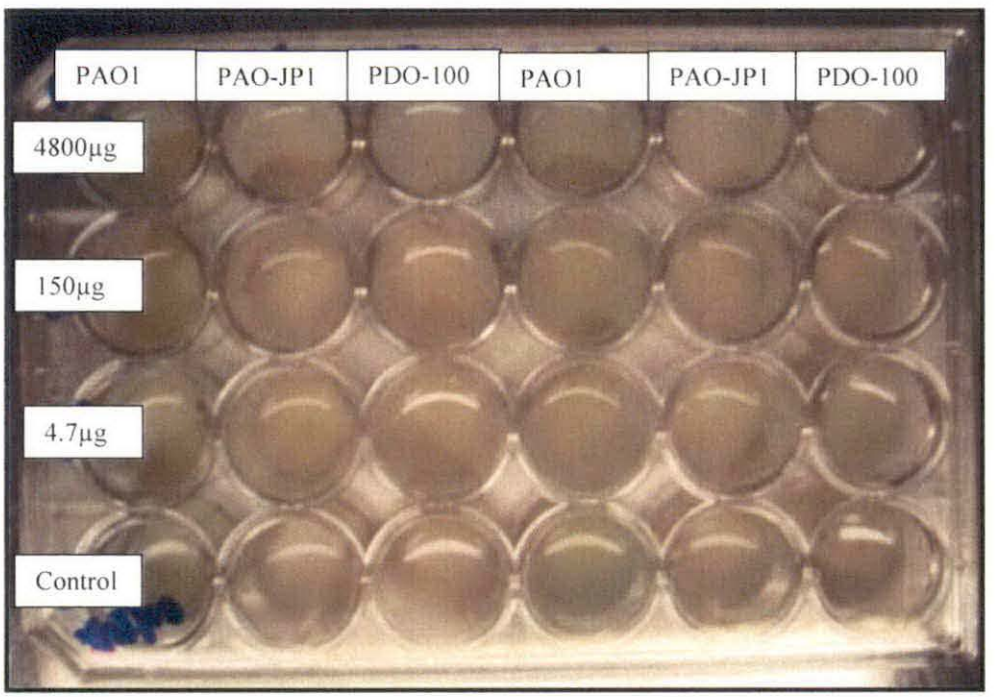


Figure 17. Set up of the experiment for the SEM.

The bacteria on the nitrocellulose membrane substrate were fixed with 2.5% glutaraldehyde for 3 hours at 42°C. Fixation with glutaraldehyde kills the bacteria and stabilizes the structure of the bacteria (preserve the bacteria). After the fixation with glutaraldehyde, the membranes were stained with 1% osmium tetroxide, and then left at room temperature for 2 hours. Osmium tetraoxide is a lipid stain that embeds a heavy metal directly into the bacterial membrane creating a high secondary electron emission. The samples were then dehydrated in a series of graded ethanol solutions starting with 25% ethanol then progressively through

50%, 75%, 90% and 100% ethanol. Dehydration is an essential process as it eliminates water from the bacteria and replaces it slowly with ethanol which has a low surface tension. It helps to reduce shrinkage and minimizes collapse and distortion of the biofilm. After dehydration with the series of graded ethanol solutions the specimen is washed with hexamethyldisilizane. Hexamethyldisilizane is an organic compound that is volatile at ambient temperature and hence can be used for air-drying bacteria. This technique is comparable to critical point drying and is economical and time saving.

Figure 18. Carbon sputter coater (Denton Vacuum).

After air drying, the specimen was mounted on an aluminum stub, and sputter coated with carbon using a sputter coater (Figure 18). The specimen was then visualized utilizing the SEM.

Fluorescence Microscopy

Fluorescence microscope is a very useful tool to observe biological specimens (Figure 19). Here the sample itself becomes the light source. The sample is made to fluoresce. This can be achieved by fluorescent dyes, immunoflorescence, tagging proteins etc. The basis of this technique is the phenomenon that certain materials emit energy detectable as visible light when excited with light of a specific wavelength. The sample can either be fluorescing in its natural form or treated with fluorescing chemicals as mentioned earlier. The advantage of fluorescence microscopy is that it is capable of imaging the distribution of a single molecular species based solely on the properties of fluorescence emission. Fluorescence microscopes have filters that contain a combination of dichroic mirrors and filters which excite fluorescent chromophores and divert the resulting secondary fluorescence to the eyepieces or camera tube. Mercury or xenon arc lamps (the light source) are used to generate enough excitation light intensity to furnish secondary fluorescence emission capable of detection.

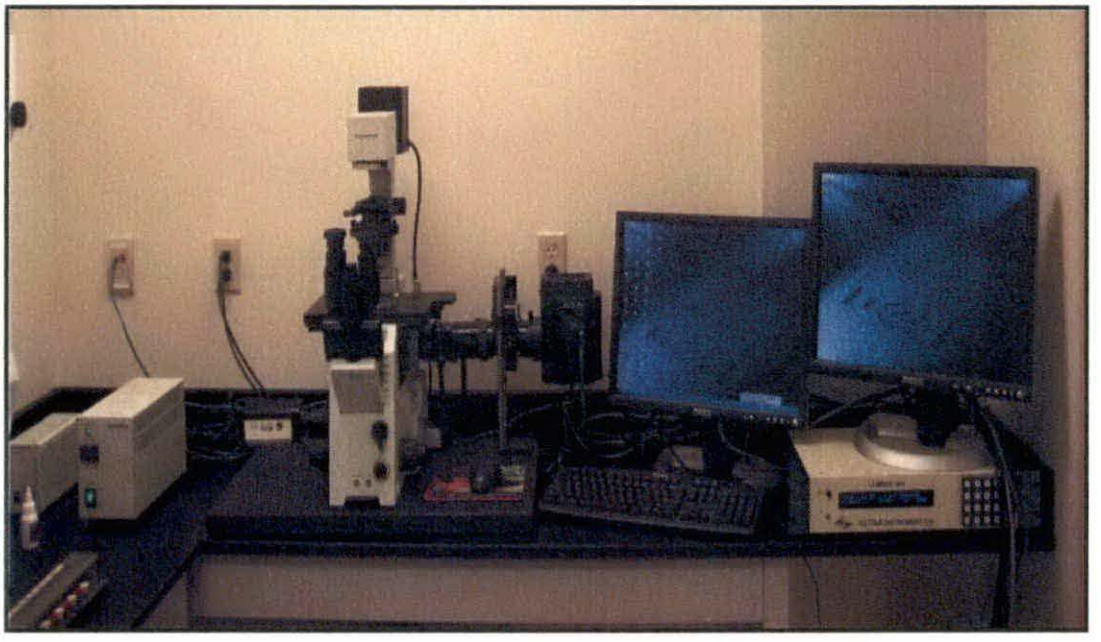


Figure 19. Fluorescence microscope (Olympus).

In this experiment, the biofilms were first cultured in 6-well plates (Figure 20). Glass cover slips were used as the substratum. One glass coverslip was placed inside each of the wells. Two mL of TSB was dispensed into each well. Then 200 μL of three different concentrations of ginseng was added to the wells. 100 μL of PAO1 (wild type), PAO-JP1 (Δ *lasI)* and PDO-100(Δ *rhlI*) cultures exhibiting an optical density at 595 nm (OD_{595}) of 0.4 to 0.6 was dispensed into the wells. There were also control wells for each strain with TSB.

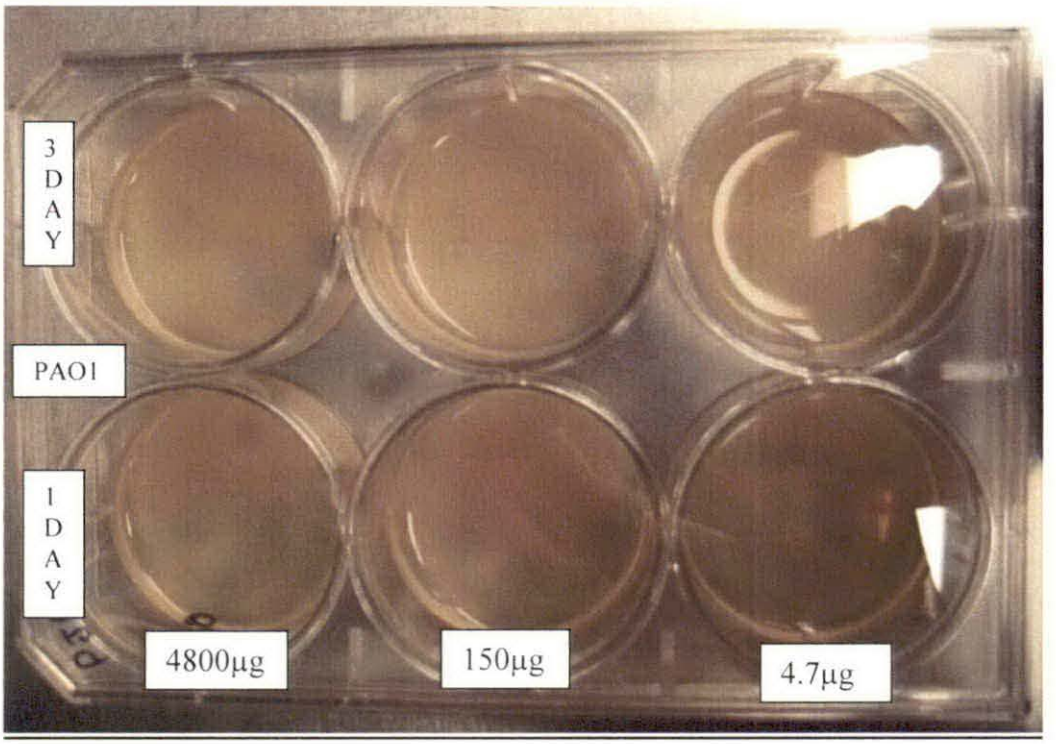


Figure 20. Set up of the experiment for the Florescence microscopy.

This plate was then incubated at 37°C for 1, 3, 7 and 10 days, respectively, and then the coverslips were mounted on a glass slide. A drop of mounting media (Biomeda Gel/Mount) was used to seal each coverslip to the slide. After the slides were prepped, they were stored in a covered box in the refrigerator so that the mounting media could harden and also to prevent photobleaching. The mounting media is used because it prevents rapid loss of fluorescence during microscopic examination, it retains its anti-fading ability during long-term storage, and it inhibits photobleaching and is optically clear.

Confocal Laser Scanning Microscopy

Confocal microscopy is a very popular tool to look at biological specimens due to its ease and high-quality images (Figure 21). Unlike florescence microscopy, here illumination is achieved by scanning one or more focused beams of light, usually from a laser or arc-discharge source, across the specimen. This point of illumination is brought to focus on the specimen by the objective lens, and laterally scanned using some form of scanning device under computer control. The sequences of points of light from the specimen are detected by a photomultiplier tube through a pinhole (or in some cases, a slit), and the output is built into an image and displayed by the computer. High-resolution images are achieved by optical sections. Data can be collected from fixed and stained specimens in single, double, triple, or multiple-wavelength illumination modes, and the images collected with the various illuminations and labeling strategies will be in register with each other. Live cell imaging and time-lapse sequences are possible, and digital image processing methods applied to sequences of images allow z-series and three-dimensional representation of specimens, as well as the time-sequence presentation of 3D data as four-dimensional imaging.

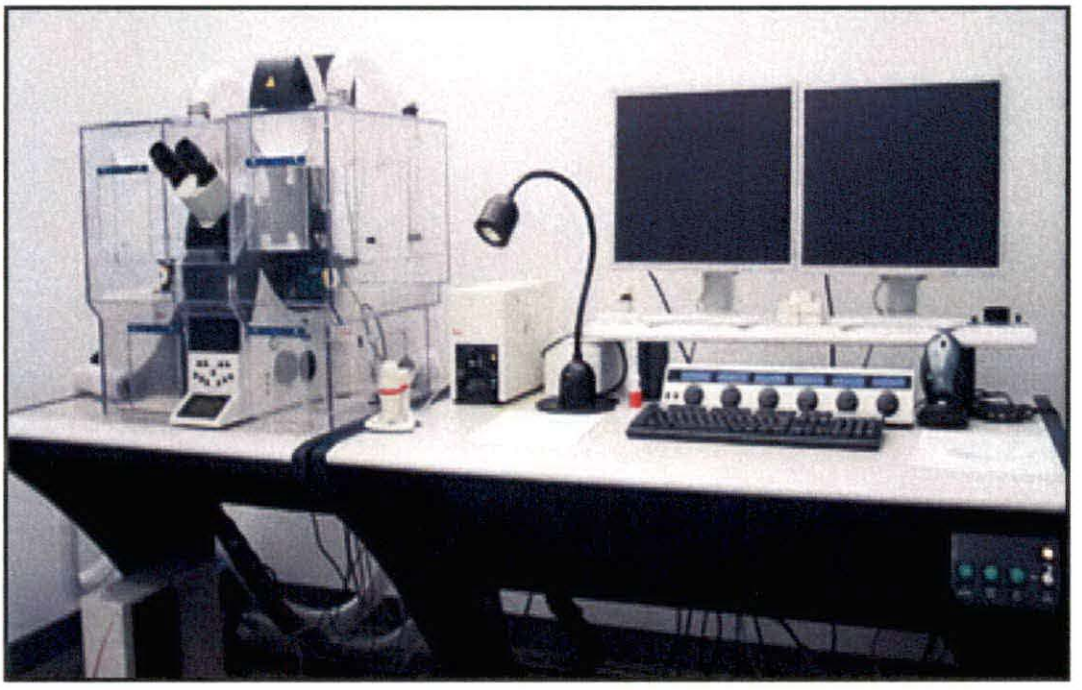


Figure 21. Confocal scanning laser microscope (Leica SP5 AOBS, University of Kentucky).

In this experiment, the biofilms were first cultured in 6-well plates (Figure 22). Glass cover slips were used as the substratum. One glass coverslip was placed inside each of the wells. Two mL of TSB was dispensed into each well. Then 200 µL of three different concentrations of ginseng was added to the wells. After that 100µL of PAO1 (wild type), PAO-JP1 (Δ *lasI)* and PDO-100(Δ *rhlI*) cultures exhibiting an optical density at 595nm (OD_{595}) of 0.4 - 0.6 was dispensed in the wells. There were also control wells for each strain with TSB.

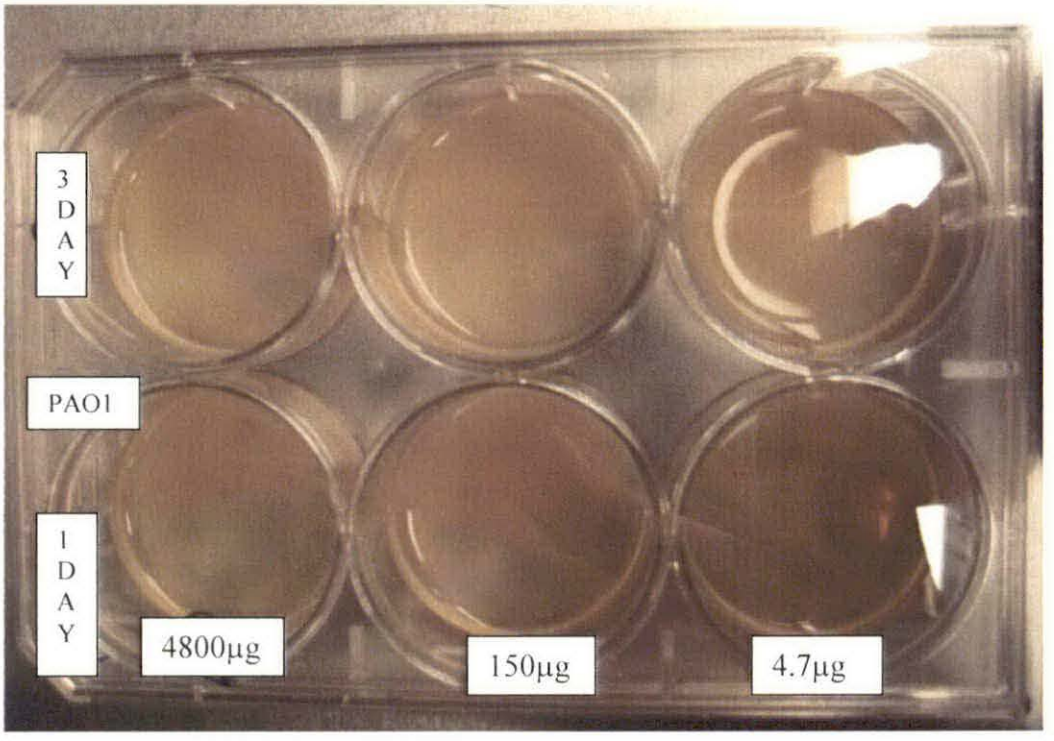


Figure 22. Set up of the experiment for the florescence microscopy.

The plates were then incubated at 37°C for 1, 3, 7 and 10 days, respectively, and then the coverslips were mounted on a glass slide. A drop of mounting media (Biomeda Gel/Mount) was used to seal each coverslip to the slide. After the slides were prepped, they were stored in a covered box in the refrigerator so that the mounting media could harden and also to prevent photobleaching. The mounting media is used because it prevents rapid loss of fluorescence during microscopic examination, it retains its anti-fading ability during long-term storage, it inhibits photobleaching and is optically clear.

Statistical analysis of biofilm thickness

For this experiment, Analysis of variance model was used. This was essential because of the fundamental variability in biofilm experiments. The analysis of variance model helped in comparing biofilm thickness in an objective manner and helped to make valid statements about the development of each biofilm. Here lowercase and uppercase letters were used to define fixed and random factors, respectively. The experimental factors were as follows: bacterial strain, *b* (fixed factor at three levels: 1, wild type (PAO1); 2, PJP1 (Δ *lasI* mutant); 3, PDO100 (Δ *rhlI* mutant); time, *t* (fixed factor at four levels: 1, 3, 7,and 10 day) and ginseng concentration, g (fixed factor at 3 levels: 1,4.7 μg/mL; 2, 150 μg/mL; 3,4800 μg/mL. In order to fully profit from the univariate nature of the measured variable, the average thickness of the two experimental rounds was subjected to a one way analysis of variance ANOVA for each of the factors first.

ANOVA for PAO1 (wild type)

Here two factors ginseng concentrations and days were analyzed for the bacterial strain PAO1 (wild type) in order to see their effect on the biofilm thickness.

The model thus became:

$$Y_{dcj} = \mu + t_d + g_c + \varepsilon_{dcj}$$

With d= day 1, day 3, day 7, day 10, c= 1, 4.7 μg/mL; 2, 150 μg/mL; 3, 4800 μg/mL and j= 12 responses for the biofilm thickness, Table 6.

μ = The common effect for the experiment, which was the average biofilm thickness by four different days

g_c= The treatment effect, which was the three different concentration of ginseng.

t_d = The treatment effect, which was the four different days.

ε_{dcj} = The random error in the experiment.

Through this mathematical model it was seen that there were two hypotheses to test. These hypotheses tested if the different days and the different concentrations of ginseng have an effect on the biofilm formation (thickness) for the bacterial strain PAO1.

Performance of ANOVA

To test these two hypotheses an analysis of variance (ANOVA) was done and the variation of means were compared.

The null hypothesis was that the four different days had the same effect on the biofilm formation. The alternative hypothesis was that not all the days had the same effect on the biofilm formation.

$H_0 = t_1 = t_3 = t_7 = t_{10}$
$H_1 = t_1 \neq t_3 \neq t_7 \neq t_{10}$

The second null hypothesis was that the three different concentrations of ginseng had the same effect on the biofilm formation. The alternative hypothesis was that not all the concentrations of ginseng had the same effect on the biofilm formation.

$H_0 = g_{4.7} = g_{150} = g_{4800}$
$H_1 = g_{4.7} \neq g_{150} \neq g_{4800}$

From the results for the ANOVA test, using the general linear model, the p-value for the ginseng concentration was (p value=0.000) which was statistically significant because the null hypothesis was rejected if the p-value was less than 0.05. The p-value for days was (p value=0.005) which was statistically significant because the null hypothesis was rejected if the p-value was less than 0.05. From these results it was concluded that there was a significant effect of ginseng concentration and days on biofilm formation for PAO1 (wild type). Therefore, the alternative hypotheses were favored.

Validation of the Assumptions of the Model

As the p-value after the general linear model showed that there was a significant effect of ginseng concentration and days on the biofilm thickness the assumption were validated for the model.

Table 2. Validation of assumption for ANOVA for PAO1.

Assumption	**Test**	**Value**	**Validation**
Normality Assumption	Anderson-Darling	0.339>0.05 p-value at 95% confidence interval	Not Violated
Independence Assumption	Autocorrelation Function	0.419 <0.565	Not Violated
Constant Variance	Bartlett's test	0.468>0.05	Not Violated

ANOVA for PDO100 (Δ *rhlI* mutant)

Here two factors Ginseng concentrations and days had been analyzed for the bacterial strain PDO100 (Δ *rhlI* mutant) in order to see their effect on the biofilm thickness.

The model thus became:

$$Y_{dcj} = \mu + t_d + g_c + \varepsilon_{dcj}$$

With d= day 1, day 3, day 7, day 10, c= 1, 4.7 μg/mL; 2, 150 μg/mL; 3, 4800 μg/mL and j= 12 responses for the biofilm thickness, Table 6.

μ = The common effect for the experiment, which was the average biofilm thickness by four different days

g_c= The treatment effect, which was the three different concentration of ginseng.

t_d = The treatment effect, which was the four different days.

ε_{dcj} = The random error in the experiment.

Through this mathematical model it was seen that there were two hypotheses to test. These hypotheses tested if the different days and the different concentrations of ginseng had an effect on the biofilm formation (thickness) for the bacterial strain PDO100.

Performance of ANOVA

To test these two hypotheses an analysis of variance (ANOVA) was done and the variation of means were compared.

The null hypothesis was that the four different days had the same effect on the biofilm formation. The alternative hypothesis was that not all the days had the same effect on the biofilm formation.

