ANALYZING INHIBITORY AND DISRUPTIVE EFFECT OF BORON DERIVATIVES ON *Pseudomonas aeruginosa* (ATCC- 27853) AND *Escherichia coli* (MG1655/K12) BIOFILMS ON SURFACES IN WATER SUPPLIES

by

Parinaz Zarezadeh Ardeshir

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2016

ANALYZING INHIBITORY AND DISRUPTIVE EFFECT OF BORON DERIVATIVES ON *Pseudomonas aeruginosa* (ATCC- 27853) AND *Escherichia coli* (MG1655/K12) BIOFILMS ON SURFACES IN WATER SUPPLIES

APPROVED BY:

Prof. Dr.Fikrettin Şahin (Thesis Supervisor) .....

Prof. Dr. Meral Birbir .....

Assist. Prof. Dr. Emrah Nikerel .....

DATE OF APPROVAL: ..../..../2016

This thesis is dedicated to my beloved family

and my lovely husband.

ACKNOWLEDGEMENTS

First, I would like to express my greatest and hearty gratitude to my supervisor, Professor Fikrettin Şahin for giving me the opportunity of doing research in Yeditepe university. His great support both morally and financially and endorsement throughout my study in Turkey made this research possible.

I would like to show my depth appreciation and respect to Assist.Prof. Dr. Emrah Nikerel for his endless guidance, patient, care and insightful comments. Without his help, this work would not be accomplished.

I would also thank my colleagues Dr. Ahmet Katı, Fatma Özen and Sadık Kalayıcı for their support and assistance during my research work.

My warmest appreciations go to my graceful and unique family, my dear parents Narges and Hossein, my beloved brothers Ardeshir, Sirous and Behnam, my sisters Sonia and Behnaz for their inspiration during my thesis journey.

At last but not least, I would like to express my sincere and heartfelt gratitude to my beloved husband, Arash, who has supported me during my studies. I would like to thank him for reminding my goals in life, encouraging to continue in tough situations and his continued love.

Parinaz Zarezadeh Ardeshir

Istanbul, December 2016

ABSTRACT

ANALYZING INHIBITORY AND DISRUPTIVE EFFECT OF BORON DERIVATIVES ON *Pseudomonas aeruginosa* (ATCC- 27853) AND *Escherichia coli* (MG1655/K12) BIOFILMS ON SURFACES IN WATER SUPPLIES

Biofilm is a multi-stage composition of one or more microorganisms and extracellular matrix which adhere to the wet surfaces and supported by matrix against any external perturbation. Every year, people get serious sicknesses or even die because of chronic infections typically induced by biofilm. Hygienic water is the basis for healthy society. It is impossible to continue life without hygienic water. Most people use swimming pools or take showers in their own houses as well as public places. Additionally, water tanks are being used for washing or as commodity water, in particular in poor countries. Numerous studies have shown bacterial growth and biofilm formation on water tanks, swimming pools, etc. Moreover, the bacterial growth can seriously cause diseases and harm the society. *Pseudomonas aeruginosa* and *Escherichia coli* are microorganisms which are gram negative and opportunist can proliferate easily and directly cause illness like sepsis, dysentery and even can cause death. Preventing or at least controlling biofilm formation is of great value from not only environmental but also social health perspectives. Biocidal agents such as boron derivatives are promising ways to control biofilm formation without harming human body. In this project, it was aimed to prevent biofilm formation and inhibit bacterial growth in places such swimming pools, bathrooms etc with special biocidal compounds which are non-toxic and environmentally friendly. In addition, the disruptive effect of these kinds of chemicals were be analyzed and tested on biofilm to make safe and healthy places for a human. It is expected to find a new way to prevent biofilm formation from these two bacteria in places such as swimming pool and water tanks that the water would be still for a long time. Therefore, disruption and inhibitory effects of different boron derivatives including boric acid (BA), sodium tetraborate decahydrate (STD) and disodium octaborate tetrahydrate (DOT) on biofilm formation by two bacterial species, *Pseudomonas aeruginosa* and *Escherichia coli*, were investigated *in-vitro* micro-well bioassay in this study.The results showed that 25 mg/ml concentration of STD and DOT have better potential to disrupt and/or inhibit both of bacterial biofilm

formation tested. Further studies are needed to confirm that boron derivatives may be beneficial to use in the drug industry and construction material such as ceramic in order to control biofilm formation and related health problems in the future.

Keywords: Biofilm, *Pseudomonas aeruginosa*, *Escherichia coli*, Disruption, Inhibition.

ÖZET

BOR BILEŞİKLERİNİN, SU KAYNAKLARINDA *Pseudomonas aeruginosa* (ATCC-27853) VE *Escherichia coli* (MG1655/K12) TARAFINDAN ÜRETİLEN BİYOFİLMLERİN İNHİBASYONU VE BOZUNUMU ÜZERİNE ETKİLERİNİN İNCELENMESİ

Biyofilm, bir yada birden fazla mikroorganizmanın çok aşamalı oluşturduğu ve ıslak yüzeylere yapışan, dış etmenlere karşı mikroorganizmaları koruyan hücredışı bir matristir. Her sene, ölümle de sonuçlanabilen birçok hastalık ve kronik hastalıklar biyofilmler tarafından tetiklenir. Hijyenik su sağlıklı bir toplumun temelidir ve hayatın devamı için vazgeçilmezdir. İçme ve gıda amaçlı kullanımın yanısıra, birçok insan yüzme havuzlarını ve/veya gerek evlerinde, gerekse umuma açık yerlerde duşları kullanmaktadırlar. Bunların yanısıra, su depolama tankları, özellikle şebeke suyunun düzensiz geldiği şehirlerde veya gelir düzeyi daha düşük bölgelerde, günlük su ihtiyacını karşılamaktadırlar. Birçok çalışma, su depolarında ve yüzme havuzları gibi ıslak alanlarda bakteriyel büyüme ve biyofilm oluşumunu ve bunun toplum sağlığına olan olumsuz etkisini belirtmiştir. *Pseudomonas aeruginosa* and *Escherichia coli* gram negatif, çabuk üreyen fırsatçı mikroorganizmalardır. Bu organizmalar sepsis ve dizanteri gibi enfeksiyon hastalıklara sebep olurken ölüme bile götürebilirler. Bu mikroorganizmların sebep olduğu biyofilm oluşumunu önlemek ya da en azından kontrol altında tutabilmek, yalnızca toplum sağlığı için değil, ekonomik olarak da büyük önem taşımaktadır. Boron türevleri olarak biyosidal ürünleri, insan vücuduna zarar vermeden biyofilm büyümesinin kontrolü için kullanılabilmekte, bu alanda gelecek vadetmektedir. Bu projede, toksik olmayan, çevre dostu biyosidal ürünleri ile uzun süre durağan olduğu seramik yüzeylerde biyofilm oluşumu ve bakteriyel büyümenin önlenmesi amaçlanmıştır. Bunun yanısıra, insanlara sağlıklı bir çevre sunabilmek için bu biyosidal ajanların, biyofilmler üzerine yıkıcı etkileri analiz ve test edimiştir. Bu şekilde, bu iki bakterinin, yüzme havuzu ve su depoları gibi yerlerde biyofilm oluşumunu önleyecek yeni yöntemlerin bulunması amaçlanmıştır. Bu çalışmada *Pseudomonas aeruginosa* ve *Escherichia coli* bakteri türleri tarafından *in-vitro* koşullarda mikro-well içerisinde üretilen biyofilmlerin inhibasyon ve bozunumu üzerine farklı konsantrasyonlarda borik asit, STD ve DOT bileşiklerinin etkileri incelenmiştir. Elde

edilen sonuçlara göre 25 mg/ml STD ve DOT konsantrasyonun test edilen bakteriel biyofilm uluşumu üzerinde en etkili inhibasyon ve bozunuma neden olduğu saptanmıştır. Gelecekte yapılacak daha ileri düzeyde çalışmalar ile seramik gibi ilaç ve inşaat sektörlerinde kullanılan bor katkılı yüzey malzemelerinin eliştirilmesi sonucu biyofilm oluşumundan kaynaklanan sağlık ve diğer biyolojik sorunların çözümü mümkün olabilir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

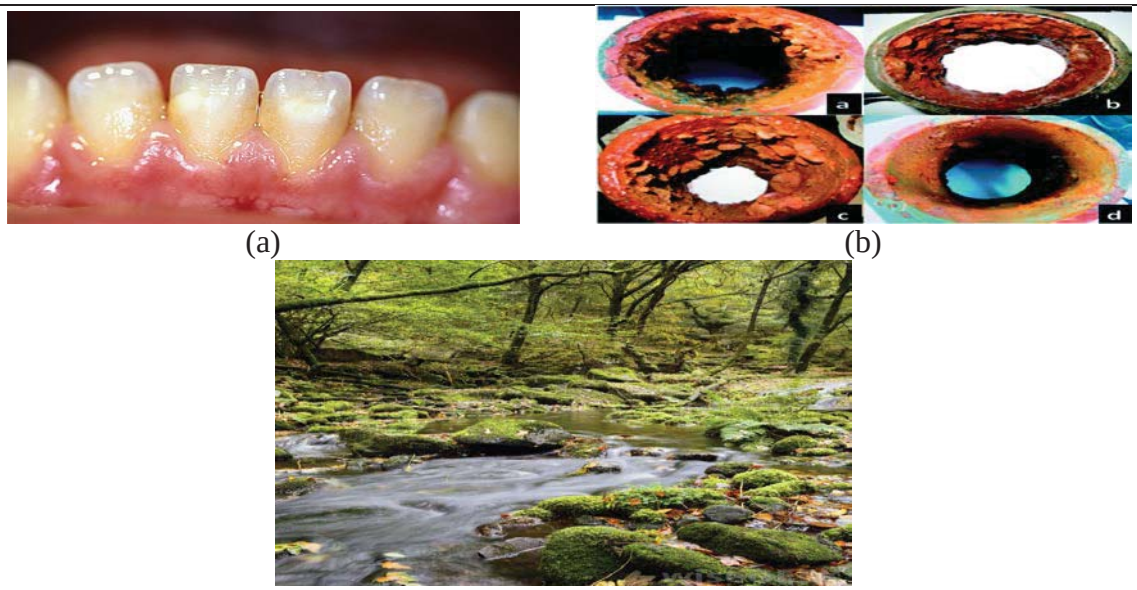
AHLs	Acyl homoserine lactones
BA	Boric acid
CF	Cycstic Fibrosis
CFU	Colony-forming unit
°C	degrees Celsius
CV	crystal violet
DOT	Disodium octaborate tetrahydrate
eDNA	Extracellular deoxyribonucleic acid
EPS	Extracellular polymeric substances
hrs	hours
mL	milliliters
min	minutes
mg	Milligram
nm	nanometer
OD	Optical density
PBS	physiological buffered saline
PH	Potential for Hydrogen ion concentration
QS	quorum sensing
Rpm	Rotation per minute
STD	Sodium tetraborate decahydrate
TSB	Tryptone soy broth
TSA	Tryptone soy agar