$H_0 = t_1 = t_3 = t_7 = t_{10}$
$H_1 = t_1 \neq t_3 \neq t_7 \neq t_{10}$

The second null hypothesis was that the three different concentrations of ginseng had the same effect on the biofilm formation. The alternative hypothesis was that not all the concentrations of ginseng had the same effect on the biofilm formation.

$H_0 = g_{4.7} = g_{150} = g_{4800}$
$H_1 = g_{4.7} \neq g_{150} \neq g_{4800}$

From the results for the ANOVA test, using the general linear model, the p-value for the ginseng concentration was (p value=0.019) which was statistically significant because the null hypothesis was rejected if the p-value was less than 0.05. The p-value for days was (p value=0.040) which was statistically significant because the null hypothesis was rejected if the p-value is less than 0.05. From these results we can conclude that there was a significant effect of ginseng concentration and days on biofilm formation for PDO100 (Δ *rhlI* mutant). Therefore the alternative hypotheses were favored.

Validation of the Assumptions of the Model

As the p-value after the general linear model showed that there was a significant effect of ginseng concentration and days on the biofilm thickness the assumption were validated for the model.

Table 3. Validation of assumption for ANOVA for PDO100.

Assumption	Test	Value	Validation
Normality Assumption	Anderson-Darling	0.062>0.05 p-value at 95% confidence interval	Not Violated
Independence Assumption	Autocorrelation Function	0.222 <0.565	Not Violated
Constant Variance	Bartlett's test	0.647>0.05	Not Violated

ANOVA for PJP1 (Δ lasI mutant)

Here two factors ginseng concentrations and days had been analyzed for the bacterial strain PJP1 (Δ lasI mutant) in order to see their effect on the biofilm thickness.

The model thus became:

$$Y_{dcj} = \mu + t_d + g_c + \varepsilon_{dcj}$$

With d= day 1, day 3, day 7, day 10, c= 1, 4.7 μg/mL; 2, 150 μg/mL; 3, 4800 μg/mL and j= 12 responses for the biofilm thickness, Table 6.

μ = The common effect for the experiment, which was the average biofilm thickness by four different days

g_c= The treatment effect, which was the three different concentration of ginseng.

t_d = The treatment effect, which was the four different days.

ε_{dcj} = The random error in the experiment.

Through this mathematical model it was seen that there were two hypotheses to test. These hypotheses tested if the different days and the different concentrations of ginseng have an effect on the biofilm formation (thickness) for the bacterial strain PJP1.

Performance of ANOVA

To test these two hypotheses an analysis of variance (ANOVA) was done and the variation of means were compared.

The null hypothesis was that the four different days had the same effect on the biofilm formation. The alternative hypothesis was that not all the days had the same effect on the biofilm formation.

$H_0 = t_1 = t_3 = t_7 = t_{10}$
$H_1 = t_1 \neq t_3 \neq t_7 \neq t_{10}$

The second null hypothesis was that the three different concentrations of ginseng had the same effect on the biofilm formation. The alternative hypothesis was that not all the concentrations of ginseng had the same effect on the biofilm formation.

$H_0 = g_{4.7} = g_{150} = g_{4800}$
$H_1 = g_{4.7} \neq g_{150} \neq g_{4800}$

From the results for the ANOVA test, using the general linear model, the p-value for the ginseng concentration was (p value=0.010) which was statistically significant because the null hypothesis was rejected if the p-value was less than

0.05. The p-value for days was (p value=0.006) which was statistically significant because the null hypothesis was rejected if the p-value was less than 0.05. From these results we can conclude that there was a significant effect of ginseng concentration and days on biofilm formation for PJP1 (Δ *lasI* mutant). Therefore the alternative hypotheses were favored.

Validation of the Assumptions of the Model

As the p-value after the general linear model showed that there was a significant effect of ginseng concentration and days on the biofilm thickness the assumption were validated for the model.

Table 4. Validation of assumption for ANOVA for PJP1.

Assumption	**Test**	**Value**	**Validation**
Normality Assumption	Anderson-Darling	0.138>0.05 p-value at 95% confidence interval	Not Violated
Independence Assumption	Autocorrelation Function	0.14 <0.565	Not Violated
Constant Variance	Bartlett's test	0.380>0.05	Not Violated

ANOVA for all three factors

Here all the three factors ginseng concentrations, bacterial strain and days had been analyzed to see their effect on the biofilm thickness.

The model thus became:

$$\mathbf{Y}_{dicj} = \boldsymbol{\mu} + \boldsymbol{b}_i + \boldsymbol{t}_d + \mathbf{g}_c + \boldsymbol{\varepsilon}_{dicj}$$

With i= PAO1 (wild type); 2, PJP1 (Δ *lasI* mutant); 3, PDO100 (Δ *rhlI* mutant), d= day 1, day 3, day 7, day 10, c= 1, 4.7 μg/mL; 2, 150 μg/mL; 3, 4800 μg/mL and j= 36 responses for the biofilm thickness, Table 6.

μ = The common effect for the experiment, which was the average biofilm thickness by four different days

b_i= The treatment effect, which was the three different bacterial strain.

t_d = The treatment effect, which was the four different days.

g_c= The treatment effect, which was the three different concentration of ginseng.

ε_{dcj} = The random error in the experiment.

Through this mathematical model it was seen that there were three hypotheses to test. These hypotheses were tested if the different bacterial strains, different days and the different concentrations of ginseng had an effect on the biofilm formation (thickness).

Performance of ANOVA

To test these three hypotheses an analysis of variance (ANOVA) was done and the variation of means were compared.

The first null hypothesis was that the three different bacterial strains had the same effect on the biofilm formation. The alternative hypothesis was that not all the three different bacterial strains had the same effect on the biofilm formation.

$H_0 = b_{PAO1} = b_{PDO100} = b_{PJP1}$
$H_1 = b_{PAO1} \neq b_{PDO100} \neq b_{PJP1}$

The second null hypothesis was that the four different days had the same effect on the biofilm formation. The alternative hypothesis was that not all the days had the same effect on the biofilm formation.

$H_0 = t_1 = t_3 = t_7 = t_{10}$
$H_1 = t_1 \neq t_3 \neq t_7 \neq t_{10}$

The third null hypothesis was that the three different concentrations of ginseng had the same effect on the biofilm formation. The alternative hypothesis was that not all the concentrations of ginseng had the same effect on the biofilm formation.

$H_0 = g_{4.7} = g_{150} = g_{4800}$
$H_1 = g_{4.7} \neq g_{150} \neq g_{4800}$

From the results for the ANOVA test, using the general linear model, the p-value for the bacterial strains was (p value=0.000) which was statistically significant because the null hypothesis was rejected if the p-value was less than 0.05. The p-value for the ginseng concentration was (p value=0.000) which was statistically significant because the null hypothesis was rejected. The p-value for days was (p value=0.000) which was statistically significant because the null hypothesis was rejected. From these results it could be concluded that there was a significant effect of bacterial strains, ginseng concentration and days on biofilm formation .Therefore the alternative hypotheses were favored.

Validation of the Assumptions of the Model

As the p-value after the general linear model showed that there was a significant effect of bacterial strains, ginseng concentration and days on the biofilm thickness the assumption were validated for the model.

Table 5. Validation of assumption for ANOVA for all three factors.

Assumption	**Test**	**Value**	**Validation**
Normality Assumption	Anderson-Darling	0.717 >0.05 p-value at 95% confidence interval	Not Violated
Independence Assumption	Autocorrelation Function	0.150 <0.327	Not Violated
Constant Variance	Bartlett's test	0.409>0.05	Not Violated

The Results section will elaborate the results obtained from each of these ANOVA analysis with the help of main effects graphs.

Results

The objective of this study was to analyze the effect of ginseng on biofilm formation by wild type and mutant strains of *Pseudomonas aeruginosa.*

To achieve this objective, *Pseudomonas aeruginosa* (GFP tagged) PAO1 (wild type) and the quorum sensing-deficient mutants PJP1 (Δ *lasI* mutant) and PDO100 (Δ *rhlI* mutant) were used. For each strain, the control group was used which did not contain ginseng. The three experimental groups consisted of three different concentration of ginseng which consisted of 4.7 μg/mL, 150 μg/mL and 4800 μg/mL, respectively. These cultures were grown in TSB and then examined at days 1, 3, 7 and 10 of incubation. These bacterial samples were then analyzed by Scanning electron microscopy, fluorescence microscopy, and confocal microscopy. For all three the microscopy experiment was duplicated. Scanning electron microscopy and fluorescence microscopy images were interpreted visually and for confocal microscopy images statistical methods were used to interpret results.

Scanning Electron Microscopy Analysis

Here a representative example of one complete experiment is shown. Figure 23-34 shows image of the three strains for the control and the images for the three different concentrations of ginseng for day 1 to 10. A biofilm structure is defined as the bacterial cells and the extracellular polymeric substance.

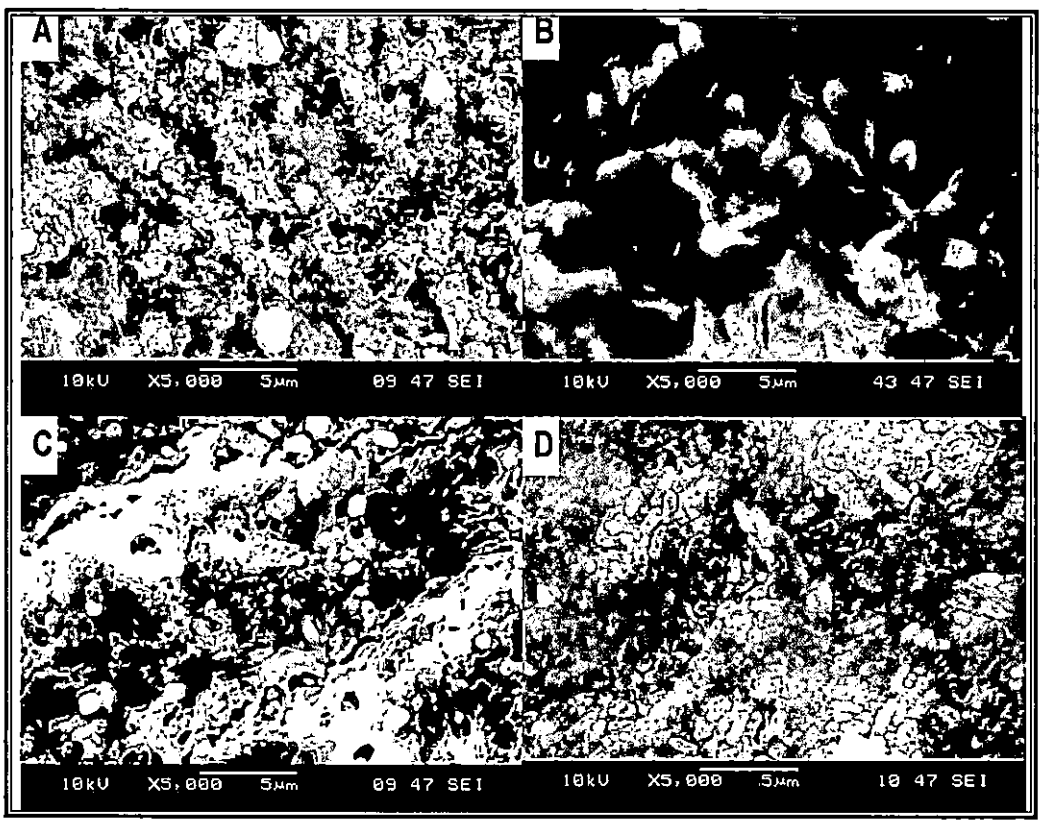


Figure 23. *Pseudomonas aeruginosa* PAO1 (wild type) biofilms on nitrocellulose membrane as a substrate under SEM after 1 day in TSB. (A) Control, (B) 4.7 µg/mL of ginseng, (C) 150 µg/mL of ginseng, (D) 4800 µg/mL of ginseng.

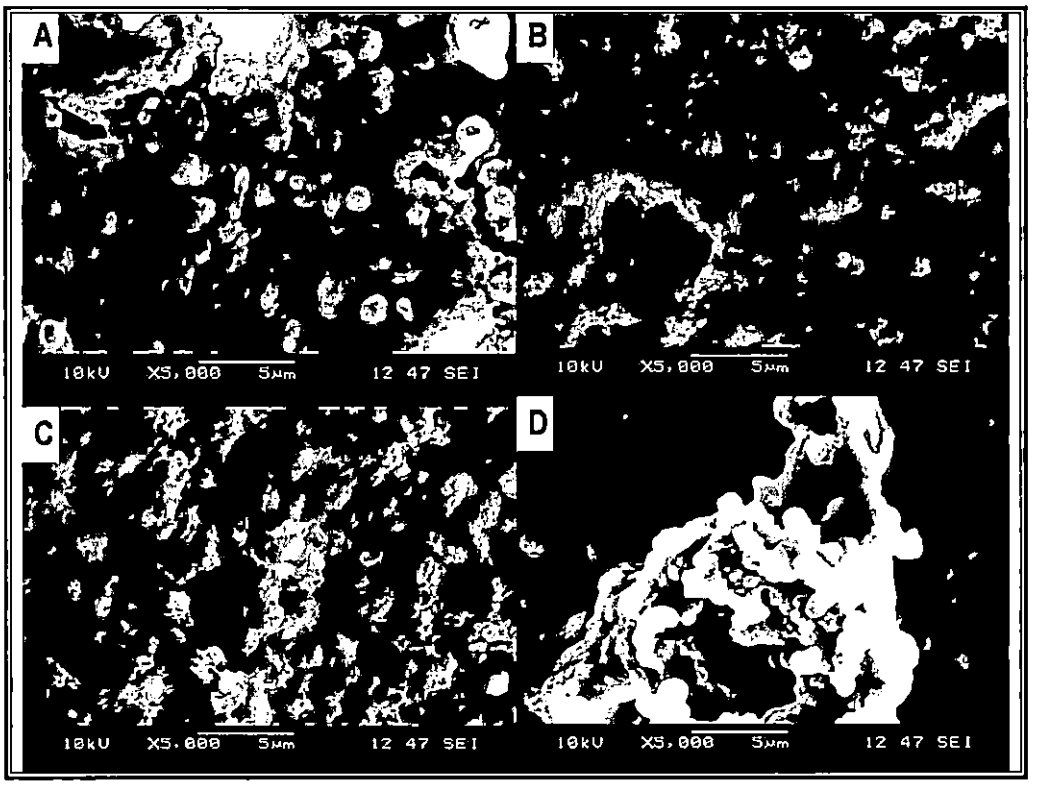


Figure 24. *Pseudomonas aeruginosa* PAO1 (wild type) biofilms on nitrocellulose membrane as a substrate under SEM after 3 days in TSB.(A) Control, (B) 4.7 μg/mL of ginseng,(C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

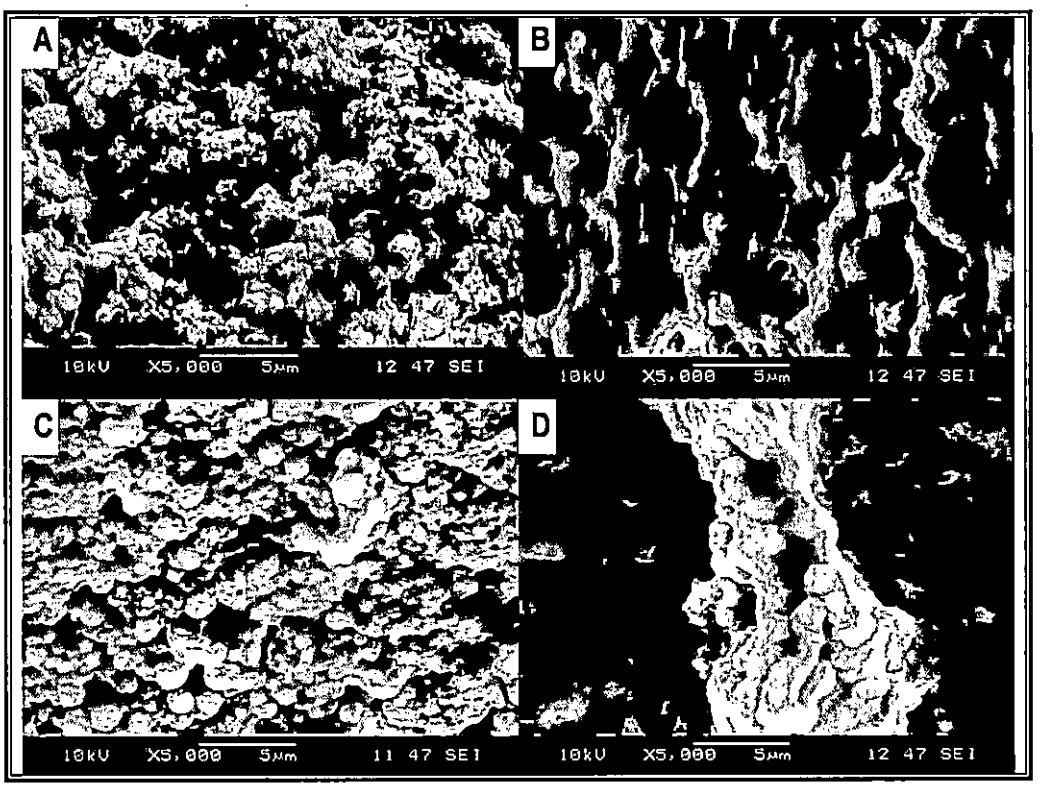


Figure 25. *Pseudomonas aeruginosa* PAO1 (wild type) biofilms on nitrocellulose membrane as a substrate under SEM after 7 days in TSB. (A) Control, (B) 4.7 μg/mL of ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

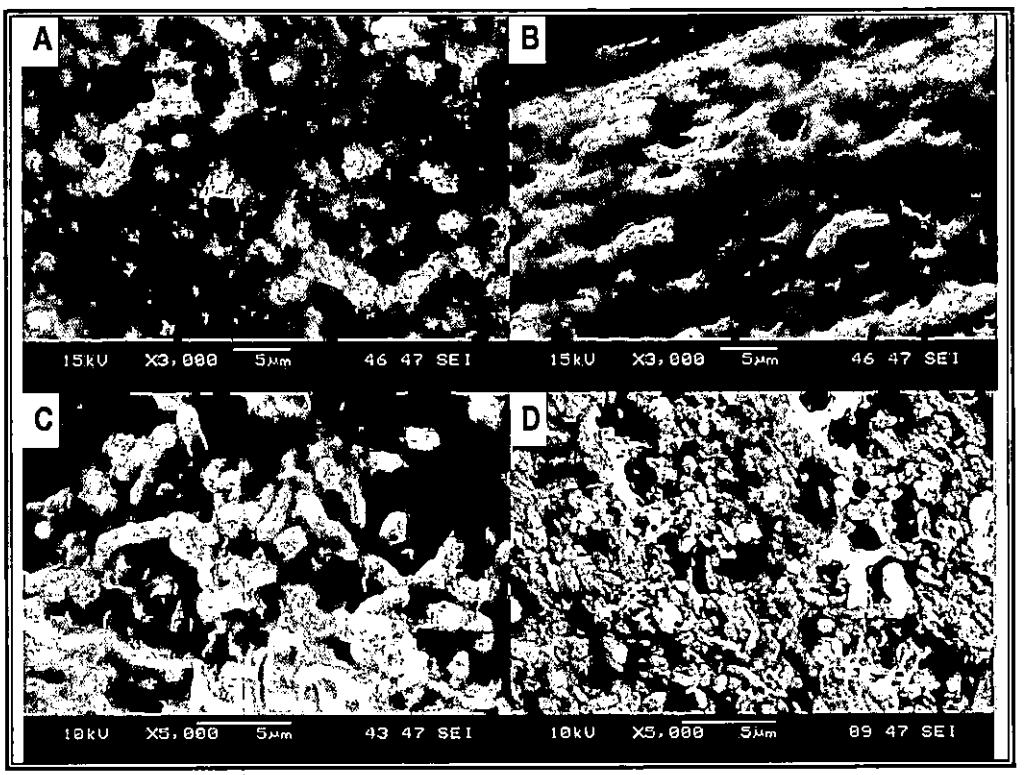


Figure 26. *Pseudomonas aeruginosa* PAO1(wild type) biofilms on nitrocellulose membrane as a substrate under SEM after 10 days in TSB.(A) Control, (B) 4.7 μg/mL of ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

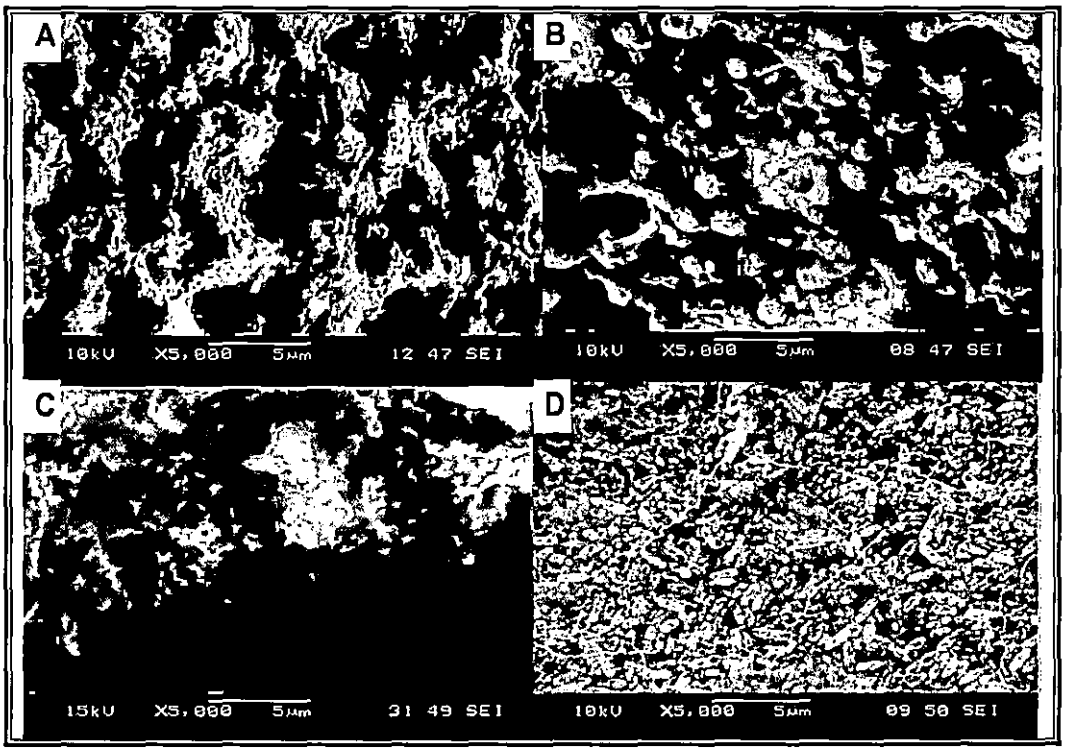


Figure 27. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on nitrocellulose membrane as a substrate under SEM after 1 day in TSB. (A) Control, (B) 4.7 μg/mL of ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

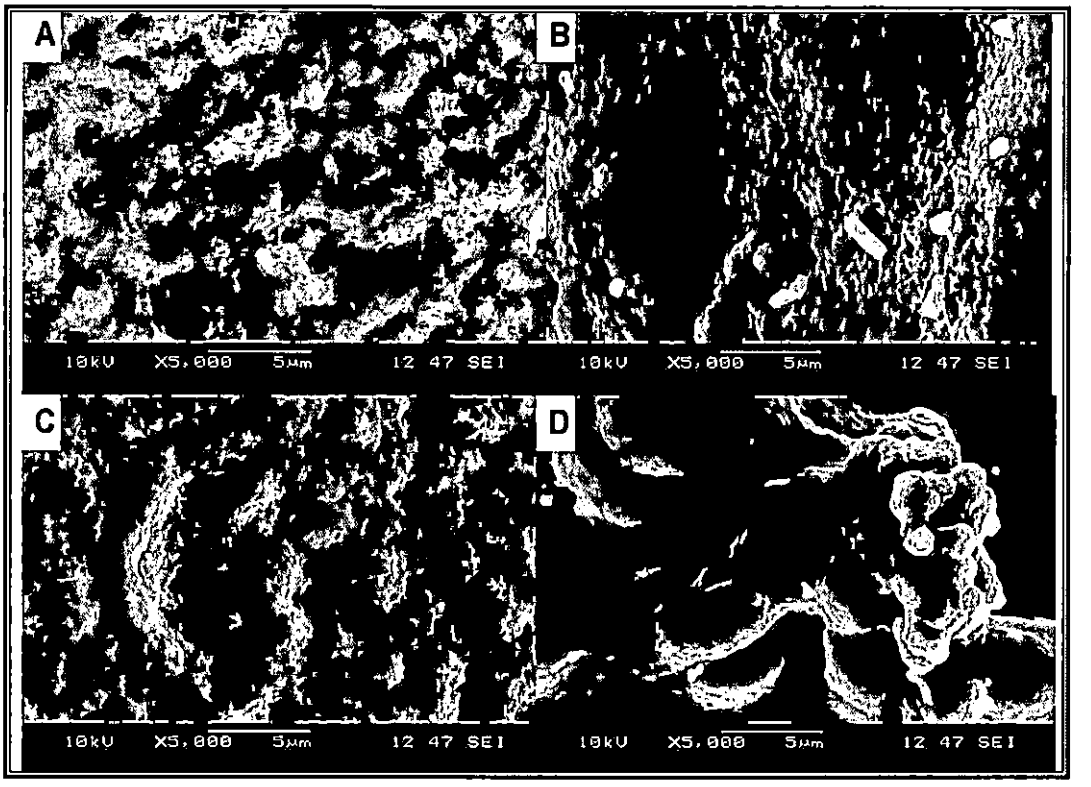


Figure 28. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on nitrocellulose membrane as a substrate under SEM after 3 days in TSB. (A) Control, (B) 4.7 µg/mL of ginseng, (C) 150 µg/mL of ginseng, (D) 4800 µg/mL of ginseng.

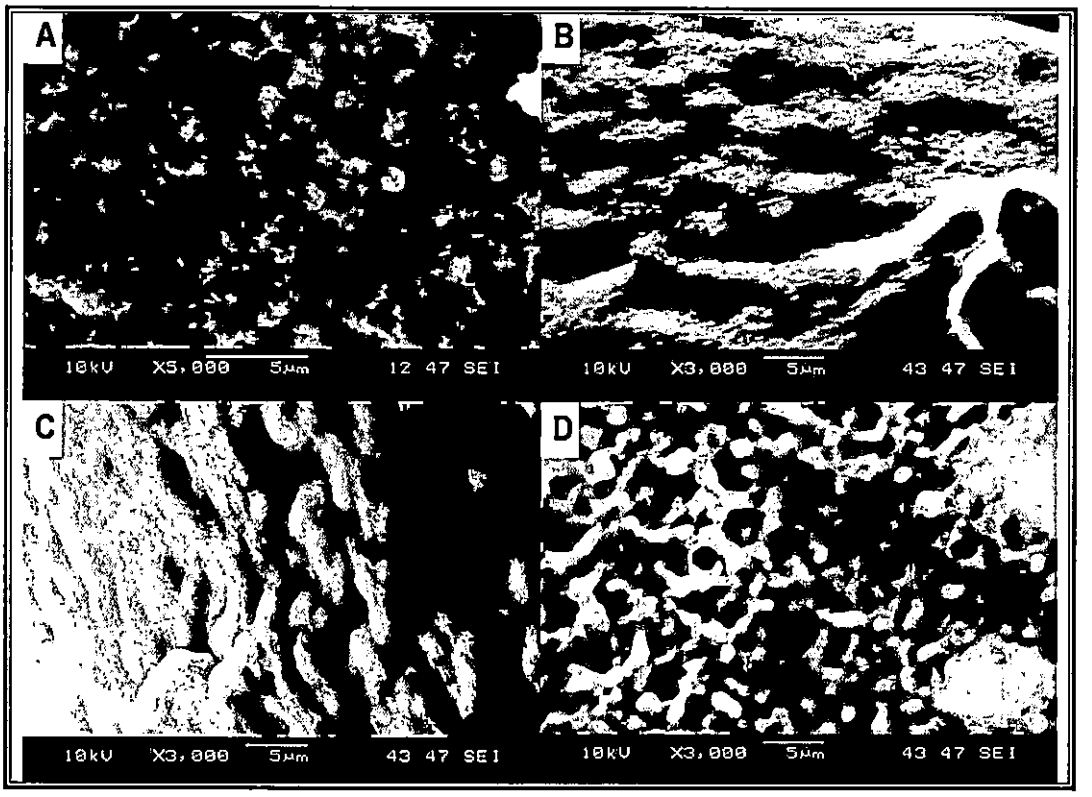


Figure 29. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on nitrocellulose membrane as a substrate under SEM after 7 days in TSB. (A) Control, (B) 4.7 µg/mL of ginseng, (C) 150 µg/mL of ginseng, (D) 4800 µg/mL of ginseng.

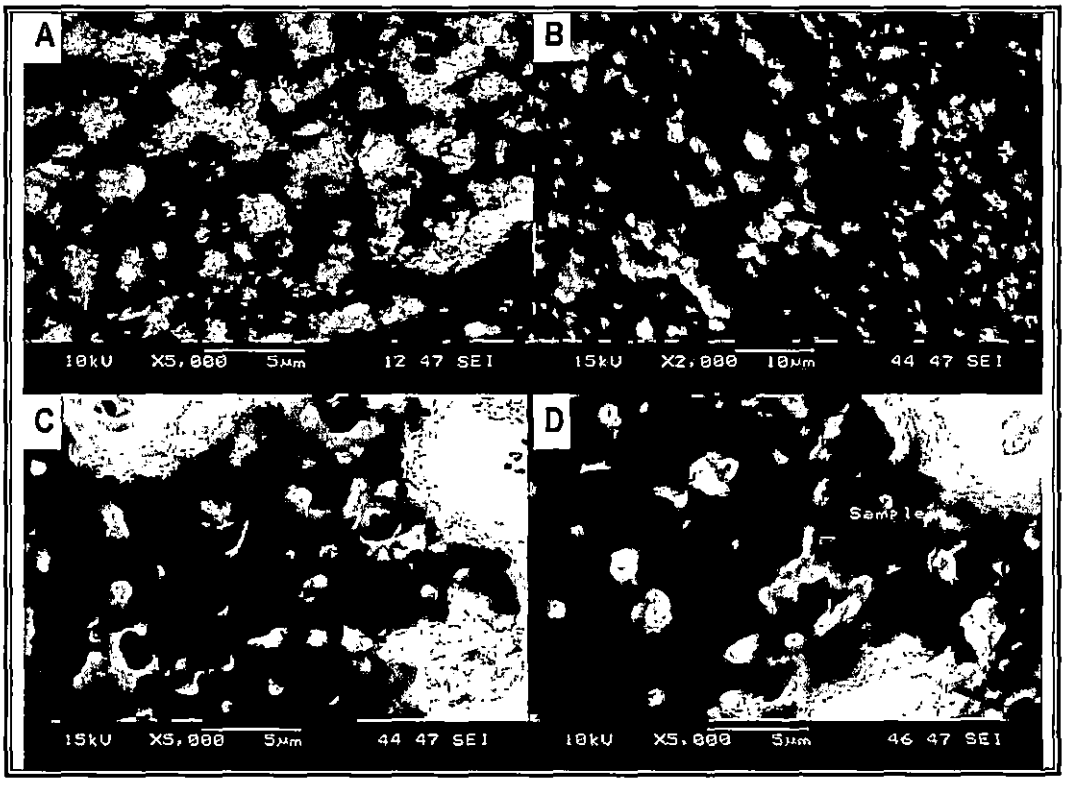


Figure 30. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on nitrocellulose membrane as a substrate under SEM after 10 days in TSB. (A) Control, (B) 4.7 µg/mL of ginseng, (C) 150 µg/mL of ginseng, (D) 4800 µg/mL of ginseng.

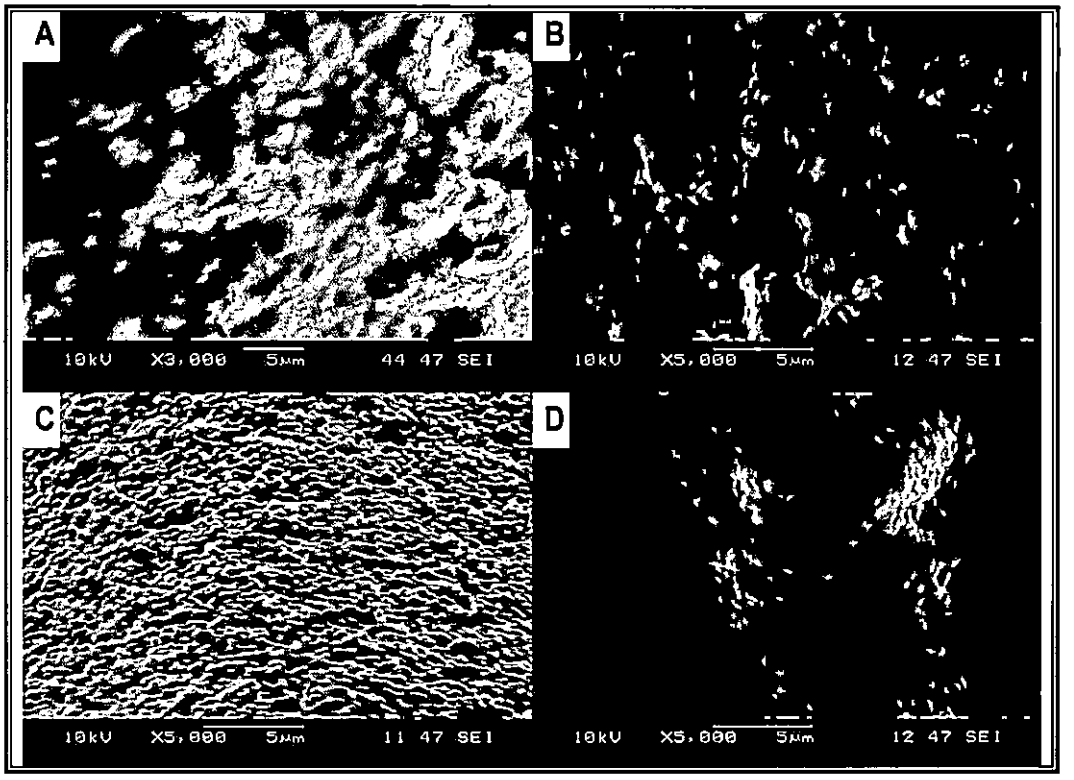


Figure 31. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on nitrocellulose membrane as a substrate under SEM after 1 days in TSB. (A) Control, (B) 4.7 µg/mL of ginseng, (C) 150 µg/mL of ginseng, (D) 4800 µg/mL of ginseng.

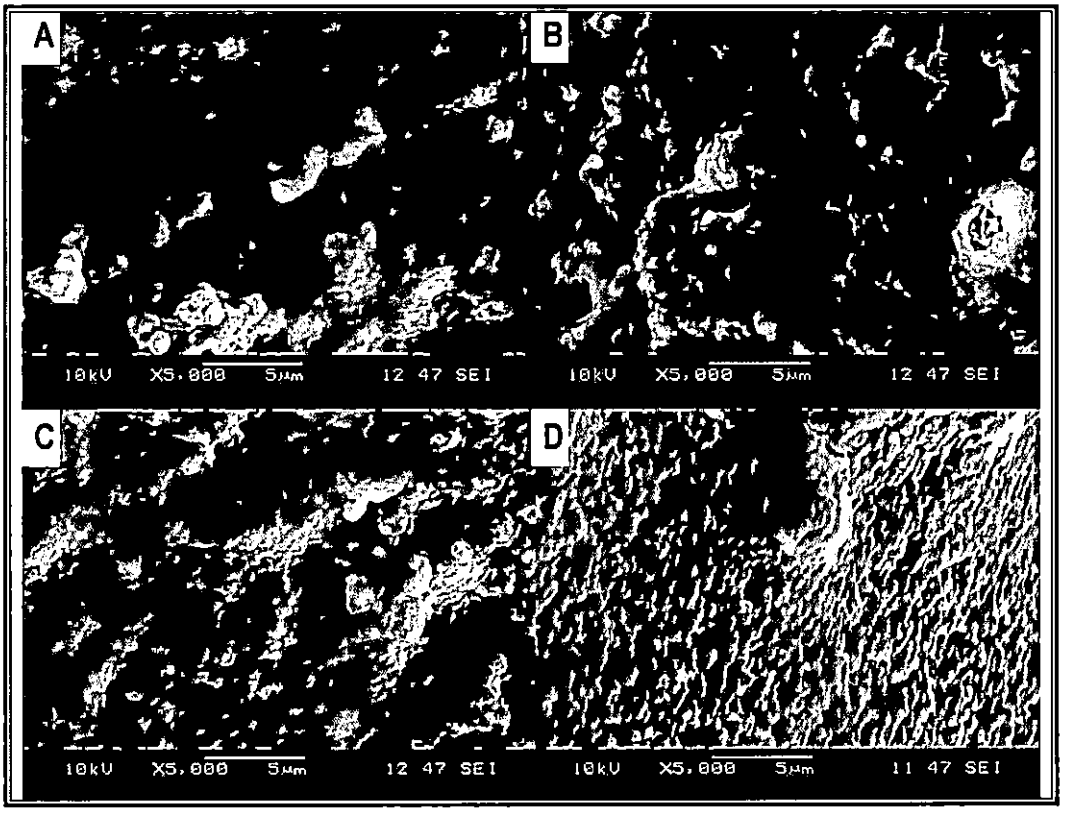


Figure 32. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on nitrocellulose membrane as a substrate under SEM after 3 days in TSB. (A) Control, (B) 4.7 µg/mL of ginseng, (C) 150 µg/mL of ginseng, (D) 4800 µg/mL of ginseng.

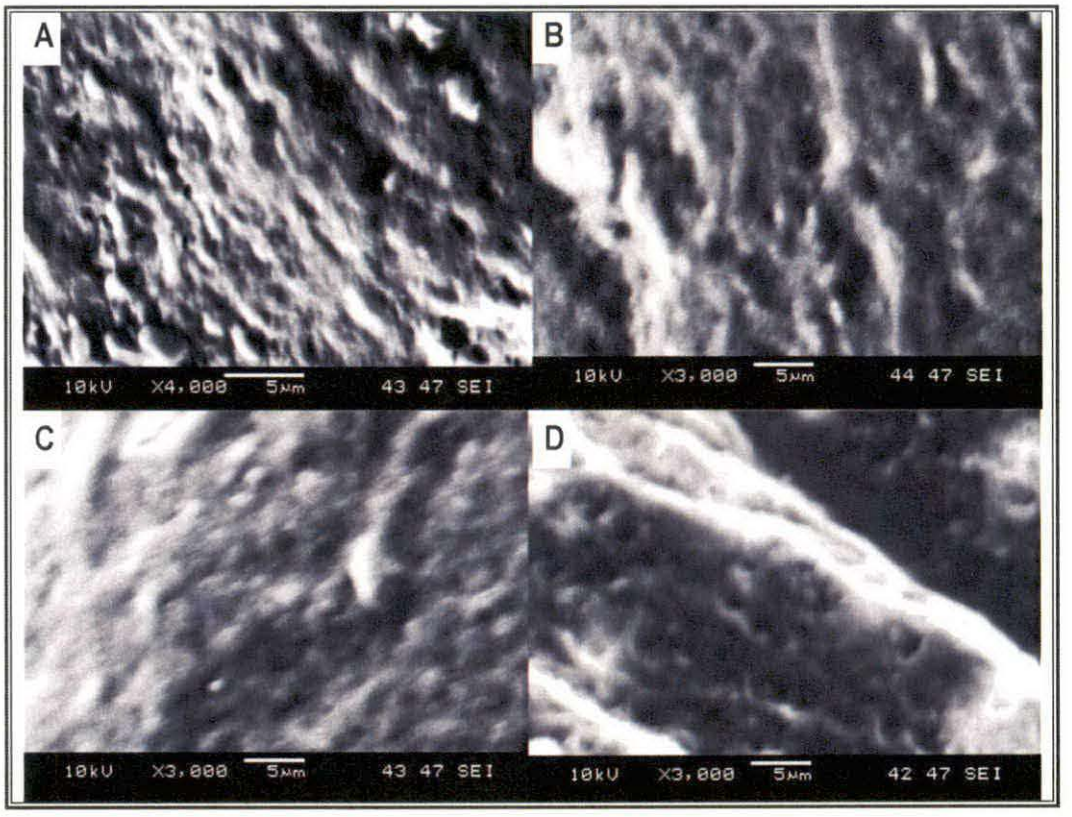


Figure 33. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on nitrocellulose membrane as a substrate under SEM after 7 days in TSB. (A) Control, (B) 4.7 μg/mL of ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

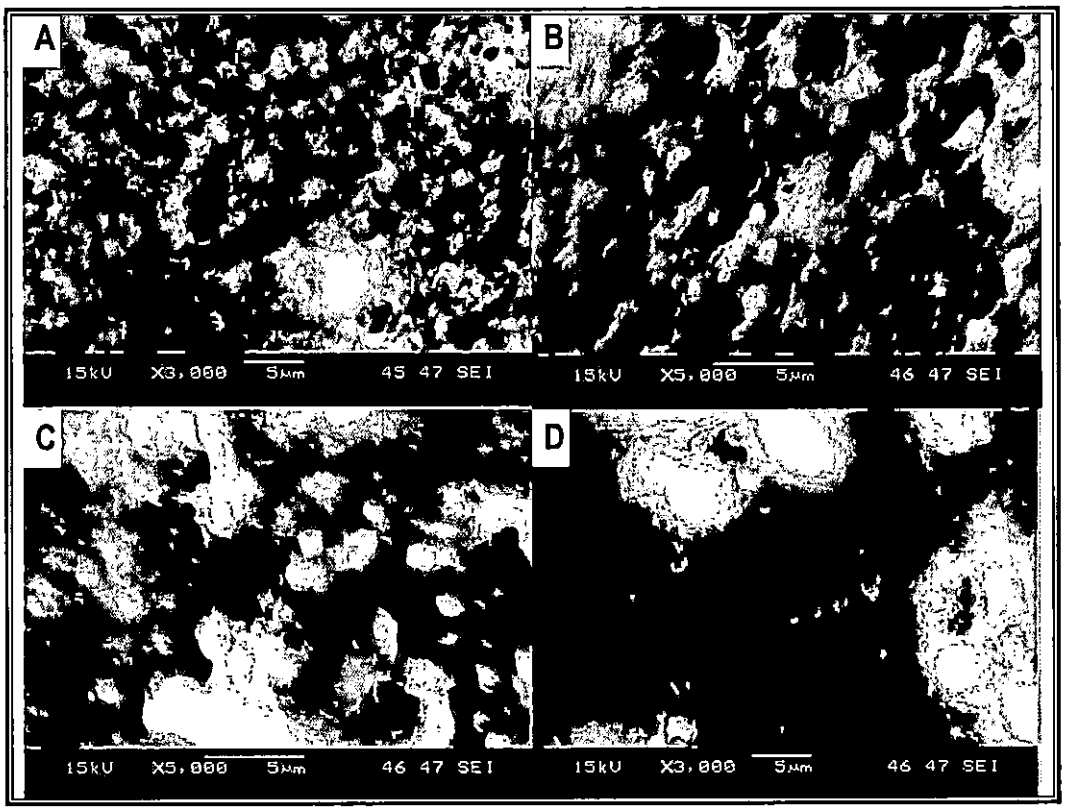


Figure 34. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on nitrocellulose membrane as a substrate under SEM after 10 days in TSB. (A) Control, (B) 4.7 μg/mL of ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

Each of the three strains was first compared to the days of incubation. From Figure 23-26, PAO1 (wild type) was analyzed from day 1 to day 10. It was seen that the biofilm formation in the controls continued to develop with increasing time of incubation. The day 10 control had the thickest biofilm formation indicating that there was still a favorable environment present for the biofilm to keep growing to day 10. Ginseng concentration of 4.7 μg/mL demonstrated less biofilm covering the bacteria until day 7. However, for the 10^{th} day its effect decreased. The ginseng

concentration of 150 and 4800 μg/mL showed less biofilm production even till day 10. From Figures 27-30, it can be seen that for PDO100 (Δ *rhlI* mutant) the biofilm formation increased with time. The ginseng concentration of 4.7 μg/mL showed inhibition of biofilm just for the day 1; however day 3, 7 and 10 seemed to have a significant biofilm formation. The ginseng concentration of 150 μg/mL showed inhibition of biofilm formation for 1st and 3rd day and then slowly declined. The ginseng concentration of 4800 μg/mL showed inhibition of biofilm throughout the 10 days with the best results on day 1. From Figures 31- 34 it was seen that for PJP1 (Δ *lasI* mutant) the biofilm formation increased with increasing days. The ginseng concentration of 4.7 μg/mL and 150 μg/mL showed inhibition of biofilm on day 1 and day 3, however day 7 and day 10 showed lack of inhibition. The ginseng concentration of 4800 μg/mL showed inhibition of biofilm to some extent even on day 10.

The three strains were compared to one another without ginseng, at each of the 4 incubation days. The PAO1 (wild type) had more biofilm growth for day 1 and day 3 when compared to PDO100 (Δ *rhlI* mutant) and PJP1 (Δ *lasI* mutant). The biofilm growth was slower for the two quorum sensing mutants.

The effect of each ginseng concentration on biofilm formation to the control culture was compared for each strain. It was seen that for PAO1 (wild type) the ginseng concentration of 150 μg/mL and 4800 μg/mL showed tremendous biofilm inhibition. For quorum sensing mutant strains PDO100 (Δ *rhlI* mutant) and PJP1 (Δ

lasI mutant), the ginseng concentration of 4800 μg/mL showed the best biofilm inhibition when compared to their respective controls.

Finally, the three strains were compared to one another under the experimental conditions (i.e., ginseng's effect on biofilm formation). The most effect of ginseng was seen in the PAO1 (wild type strain) followed by PJP1 (Δ *lasI* mutant) and then PDO100 (Δ *rhlI* mutant).

Fluorescence Microscopy Analysis

The spatial biomass distribution was analyzed in each sample which was the total GFP tagged bacterial cells with the exopolymeric substance that made up the biofilm on the substrate (coverslip). The biofilm appeared fluorescent green on a black background by visual inspection of Fluorescence microscopy images. Here a representative example of one complete experiment is shown (Figures 35-46) which was viewed in the XY plane (Top view, 600 magnification).

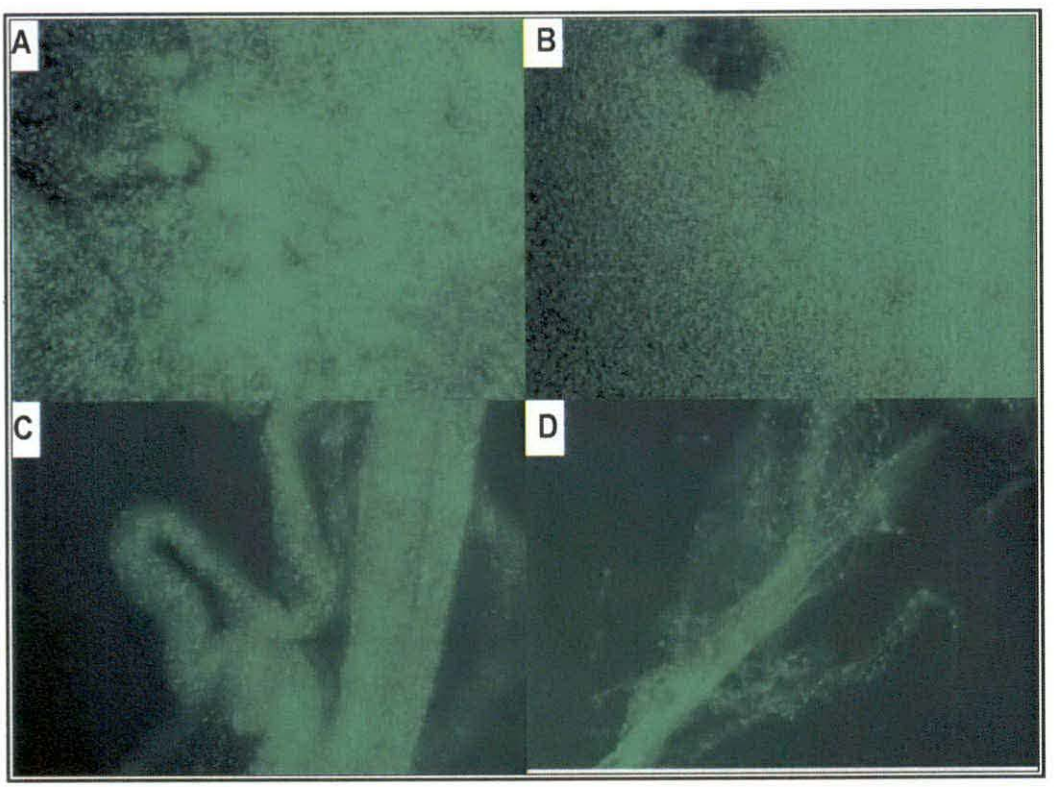


Figure 35. *Pseudomonas aeruginosa* PA01 (wild type) biofilms on a coverslip as a substrate under fluorescent microscope after 1 day in TSB (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

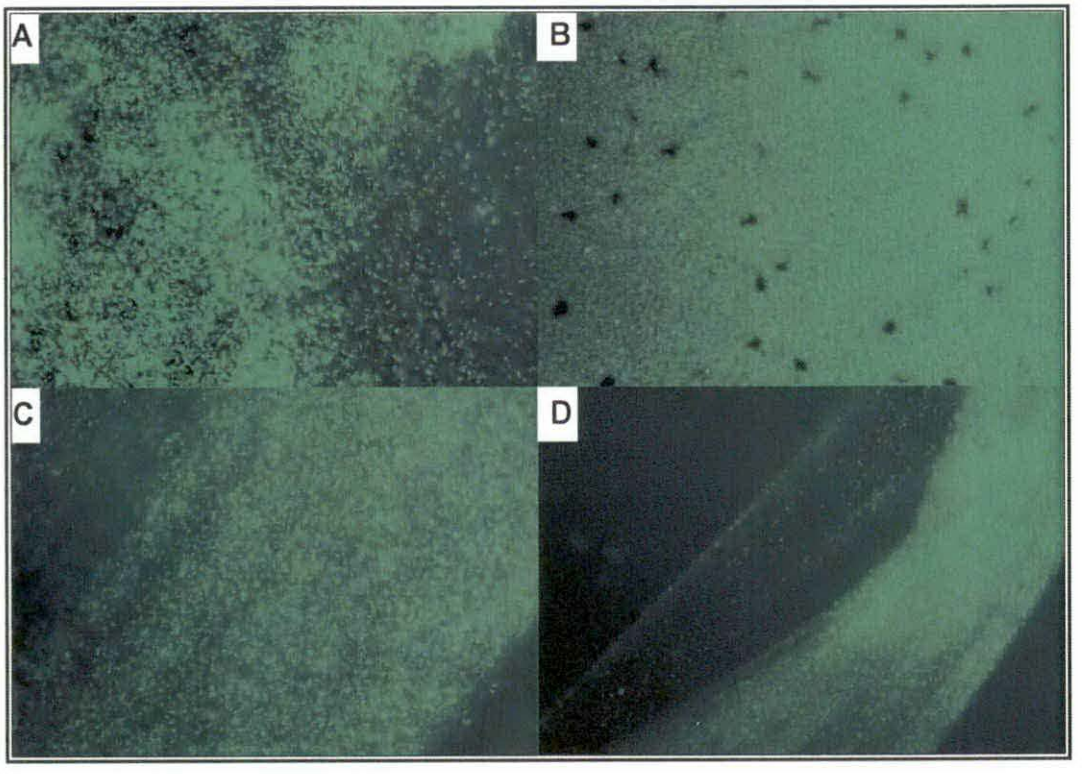


Figure 36. *Pseudomonas aeruginosa* PA01 (wild type) biofilms on a coverslip as a substrate under fluorescent microscope after 3 days in TSB. (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

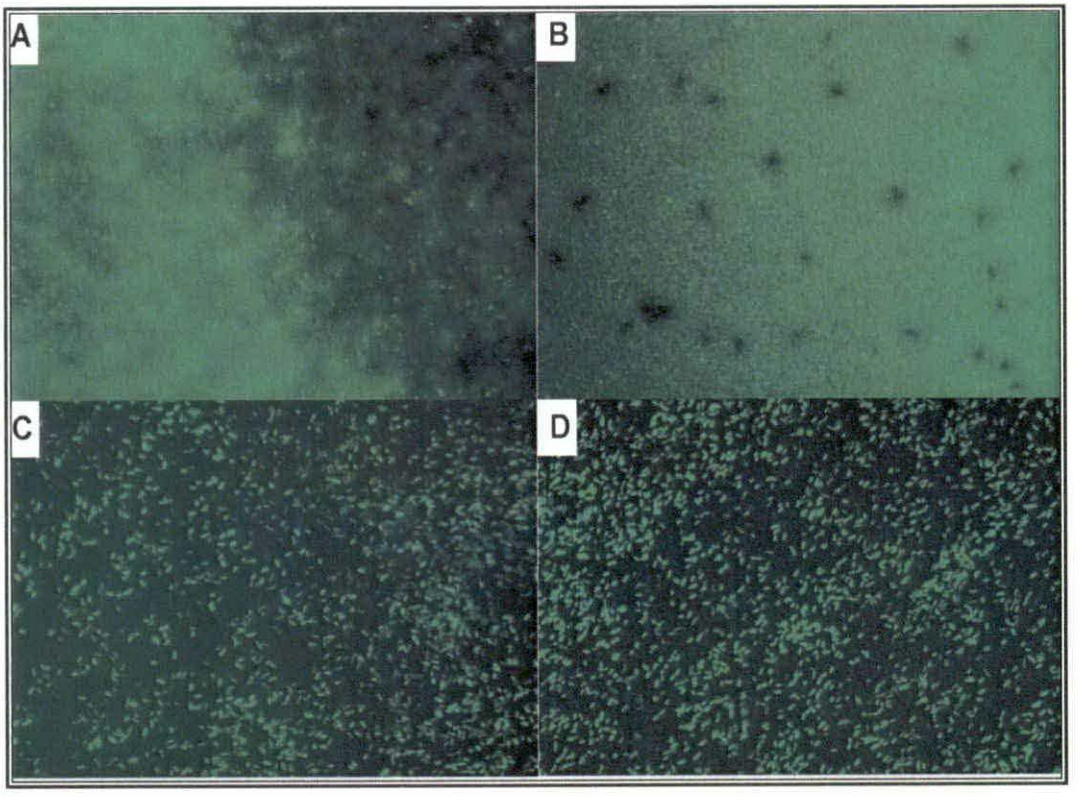


Figure 37. *Pseudomonas aeruginosa* PA01 (wild type) biofilms on a coverslip as a substrate under fluorescent microscope after 7 days in TSB. (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

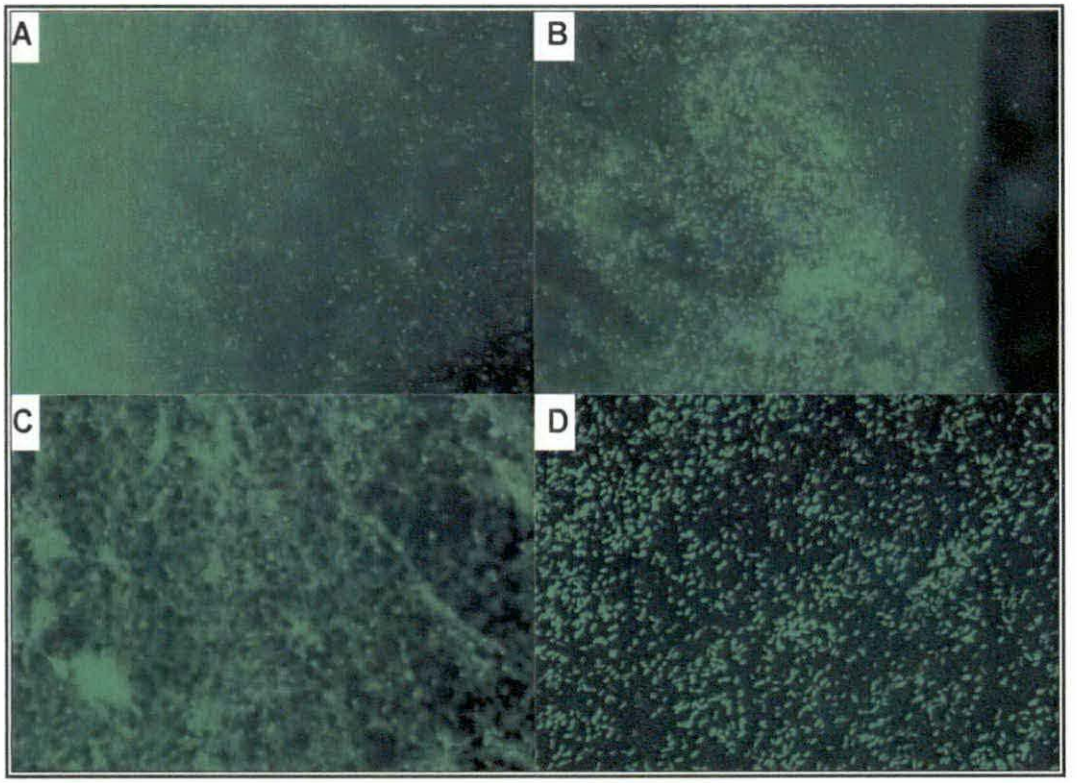


Figure 38. *Pseudomonas aeruginosa* PA01 (wild type) biofilms on a coverslip as a substrate under fluorescent microscope after 10 days in TSB. (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

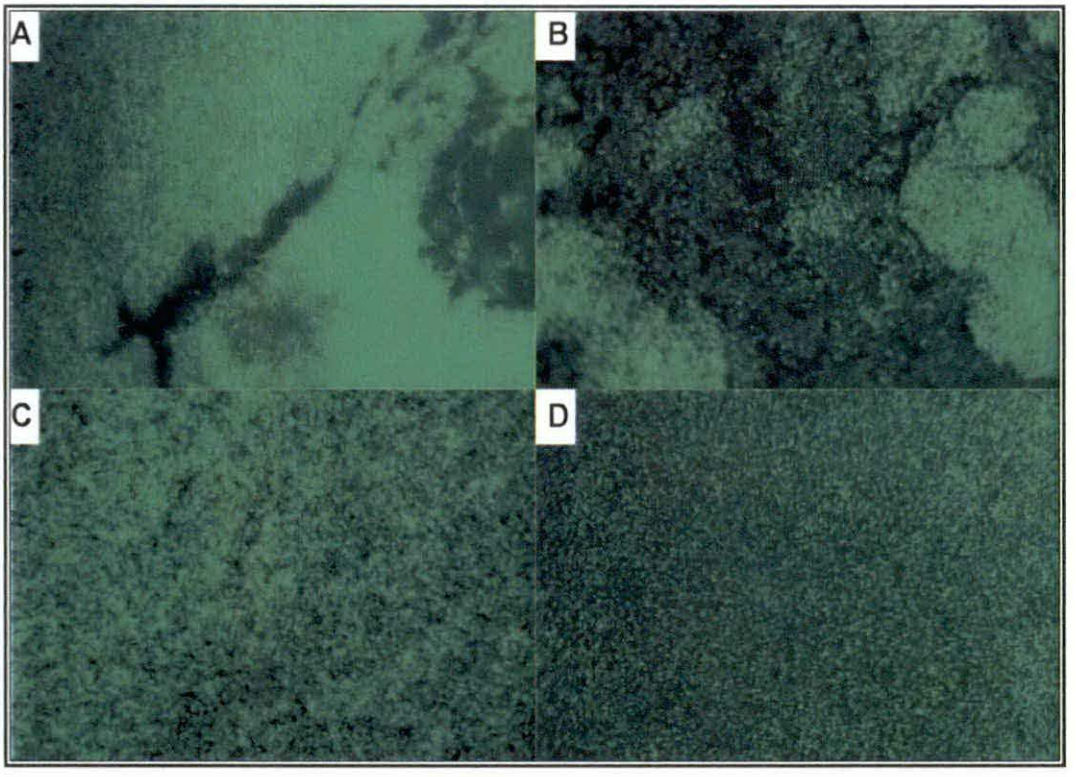


Figure 39. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on a coverslip as a substrate under fluorescent microscope after 1 day in TSB.
(A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

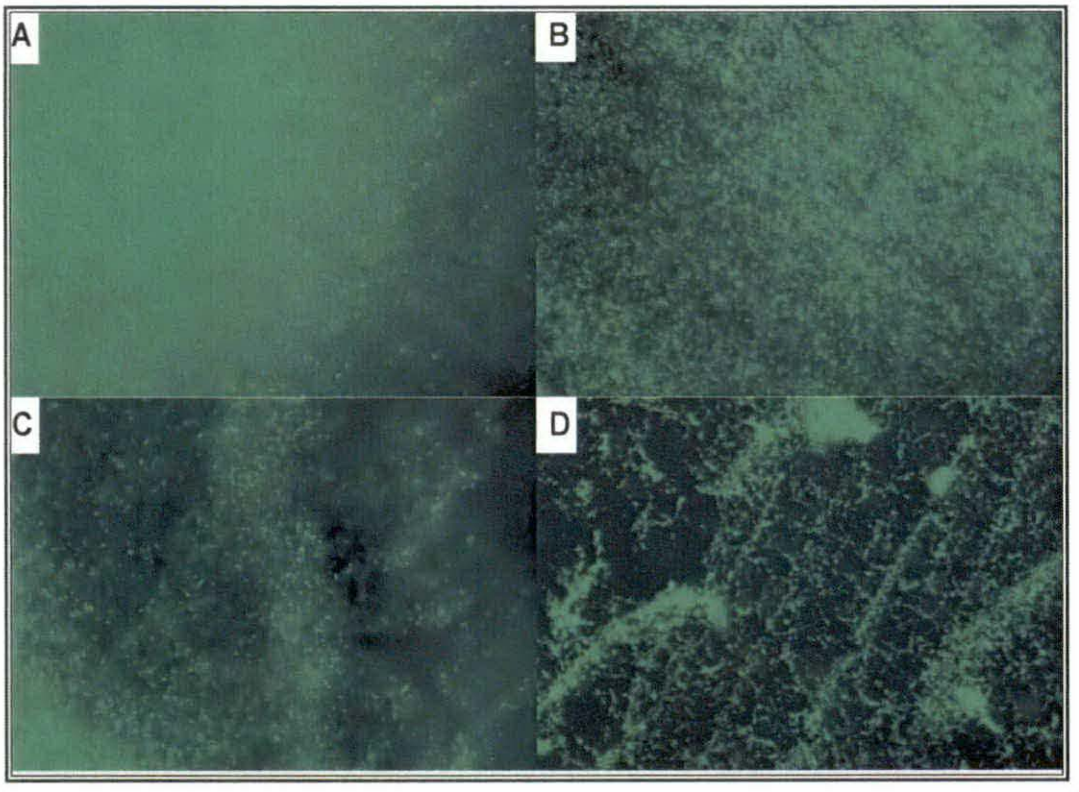


Figure 40. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on a coverslip as a substrate under fluorescent microscope after 3 days in TSB.
(A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

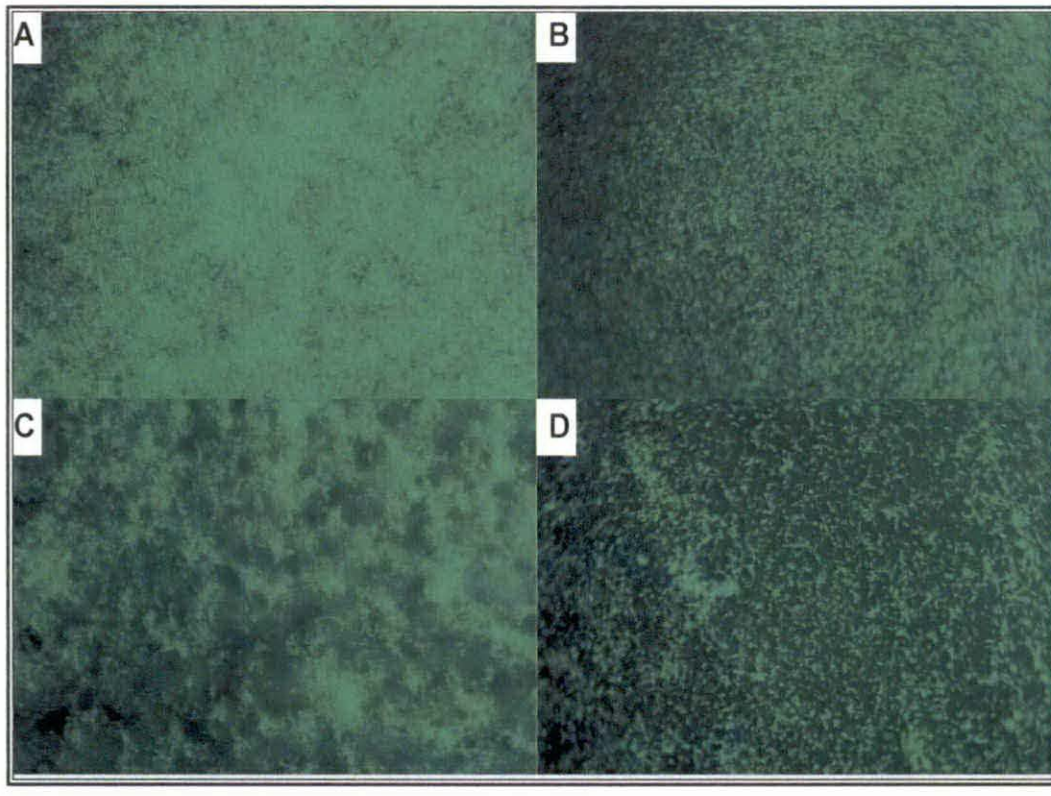


Figure 41. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on a coverslip as a substrate under fluorescent microscope after 7 days in TSB. (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

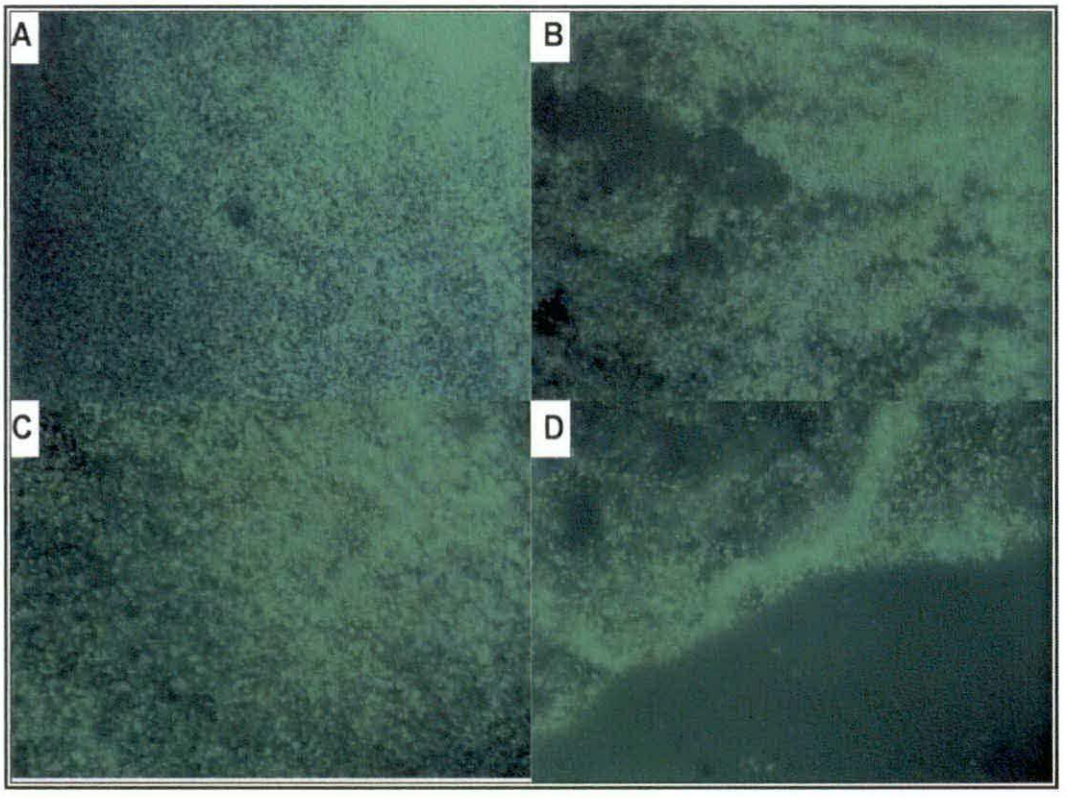


Figure 42. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on a coverslip as a substrate under fluorescent microscope after 10 days in TSB. (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

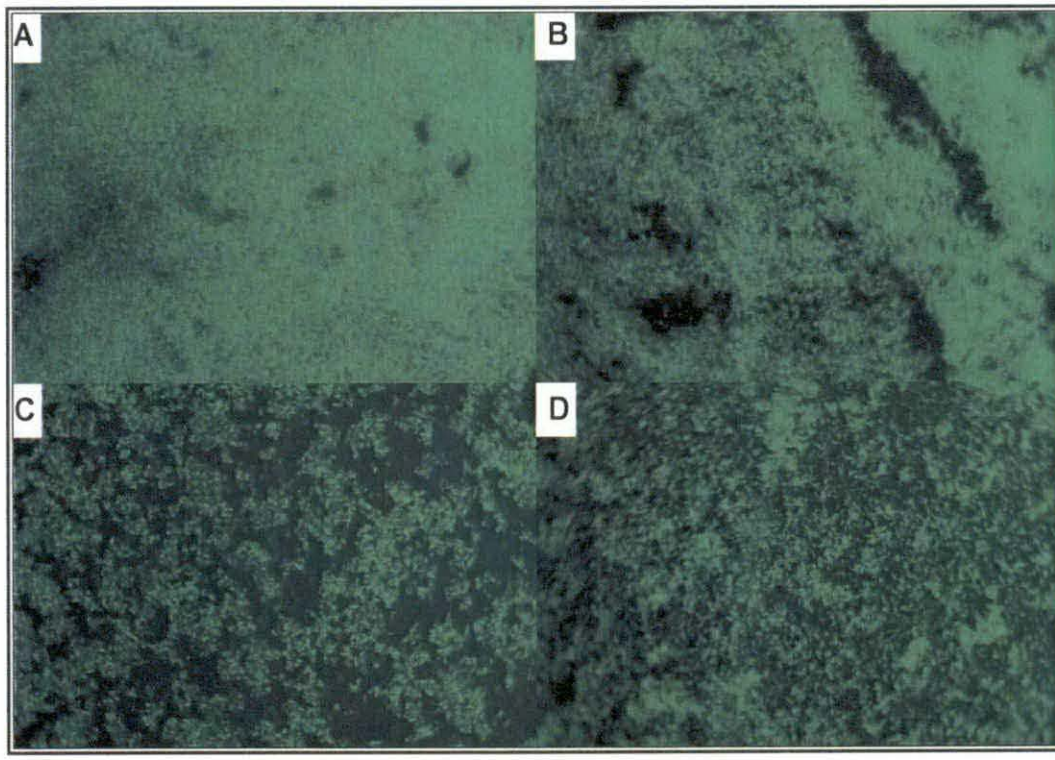


Figure 43. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on a coverslip as a substrate under fluorescent microscope after 1 day in TSB.
(A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

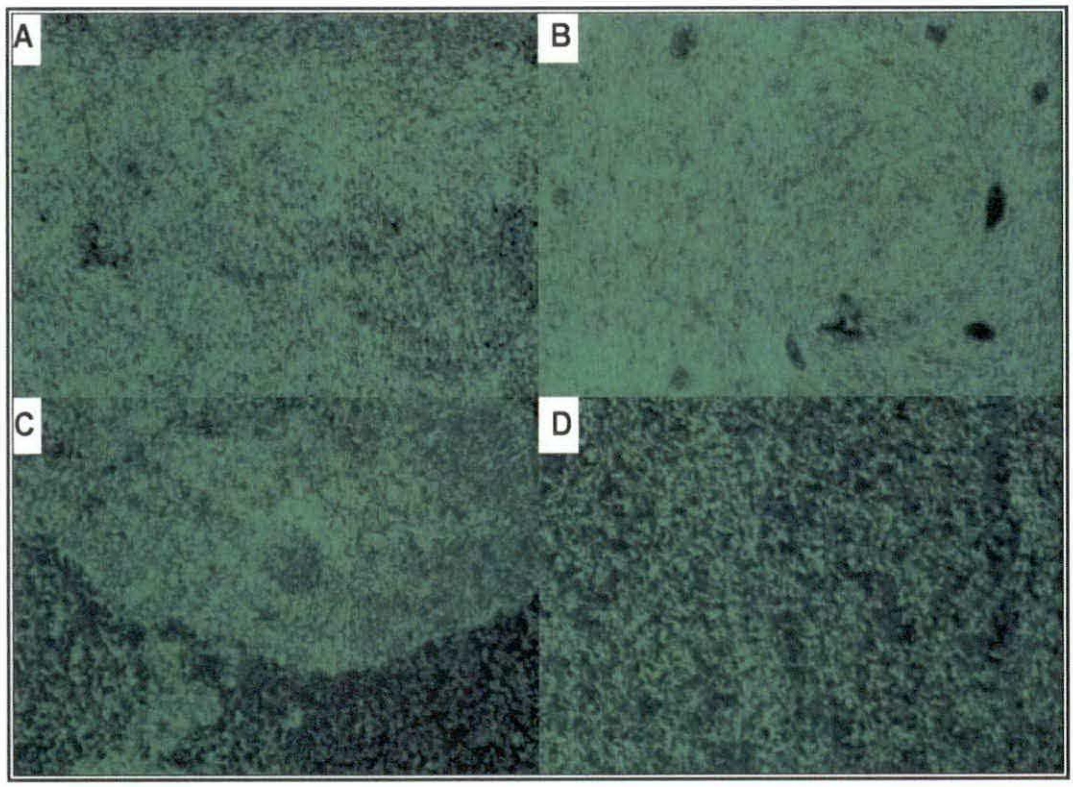


Figure 44. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on a coverslip as a substrate under fluorescent microscope after 3 days in TSB. (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

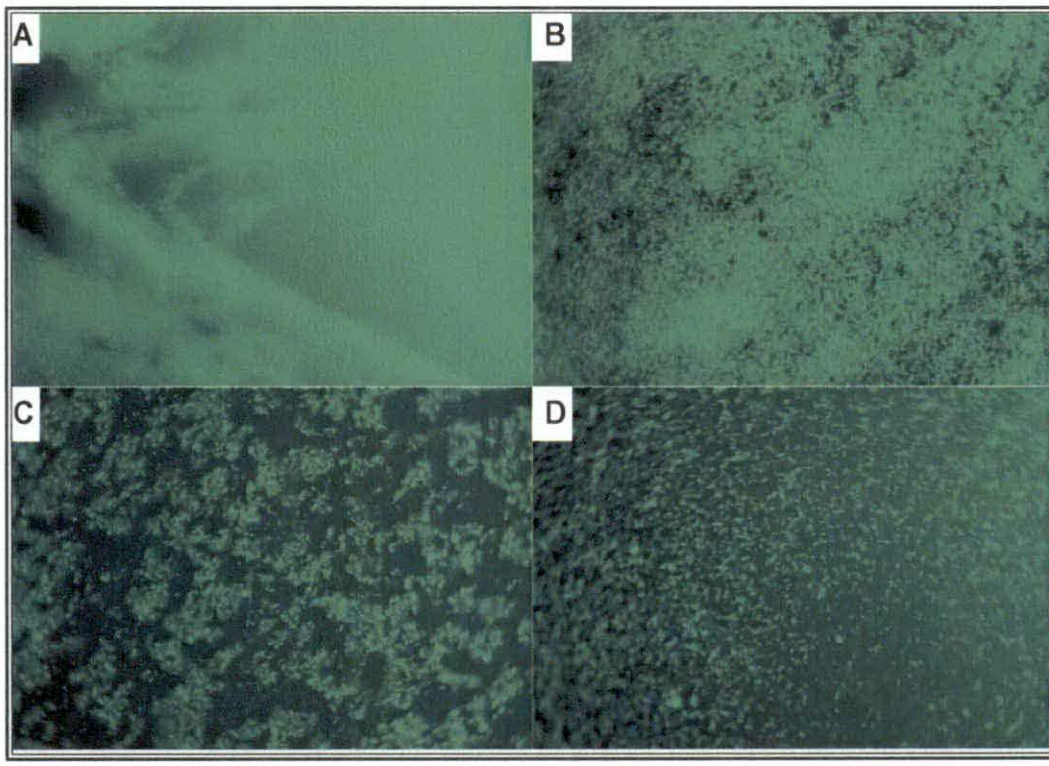


Figure 45. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on a coverslip as a substrate under fluorescent microscope after 7 days in TSB. (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

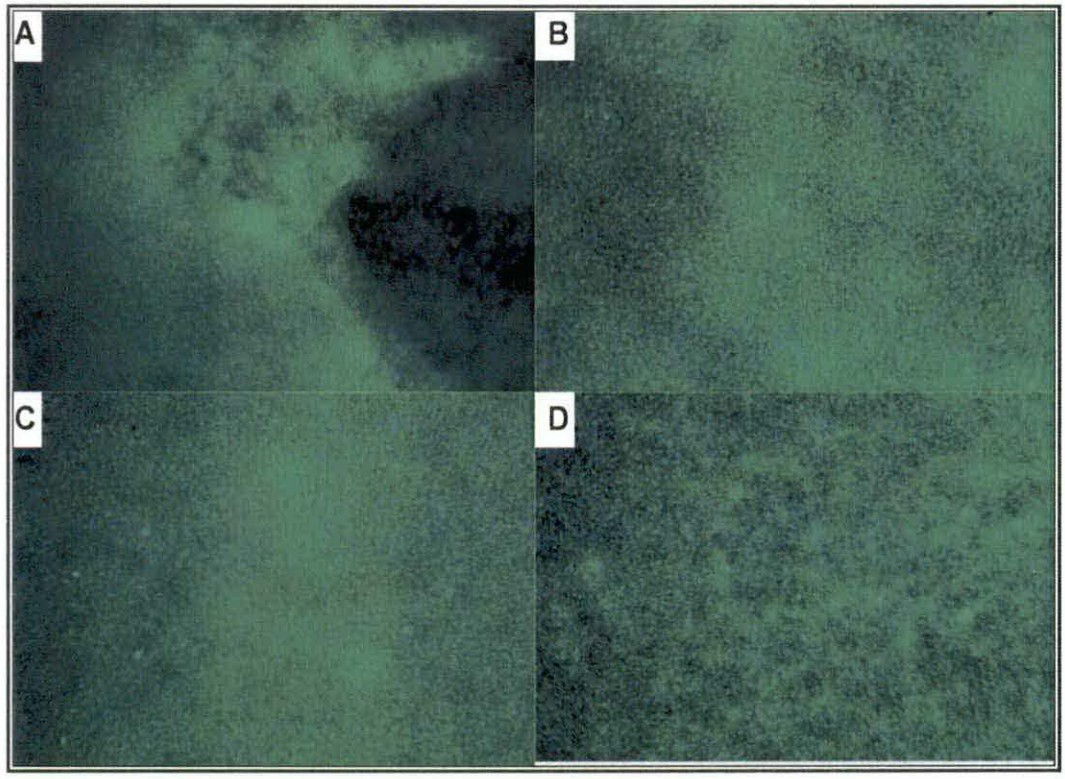


Figure 46. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on a coverslip as a substrate under fluorescent microscope after 10 days in TSB. (A) Control, (B) 4.7 μg/mL ginseng, (C) 150 μg/mL of ginseng, (D) 4800 μg/mL of ginseng.

Each of the three strains was first compared to the days of incubation. From Figures 35- 38, PAO1 (wild type) was analyzed from day 1 to day 10. It was seen that the biofilm thickness of the controls increased in thickness with the increasing days of incubation. Day 10 control indicated an increase in extracellular polymeric substance and lesser bacterial cells whereas day 1 showed more clusters of bacteria then the extracellular polymeric substance. Ginseng concentration of 4.7 μg/mL showed less biofilm covering for days 1, 3, 7, and 10 when compared to their controls. However, the ginseng concentration of 150 and 4800 μg/mL showed the

least amount of biofilm production even till day 10. From Figures 39- 42 it can be seen that for PDO100 (Δ *rhlI* mutant) the biofilm formation goes on increasing with increasing days of incubation. The ginseng concentration of 4.7 μg/mL showed inhibition of biofilm just for day 1; however, the 3rd, 7th and 10th day seem to have a pretty good biofilm formation but not as much as the control. The ginseng concentration of 150 μg/mL showed inhibition of biofilm formation for day 1 and 3 and then slowly declined. The ginseng concentration of 4800 μg/mL showed inhibition of biofilm throughout the 10 days with the best results on day 1 and day 3. In Figures 43- 46, it can be seen that for PJP1 (Δ *lasI* mutant) the biofilm formation goes on increasing with increasing days of incubation. The ginseng concentration of 4.7 μg/mL showed some effect only on day 1 and 150 μg/mL concentration of ginseng shows inhibition of biofilm on day 1, 3 and 7. The ginseng concentration of 4800 μg/mL showed inhibition of biofilm to some extent even on day 10.

The three strains were compared to one another without ginseng, at each of the 4 incubation days. The PAO1 (wild type) had more biofilm growth for day 1 when compared to PDO100 (Δ *rhlI* mutant) and PJP1 (Δ *lasI* mutant). However for day 3, 7 and 10 the biofilm growths were similar in all three strains.

The effect of each ginseng concentration on biofilm formation to the control culture was compared for each strain. It was seen that for PAO1 (wild type) the ginseng concentration of 150 μg/mL and 4800 μg/mL showed tremendous biofilm

inhibition. For quorum sensing mutant strains PDO100 (Δ *rhlI* mutant) and PJP1 (Δ *lasI* mutant), the ginseng concentration of 4800 μg/mL showed the best biofilm inhibition when compared to their respective controls.

Finally, the three strains were compared to one another under the experimental conditions (i.e., ginseng's effect on biofilm formation). The most effect of ginseng was seen in the PAO1 (wild type strain) followed by PJP1 (Δ *lasI* mutant) and then PDO100 (Δ *rhlI* mutant).

The florescence microscopy results were similar to the SEM results.

Confocal Laser Scanning Microscopy Analysis

By using confocal microscopy, biofilm thickness was analyzed (Figures 47-58). The biofilm thickness was measured for each of the samples. The biofilm thickness was measured using the XZY plane which looked at the side view thus visualizing the cross section for the thickness of the biofilm. The thickness of the biofilm appears fluorescent green on a black background by visual inspection of confocal microscopy images. A live scan of the image was done and then a snap shot was acquired of the image. The biofilm thickness was then measured using the confocal microscopy software. Here a representative example of one complete experiment is shown. Statistical data for the biofilm thickness was also analyzed using Minitab, release 15. The statistical analysis gives a mathematical interpretation of the images and will help in supporting the descriptive results from the scanning electron microscopy and fluorescence microscopy. Analysis of variance (ANOVA) will be the model used for the statistical analysis.

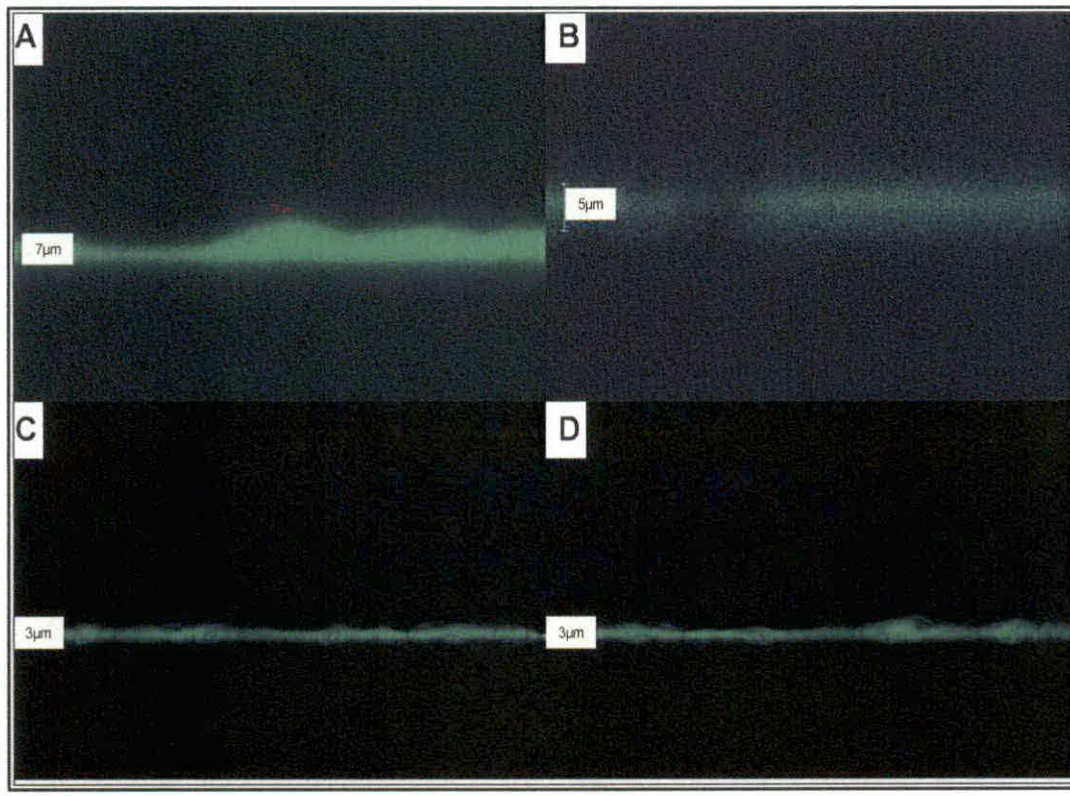


Figure 47. *Pseudomonas aeruginosa* PA01 (wild type) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 1 day in TSB.
Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (7µm), (B) 4.7 µg ginseng (5µm), (C) 150 µg of ginseng (3µm), (D) 4800 µg of ginseng (3µm).

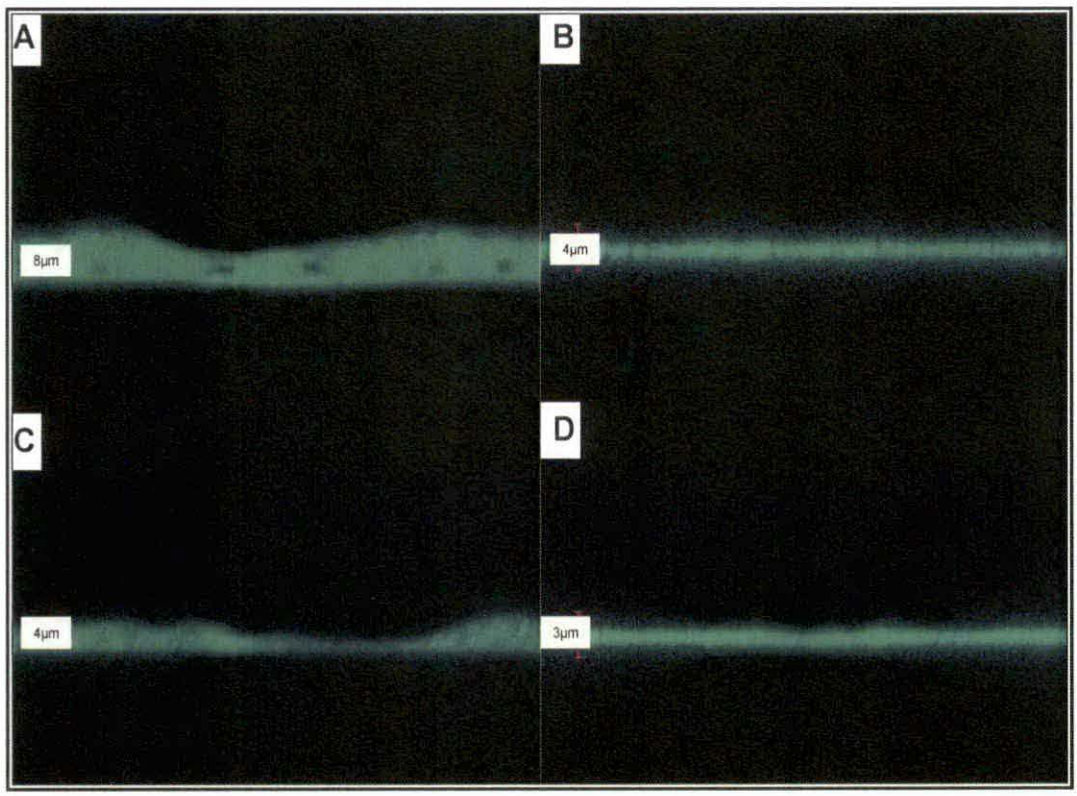


Figure 48. *Pseudomonas aeruginosa* PA01 (wild type) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 3 days in TSB.
Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (8µm), (B) 4.7 µg/mL ginseng (4µm), (C) 150 µg/mL of ginseng (4µm), (D) 4800 µg/mL of ginseng (3µm).

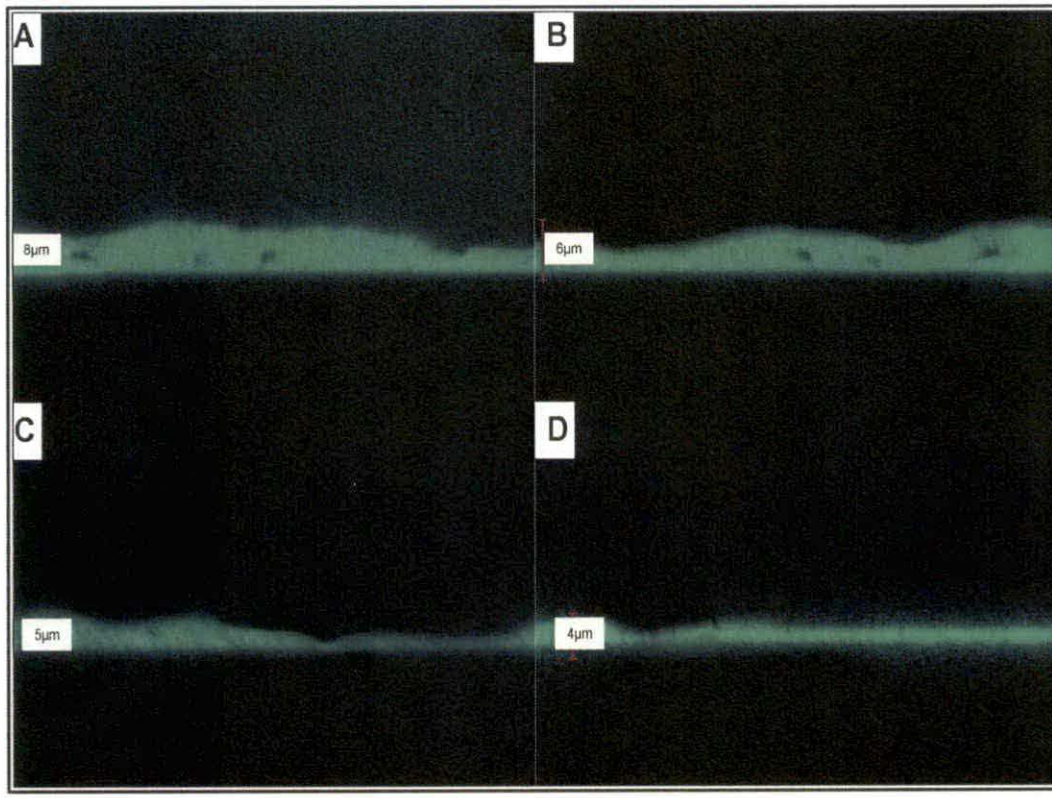


Figure 49. *Pseudomonas aeruginosa* PA01 (wild type) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 7 days in TSB.
Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (8µm), (B) 4.7 µg/mL ginseng (6µm), (C) 150 µg/mL of ginseng (5µm), (D) 4800 µg/mL of ginseng (4µm).

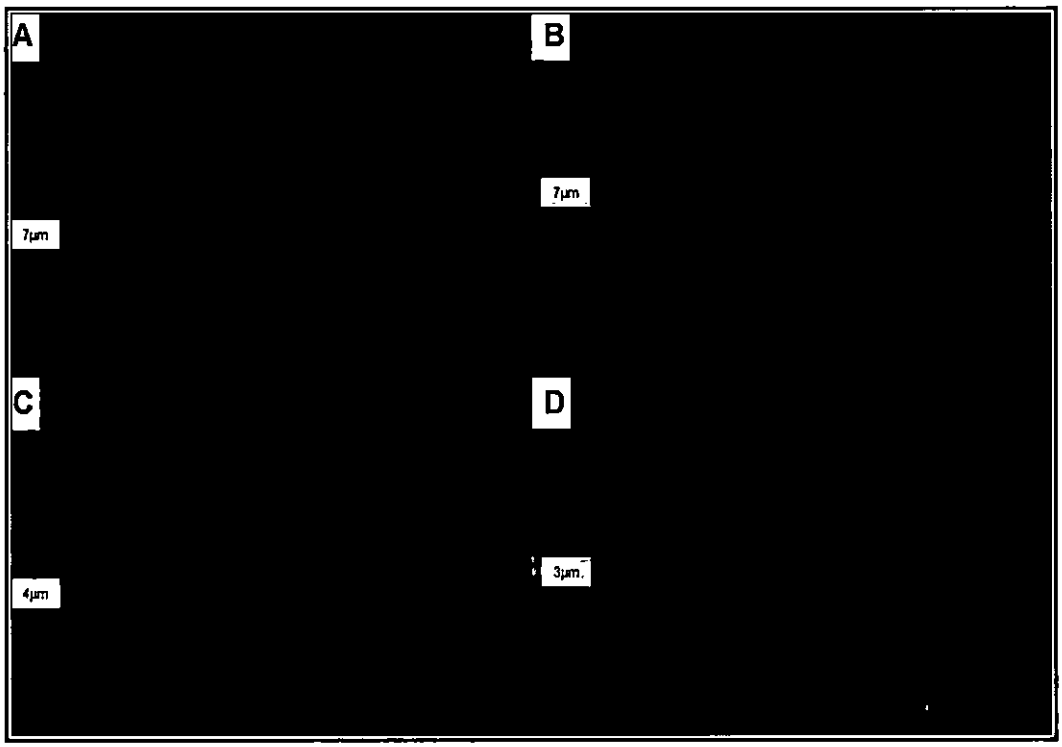


Figure 50. *Pseudomonas aeruginosa* PA01 (wild type) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 10 days in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (7µm), (B) 4.7 µg/mL ginseng (7µm), (C) 150 µg/mL of ginseng (4µm), (D) 4800 µg/mL of ginseng (3µm).

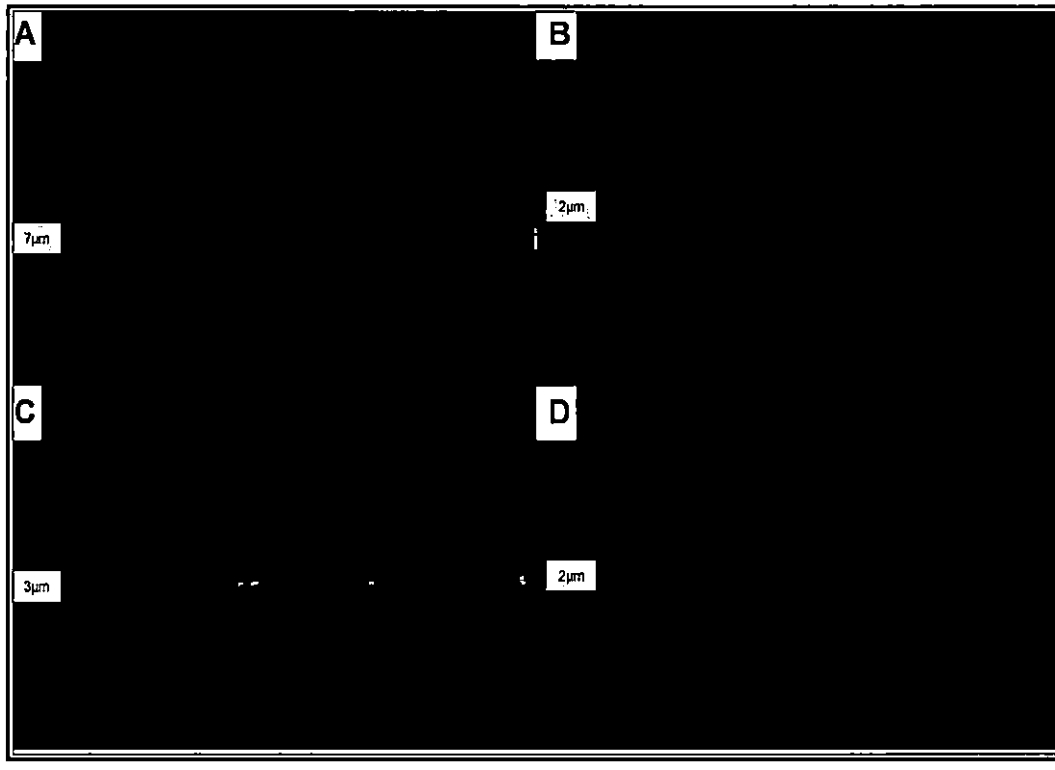


Figure 51. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 1 day in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (7µm), (B) 4.7 µg/mL ginseng (2µm), (C) 150 µg/mL of ginseng (3µm), (D) 4800 µg/mL of ginseng (2µm).

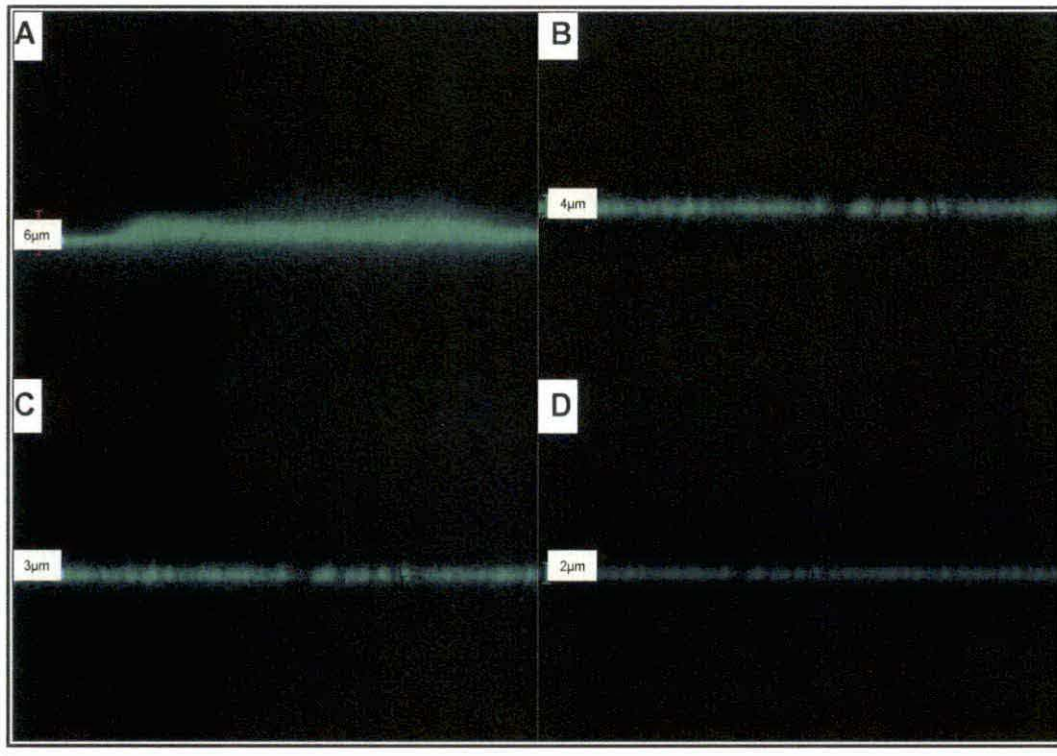


Figure 52. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 3 days in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (6µm), (B) 4.7 µg/mL ginseng (4µm), (C) 150 µg/mL of ginseng (3µm), (D) 4800 µg/mL of ginseng (2µm).

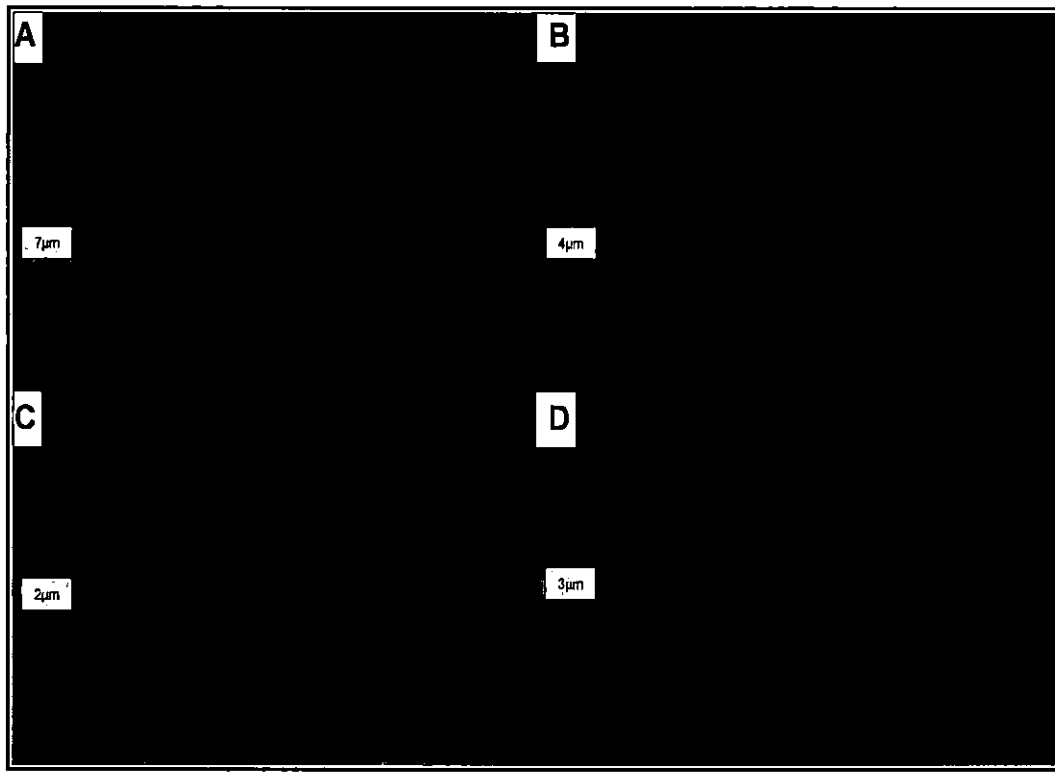


Figure 53. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 7 days in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (7μm), (B) 4.7 μg/mL ginseng (4μm), (C) 150 μg/mL of ginseng (2μm), (D) 4800 μg/mL of ginseng (3μm).

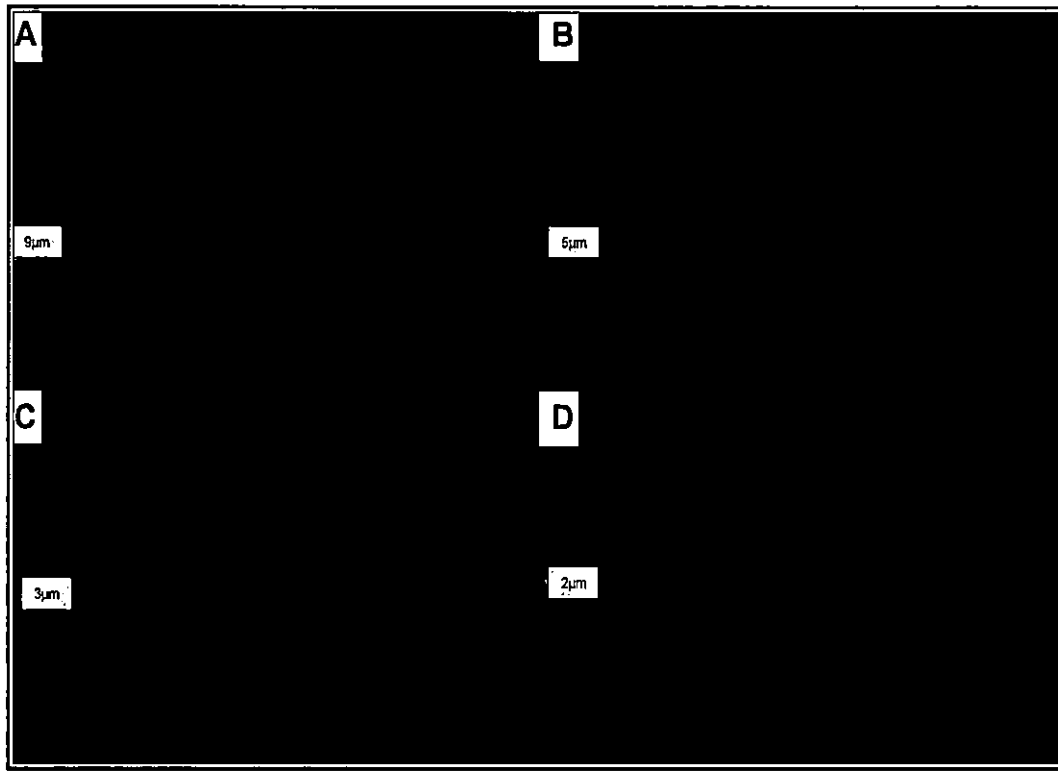


Figure 54. *Pseudomonas aeruginosa* PDO100 (Δ *rhlI* mutant) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 10 days in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (9µm), (B) 4.7 µg/mL ginseng (5µm), (C) 150 µg/mL of ginseng (3µm), (D) 4800 µg/mL of ginseng (2µm).

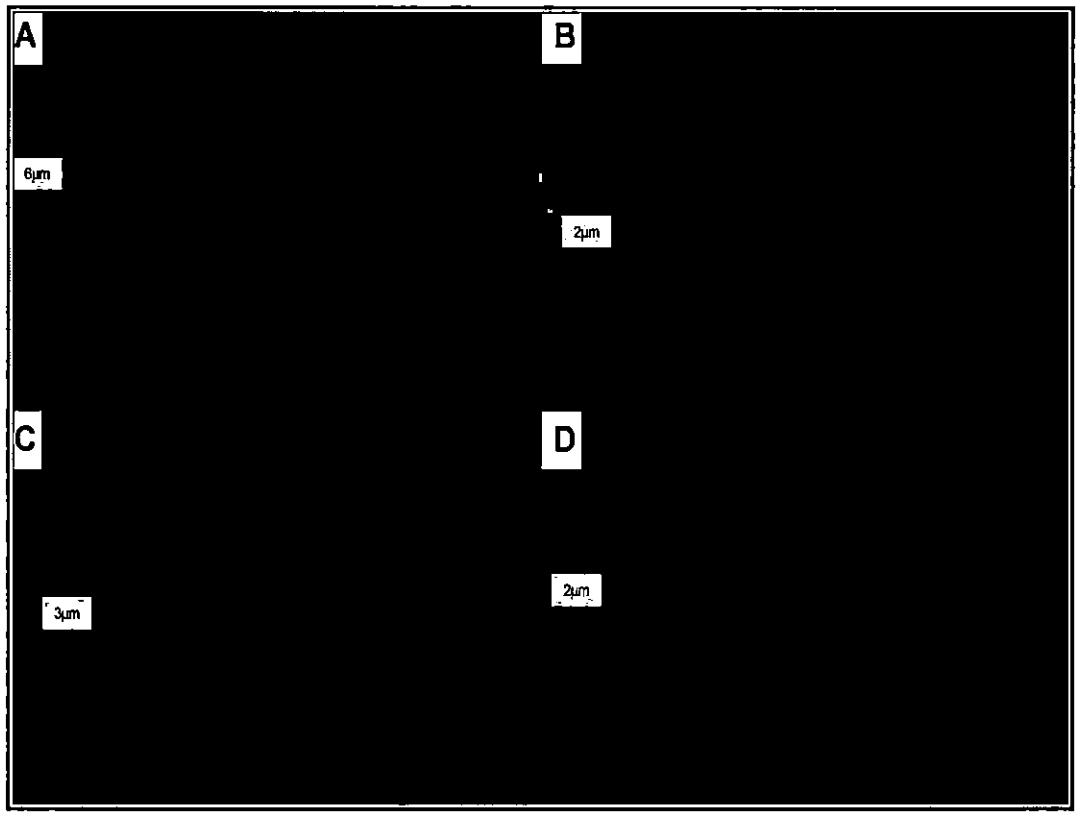


Figure 55. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 1 day in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (6µm), (B) 4.7 µg/mL ginseng (2µm), (C) 150 µg/mL of ginseng (3µm), (D) 4800 µg/mL of ginseng (2µm).

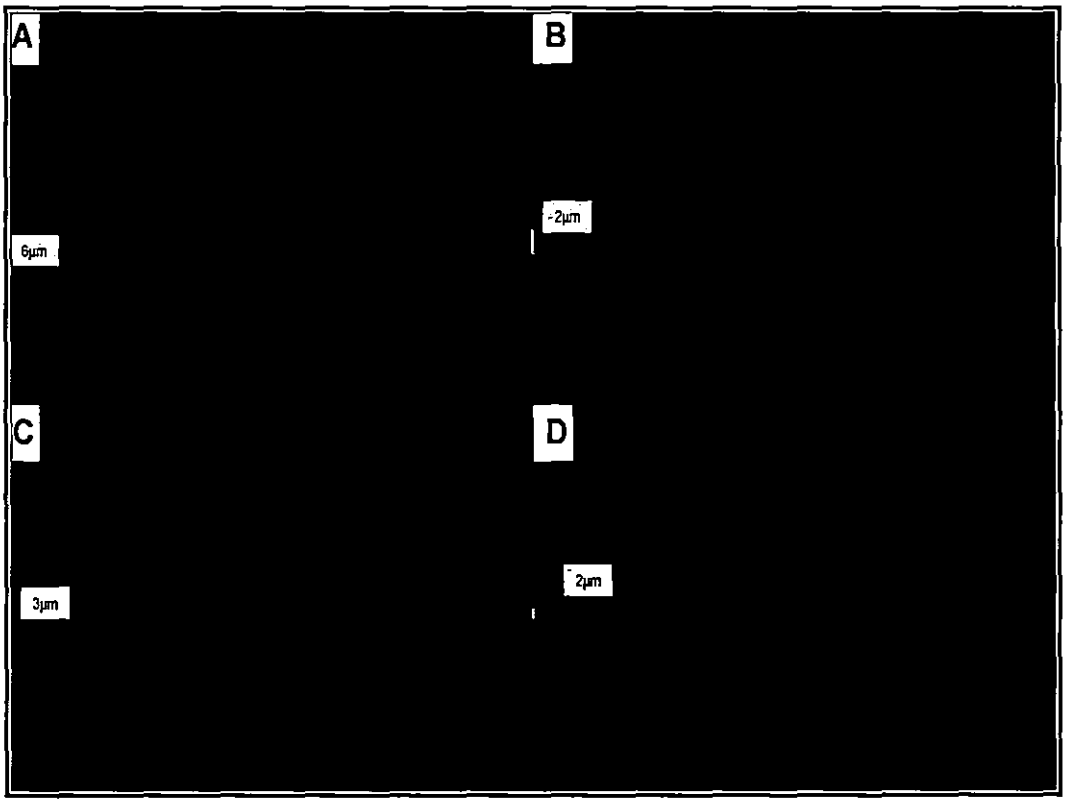


Figure 56. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 3 days in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (6µm), (B) 4.7 µg/mL ginseng (2µm), (C) 150 µg/mL of ginseng (3µm), (D) 4800 µg/mL of ginseng (2µm).

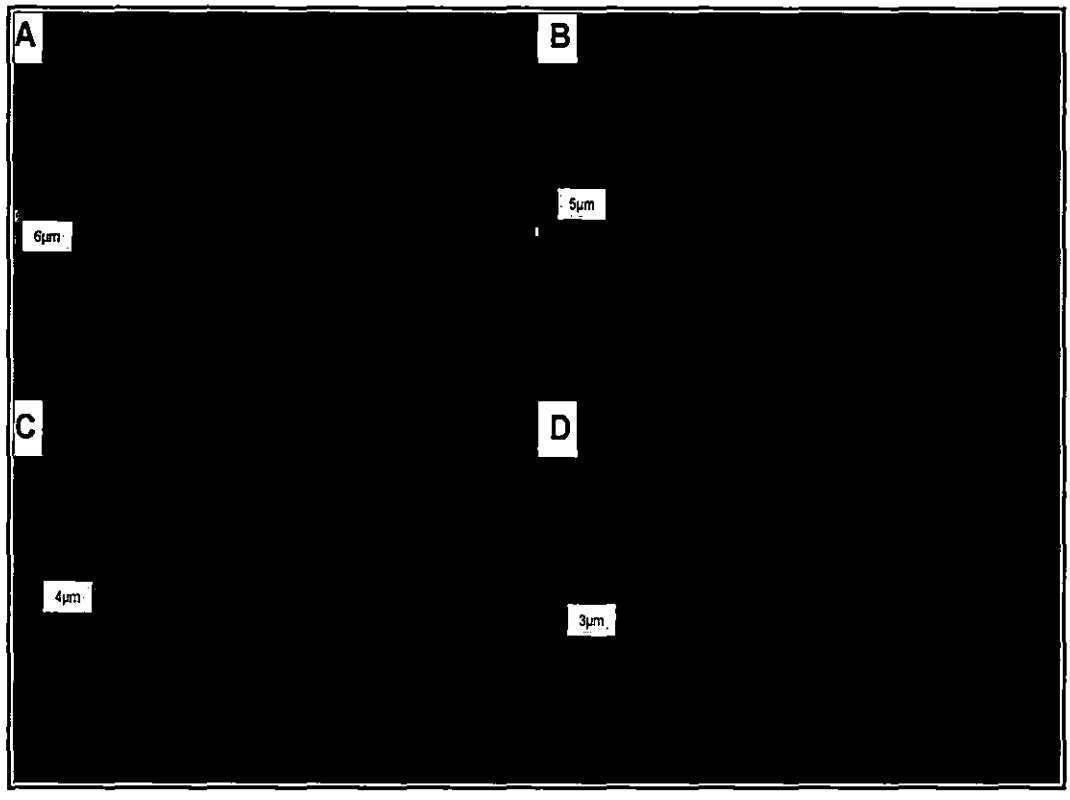


Figure 57. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 7 days in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (6µm), (B) 4.7 µg/mL ginseng (5µm), (C) 150 µg/mL of ginseng (4µm), (D) 4800 µg/mL of ginseng (3µm).

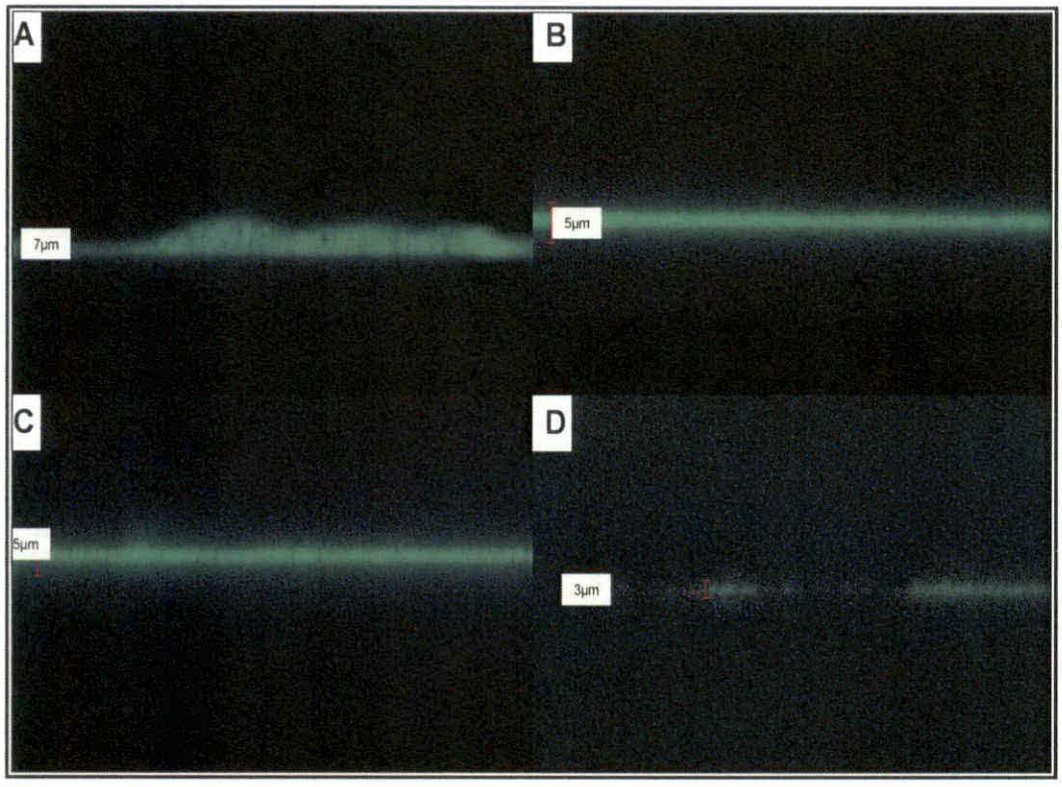


Figure 58. *Pseudomonas aeruginosa* PJP1 (Δ *lasI* mutant) biofilms on a coverslip as a substrate under Confocal laser scanning microscope after 10 days in TSB. Here the XZY plane (side view) shows the cross section visualizing the thickness of the biofilm. (A) Control (7µm), (B) 4.7 µg/mL ginseng (5µm), (C) 150 µg/ml of ginseng (5µm), (D) 4800 µg/mL of ginseng (3µm).

Figures 47 -58 show the different biofilm thickness for the three different bacterial strains with three different concentrations on four different days. The biofilm thickness was then tabulated in Table 2 for statistical analysis.

Table 6. Biofilm thickness data for bacterial strain, PAO1 (wild type). The average data represented includes ± the standard error.

			Ginseng Concentrations		
	DAY	**Control**	**4.7μg/mL**	**150μg/mL**	**4800μg/mL**
	1	7	5	3	3
	3	8	4	4	3
	7	8	6	5	4
Exp. 1	10	7	7	4	3
	1	7	4	2	2
	3	8	5	3	3
	7	8	6	4	3
Exp. 2	10	9	6	5	4
	1	7	4.5±0.5	2.5±0.5	2.5±0.5
	3	8	4.5±0.5	3.5±0.5	3±0.5
Avg of Exp. 1 and	7	8	6±0.5	4.5±0.5	3.5±0.5
2	10	8	6.5±1.5	4.5±0.5	3.5±0.5

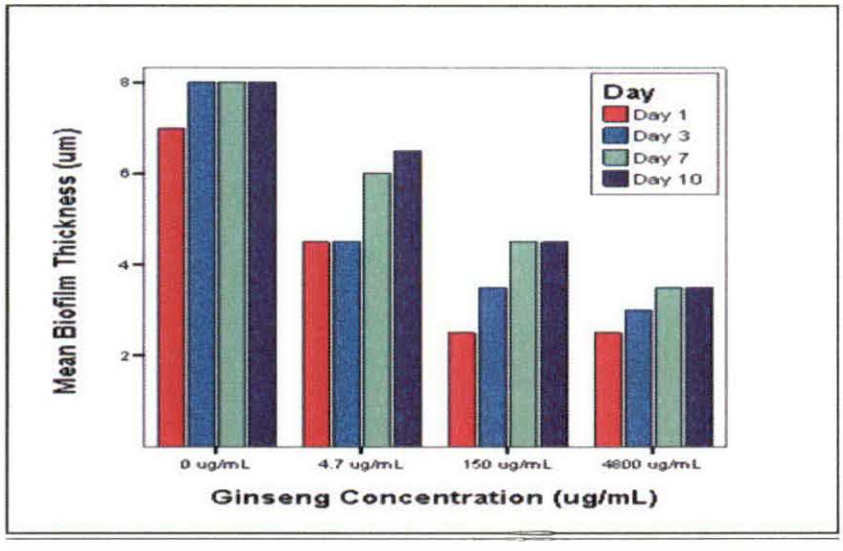


Figure 59. The biofilm thickness of the strain PAO1 analyzed using a t-test by comparing the different ginseng concentration for each day to its control. All of them were statistically insignificant.

Table 7. Biofilm thickness data for bacterial strain, PDO100 (Δ *rhlI* mutant). The average data represented includes ± the standard error.

			Ginseng Concentrations µg/mL		
	DAY	Control	4.7	150	4800
	1	7	2	3	2
	3	6	4	3	2
	7	7	4	2	3
Exp. 1	10	9	5	3	2
	1	8	3	2	2
	3	7	3	2	2
	7	9	4	3	3
Exp. 2	10	7	4	4	3
	1	7.5	2.5±0.0	2.5±1.0	2±0.5
	3	6.5	3.5±1.0	2.5±1.0	2±0.5
	7	8	4±1.0	2.5±0.5	3±1.0
Avg of Exp 1 and 2	10	8	4.5±0.5	3.5±1.5	2.5±1.5

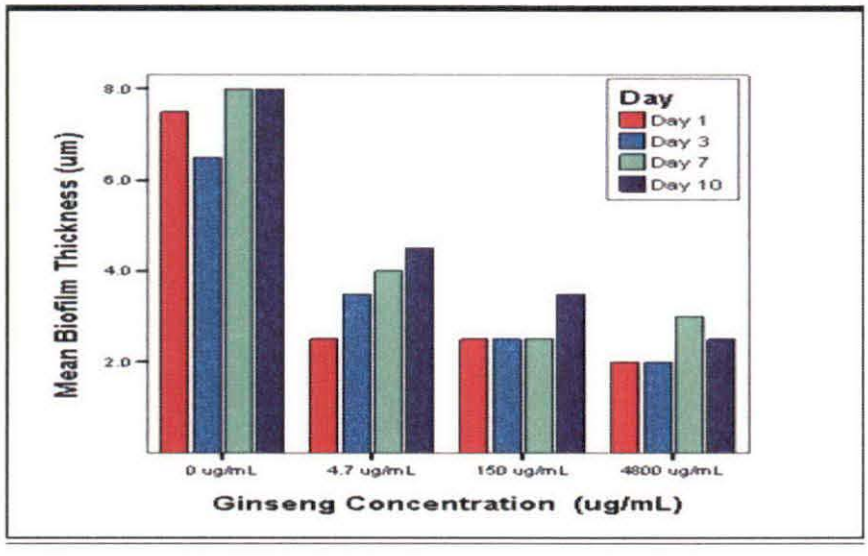


Figure 60. The biofilm thickness of the strain PDO100 analyzed using a t-test by comparing the different ginseng concentration for each day to its control. All of them were statistically insignificant.

Table 8. Biofilm thickness data for bacterial strain, PJP1 (Δ lasI mutant). The average data represented includes ± the standard error.

			Ginseng Concentrations μg/mL		
	DAY	**Control**	**4.7**	**150**	**4800**
	1	6	2	3	2
	3	6	2	3	2
	7	6	5	4	3
Exp. 1	10	7	5	5	3
	1	6	3	3	2
	3	7	4	4	3
	7	7	4	4	3
Exp. 2	10	7	5	5	3
	1	6	2.5±0.5	3±0.5	2±0.5
	3	6.5	3±0.5	3.5±0.0	2.5±0.0
	7	6.5	4.5±1.0	4±0.5	3±0.5
Avg of Exp. 1 and 2	10	7	5±0.5	5±0.5	3±0.5

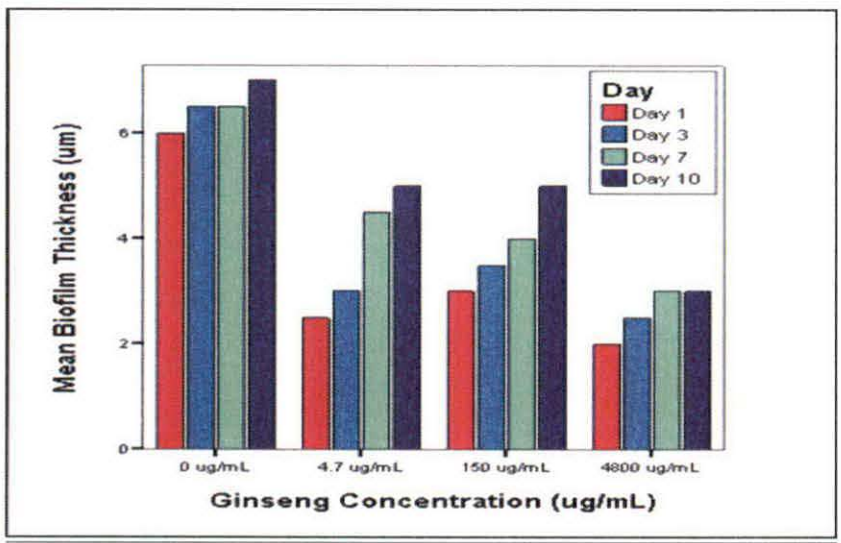


Figure 61. The biofilm thickness of the strain PJP1 analyzed using a t-test by comparing the different ginseng concentration for each day to its control. All of them were statistically insignificant.

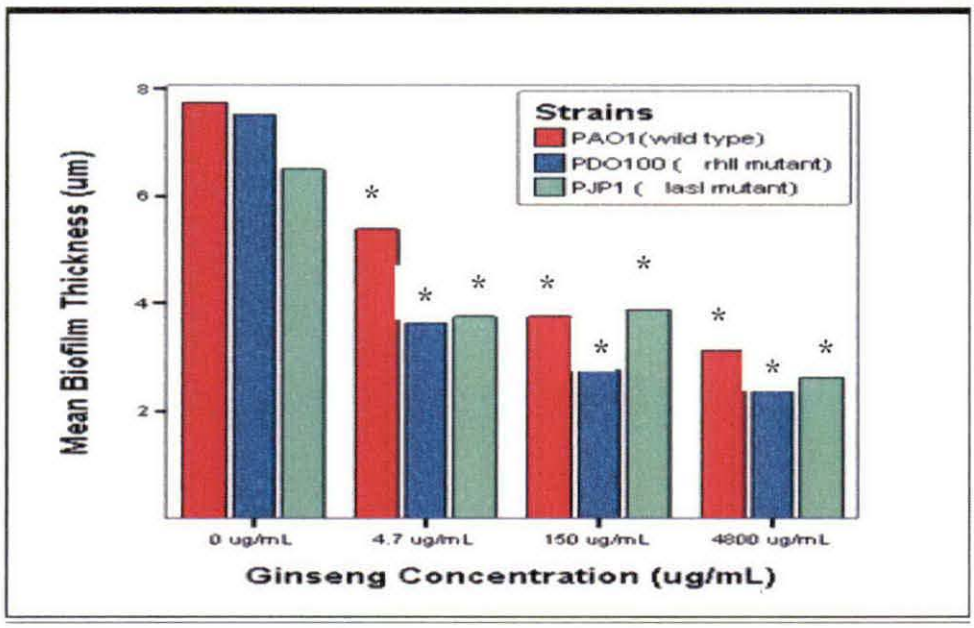


Figure 62. The biofilm thickness of all the three strains analyzed using a t-test by comparing the different ginseng concentration to its control. All the three concentrations of ginseng were statistically significant. Asterisks denote significance.

From Figures 59-61, it was seen that when the different ginseng concentrations were compared to their respective controls for each day for all the four days for each of the three strains they were statistically insignificant. However, when the biofilms were analyzed (Figure 62) by comparing the three different ginseng concentrations on the basis of each individual strains showed them to be statistically significant. Thus, showing that there was a significant effect of ginseng concentration for each strain.

ANOVA Analysis

An ANOVA analysis was done for PAO1 (wild type) to look at the effect of ginseng concentrations and days on the biofilm thickness.

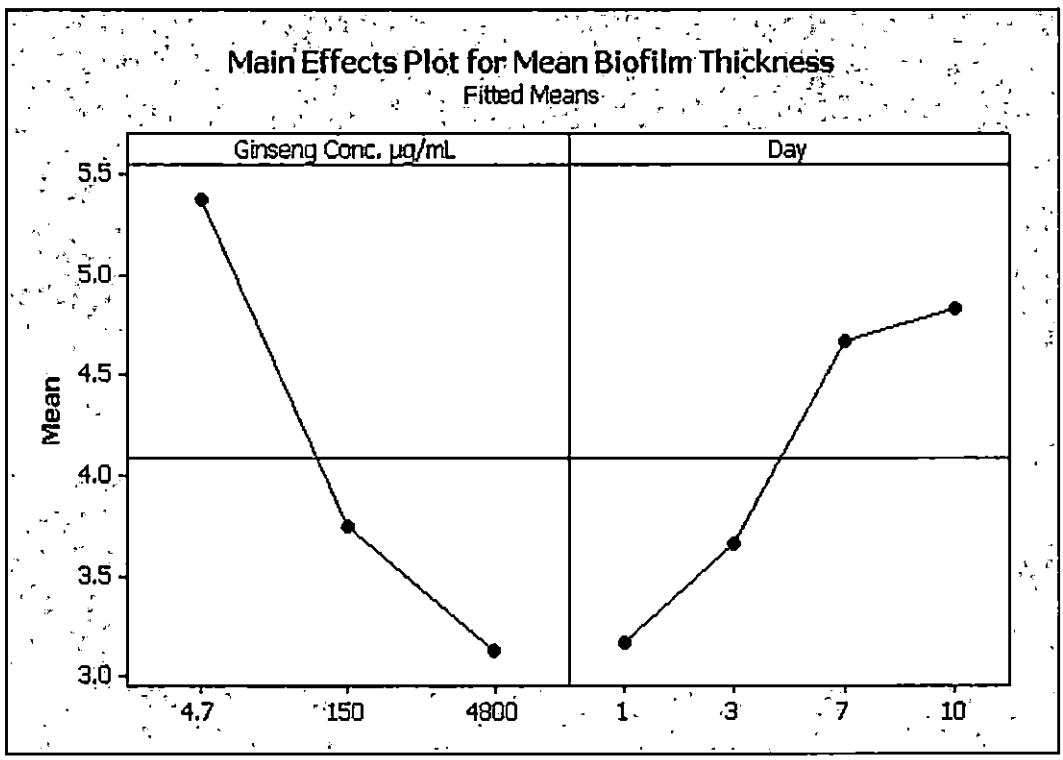


Figure 63. Main effects plot of the mean biofilm thickness for PAO1, the three different concentrations of ginseng and four different days 1, 3, 7 and 10.

From Figure 63, it was seen that the main effects plot for the ginseng concentration showed that the biofilm thickness decreased with the increasing concentration of ginseng for PAO1 (wild type). The main effects plot for day showed that the biofilm thickness increased with increasing number of days.

Further analysis showed that these differences in thickness were significant for the four different days and the three different concentrations of ginseng.

As the assumption of Independence, normality and equal variance were not violated this ANOVA model was valid. The R^2 value for this experiment was 94.98% which was good and shows that more variance was accounted for. The adjusted R^2 value was 90.79 % which was close to the R^2. This showed that other factors would not be that effective. Interactions between the factors were also not required as they were insignificant when an ANOVA, was run on them (not shown here due to their insignificance). From the ANOVA it was seen that the highest ginseng concentration worked the best on the first day showing the least biofilm growth (thickness).

An ANOVA analysis was done for PDO100 (Δ *rhlI* mutant) to look at the effect of ginseng concentrations and days on the biofilm thickness.

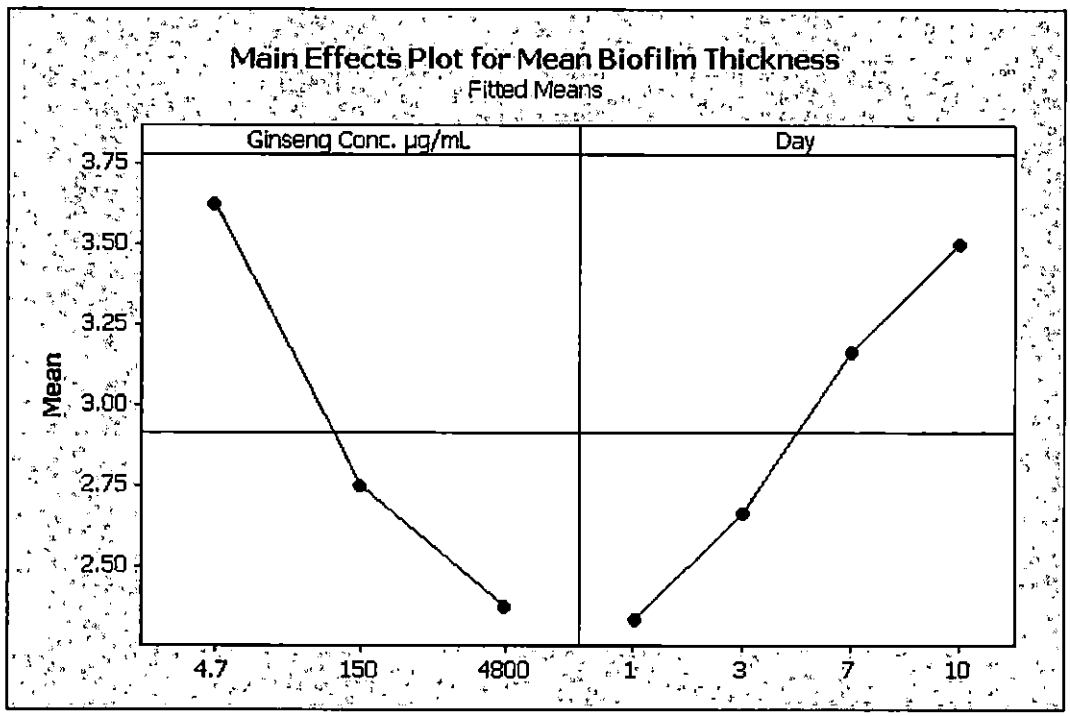


Figure 64. Main effects plot of the mean biofilm thickness for PDO100, the three different concentrations of ginseng and four different day 1,3 ,7 and 10.

From Figure 64, it can be seen that the main effects plot for the ginseng concentration showed that the biofilm thickness decreased with the increased concentration of ginseng for PDO100 (Δ *rhlI* mutant). The mean biofilm thickness was lower in PDO100 then it was in PAO1. The main effects plot for day showed that the biofilm thickness increased with increased number of days. Even here it showed that the mean biofilm thickness was lower in PDO100 then it was is PAO1. Further analysis showed that these differences in thickness were significant for the four different days and the three different concentrations of ginseng. As the

assumption of Independence, normality and equal variance were not violated this ANOVA model was valid. The R^2 value for this experiment was 82.53 % which was good and showed that more variance was accounted for. The adjusted R^2 value is 67.97 % which was not very close to the R^2. From the ANOVA, it was seen that the highest ginseng concentration worked the best on the first day showing the least biofilm growth (thickness).

An ANOVA analysis was done for PJP1 (Δ *lasI* mutant) to look at the effect of ginseng concentrations and days on the biofilm thickness.

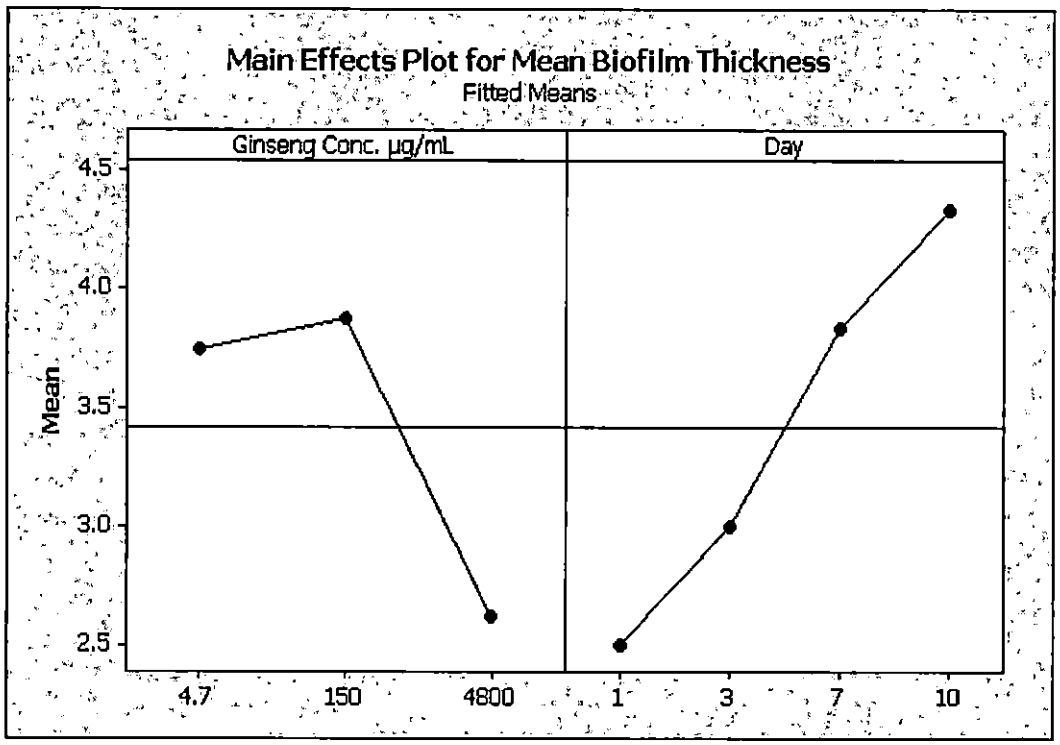


Figure 65. Main effects plot of the mean biofilm thickness for PJP1, the three different concentrations of ginseng and four different days 1, 3, 7 and 10.

From Figure 65, it was seen that the main effects plot for the ginseng concentration showed that the biofilm thickness was increased for the 150 µg/ml concentration of ginseng. The main effects plot for day showed that the biofilm thickness increased with increased number of days. Further analysis showed that these differences in thickness were significant for the four different days and the three different concentrations of ginseng.As the assumption of Independence, normality and equal variance were not violated this ANOVA model was valid. The

R^2 value for this experiment was 90.46% which was good and showed that more variance was accounted for. This model with ginseng concentration and day worked really well for the PJP1 strain. The adjusted R^2 value was 82.51 % which was not very close to the R^2. From the ANOVA it was seen that the highest ginseng concentration worked the best on the first day showing the least biofilm growth (thickness). Even though the 150 μg/mL showed an increase in the biofilm thickness.

An ANOVA analysis was done to see the biofilm thickness by the effect of all the three factors consisting of bacterial strains, ginseng concentrations and days of incubation. Each of these factors were independent of each other.

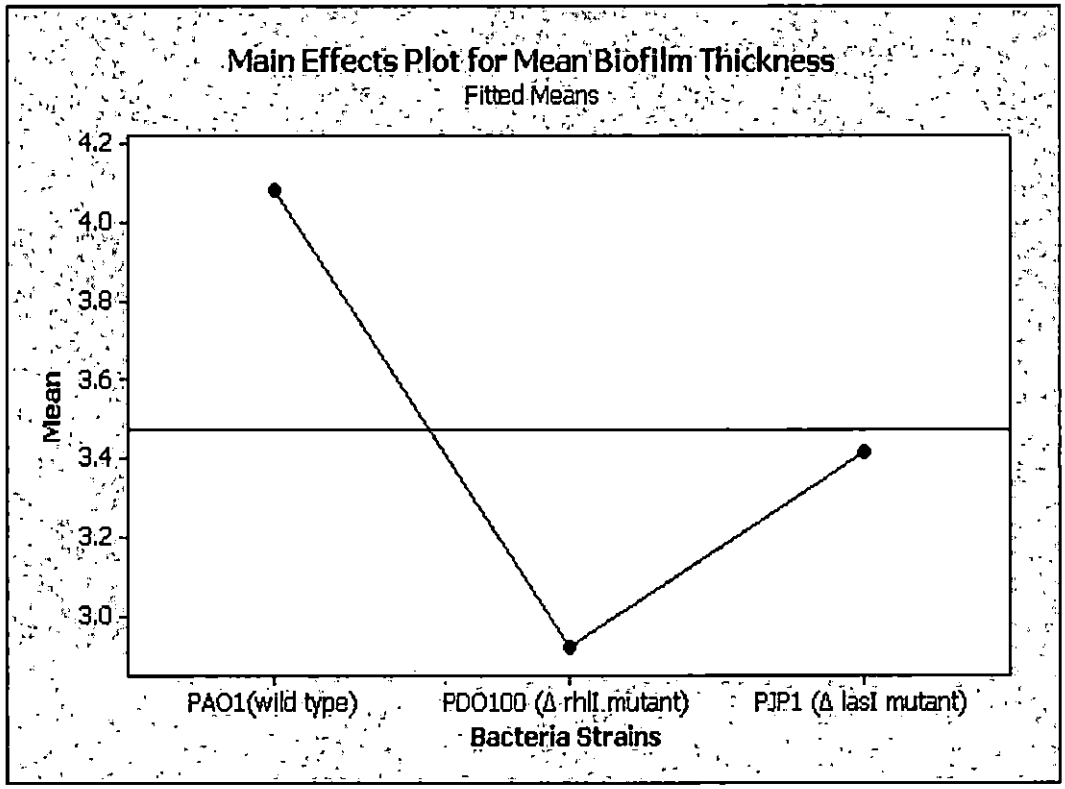


Figure 66. Main effects plot of the mean biofilm thickness for the three different strains.

From Figure 66, it can be seen that the main effects plot for the bacterial strains showed that the biofilm thickness was more in PAO1 (wild type) followed by PJP1 (Δ *lasI* mutant) and then the least in PDO100 (Δ *rhlI* mutant).

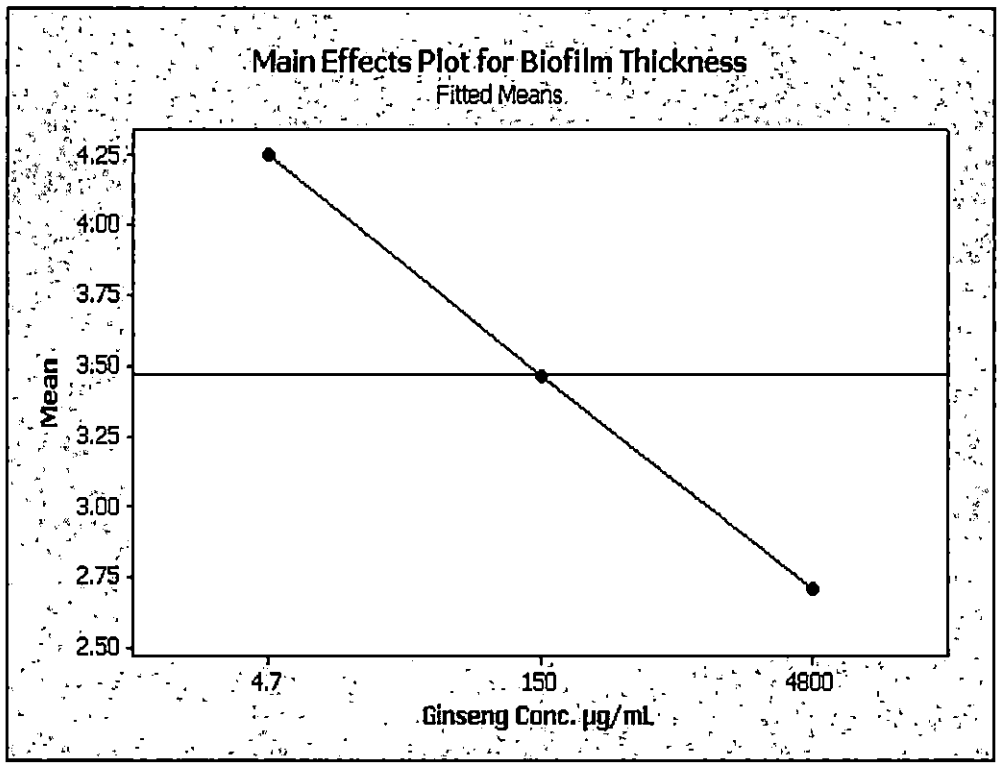


Figure 67. Main effects plot of the mean biofilm thickness for the three different ginseng concentration.

From Figure 67, the ginseng concentration showed that the biofilm thickness increased overall from the highest concentration of ginseng, 4800 µg/mL to the lowest concentration, 4.7 µg/mL.

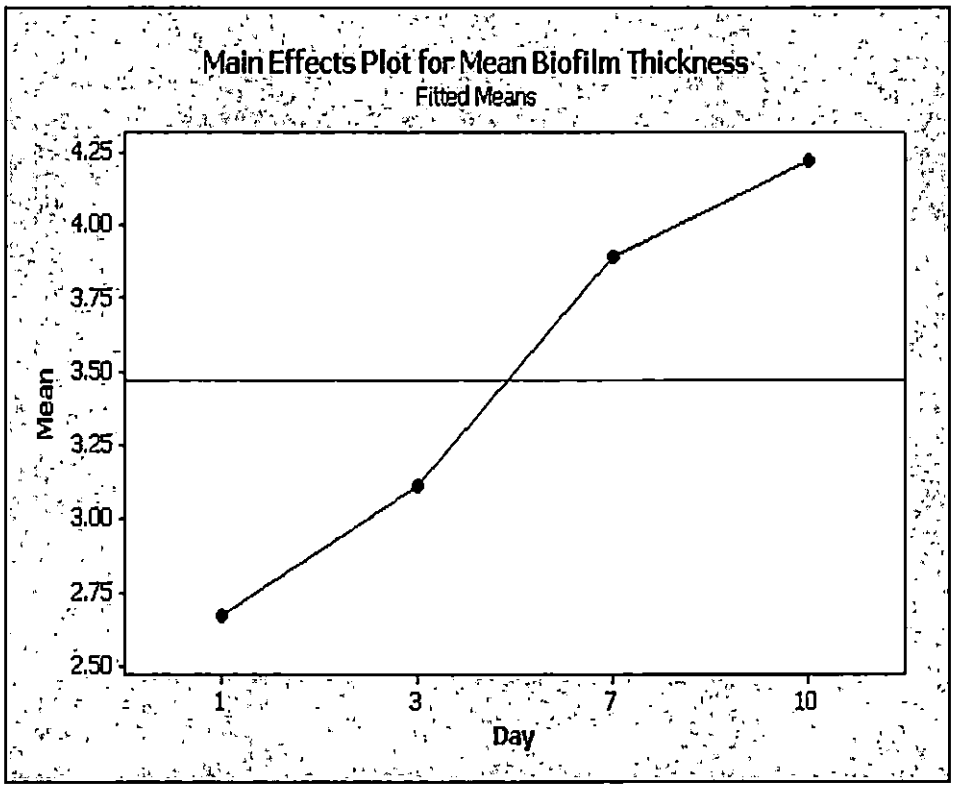


Figure 68. Main effects plot of the mean biofilm thickness for the four different days.

From Figure 68, the days showed that the biofilm thickness increased with increasing days, with lowest on day 1 to highest on day 10. Further analysis showed that these differences in thickness were significant for the three bacterial strains, four different days and the three different concentrations of ginseng.

As the assumption of independence, normality and equal variance were not violated, this ANOVA model was valid. The value for this experiment was 83.10% which was good and showed that more variance was accounted for. In general, the higher the R^2, the better the model fits the data. R^2 is always between 0 and 100%. This model with the three factors (bacterial strains, ginseng concentrations and

days) that were independent of each other worked well to analyze the results for biofilm thickness as R^2 was closer to 100%.The adjusted R^2 value was 78.87 % which was not very close to the R^2 but still indicates that the model was good. The ANOVA for all the three factors indicated the bacterial strains increased in biofilm thickness from PDO100 being the least then PJP1 and the highest being PAO1. The ginseng concentration showed that the biofilm thickness increased overall from 4800 μg/mL having the lowest to 4.7 μg/mL having the highest biofilm formation. The days showed that the biofilm thickness increased with increasing days from day 1 to day 10.

After using the three different techniques of microscopy, the confocal microscopy seemed to be the most reliable as statistical analysis was done to interpret the data in an objective fashion without bias.

Discussion

Biofilms are made up of bacteria and extracellular polymeric substance. *Pseudomonas aeruginosa* is known to form biofilms in response to certain environmental signals where free-living independent bacteria grow into interdependent aggregate architectural colonies.

The objective of this experiment was to study the effects of ginseng on biofilm formation by PAO1, PJP1 (Δ *lasI* mutant) and PDO100 (Δ *rhlI* mutant) strains *in vitro* by scanning electron microscopy, fluorescence microscopy and confocal microscopy image analysis. The biofilm formation of PAO1 using three different concentrations (4.7 μg/mL, 150 μg/mL, 4800 μg/mL) of ginseng was compared to the two quorum sensing mutants PJP1 (Δ *lasI* mutant) and PDO100 (Δ *rhlI* mutant) with the three different concentration of ginseng respectively.

Data collected from scanning electron microscopy and fluorescence microscopy images suggest that the control PAO1 (wild type) had a biofilm accumulation rate that was more rapid than those of the quorum sensing–deficient mutants. The quorum-sensing mutants lagged by 24-48 hrs. This was also demonstrated in another study done by Shih and Huang, (2002). A ginseng concentration of 4800 μg/mL showed the most inhibition of biofilm even until day 10 for all three strains. The most dramatic effect was obtained on day 1 followed by day 3. Ginseng concentration of 150 μg/mL showed inhibition of biofilm formation for all three strains at least until day 3; and that of 4.7 μg/mL concentration of

ginseng showed inhibition of biofilm dramatically on day 1. PAO1 (wild type) showed the least biofilm formation under the influence of ginseng by visually inspecting the biomass with scanning electron microscopy and fluorescence microscopy, followed by PJP1 (Δ *lasI* mutant), and then PDO100 (Δ *rhlI* mutant). However, the mean thickness of the biofilm was more in PAO1 (wild type) followed by PJP1 (Δ *lasI* mutant) and then PDO100 (Δ *rhlI* mutant). Using the confocal microscope to measure the mean biofilm thickness gave an insight on the significant difference of biofilm formation in PAO1 (wild type) versus the mutant strains (Figure 66). This suggests the possibility that quorum sensing, governing specific gene expression or regulation, may be responsible for the difference. This can specifically be seen when the controls of PJP1 (Δ *lasI* mutant) and PDO100 (Δ *rhlI* mutant) were compared to the ginseng treated group. There was significant inhibition of biofilm in the treated groups. ANOVA analysis was performed to determine if there were significant differences in all the three factors (bacterial strains, ginseng concentration and days of incubation). Biofilm thickness resulted in that all the three factors were statistically significant with p value=0.00 for each. This showed that each of the factors independently had a significant effect on the biofilm thickness. The mean thickness of the biofilm was more in PAO1 (wild type) followed by PJP1 (Δ *lasI* mutant) and then the least in PDO100 (Δ *rhlI* mutant). The biofilm thickness decreased with increasing concentration of ginseng and the biofilm thickness increased with increasing number of days of incubation.

The R^2 value for this experiment was 83.10% which was good and showed that more variance was accounted for. In general, the higher the R^2, the better the model fits the data. R^2 is always between 0 and 100%. This model with the three factors (bacterial strains, ginseng concentrations and days) that were independent of each other worked well to analyze the results for biofilm thickness as R^2 was closer to 100%.

It can be speculated that ginseng may have anti-adhesive effects on *Pseudomonas aeruginosa*. As seen in Figures 2 and 3, adhesion to the substrate is the first step to the formation of biofilm. Previous studies have revealed the inhibitory effects of an acidic polysaccharide purified from the root of *Panax ginseng* against the adhesion of *Helicobacter pylori* to gastric epithelial cells and the ability of *Porphyromonas gingivalis* to agglutinate erythrocytes (Lee, *et al.*, 2004). However, in another study, this acidic polysaccharide from *Panax* ginseng, PG-F2, was investigated further in order to characterize its anti-adhesive effects against *Actinobacillus actinomycetemcomitans*, *Propionibacterium acnes*, and *Staphylococcus aureus*, and showed no inhibitory effects. PG-F2 is a pectin-type polysaccharide which consists primarily of galacturonic and glucuronic acids along with rhamnose, arabinose, and galactose as minor components. Their results suggest that PG-F2 may exert a selective anti-adhesive effect against pathogenic bacteria, while having no effects on beneficial and commensal bacteria (Lee *et al.*, 2006).

Another speculation of biofilm inhibition could be the metabolism and biological activity of ginsenosides, the active ingredient in ginseng. On the first day, the ginseng was possibly metabolized. The microscopy images show that the biofilm growth did not stay inhibited through day 10 once inhibited on day 1. If there were doses of ginseng added to the experimental group on the different days the biofilm may have been inhibited further. A study by EA *et al.*, 2005, demonstrated that human intestinal flora metabolised ginsenoside Re, a main protopanaxatriol saponin in *Panax* ginseng mainly to ginsenoside Rh1 and ginsenoside F1, via ginsenoside Rg1, with protopanaxadiol as a minor component. A similar metabolic activity may occur but needs to be investigated.

The next step for this study could be to look at the quorum sensing genes that may be regulated or expressed during the treatment with the different ginseng concentrations. As mentioned earlier in the introduction, genes like *lasI, lasB, taxoA, rhlA, rpoS, exos* etc. are toxins, enzymes and alignate secreting factors that come under the *lasI* and *rhlI* quorum sensing system and could be studied. These studies can be done by using specific primers for these genes using quantitative PCR. The use of ginseng in this study has shown ginseng having an inhibitory effect on biofilm formation. This finding along with being an immunologically important agent as shown in other studies mean that ginseng has the potential to be a promising natural medicine, in conjunction with other forms of treatment, for CF patients with chronic *P. aeruginosa* lung infection.

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