UTIs	Urinary tract infections
UV	Ultraviolet
μL	microlitres

1. INTRODUCTION

1.1. A QUICK GLANCE TO THE BIOFILM

Biofilm is a community of microorganisms which gather together on the surfaces in moist environments or even solid surfaces by excreting a sticky, gelatin-like material collected by excreted Extracellular polymeric substances (EPS) [1]. Furthermore, the aggregation and growth of bacteria are called biofilm which may form on living or non-living surfaces. Biofilms in many cases contain many species of bacteria, as well as algae, protozoa, fungi, debris and corrosion products. It can be easily formed by bacteria and some amount of water [2]. Several microorganisms can form biofilm easily such as *Pseudomonas aeruginosa* [3], *Escherichia coli* [4], *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumonia*, *Candida albicans*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Proteus mirabilis* and *Corynebacterium* species [5]. Biofilm can be formed in many places including our body and the environment that we live such as plastics, metal, tanks, water, swimming pools, in the bath and kitchen including sinks, toilets, cutting boards, countertops and even plants, trees (Figure 1.1). Generally, infections on body surfaces, tissues of patient and fluid sites of the body, which are caused by biofilm, can be harmful such as catheters, joints and organ replacements [6].



(a) (b)

(c)

Figure 1.1. (a) Sticky teeth (teeth's plaques), (b) Biofilm in water pipes [7] and (c) Biofilm in trees and rocks

Biofilm formation requires a sequence of phases which include:

- Pre-conditioning of a gummy surface which exists in the mass of fluids that has macromolecules unintentionally or purposely covered on the surface.
- Portage of planktonic cells to the surface from the bulk liquid.
- The cells adsorption at the surface, then the detachment of them.
- Adoption of bacterial cells in an immutable way at a surface.
- Cell to cell signaling molecules genesis.
- Forwarding the substrates into the biofilm.
- Stratum metabolism by the cells which are bound to biofilm.
- Carrying the metabolized substrates out of the biofilm (7th and 8th item activities go along with the growth of a cell, duplication and producing of EPS).
- Separating or shedding of biofilm that causes biofilm to be eliminated [8,9]. (Figure 1.2)

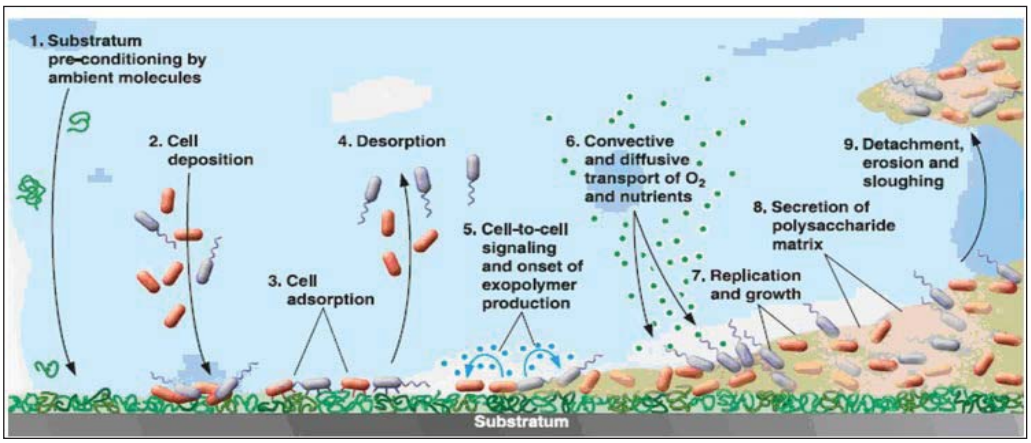


Figure 1.2. The process of biofilm formation [8].

The attachment process of microorganisms to surfaces and further biofilm formation which explained is rather complicated procedures and can be affected by several variables such as temperature, pH, available nutrients, surface chemistry and antimicrobial products present in the environment [10]. Contact of biofilm can happen on surfaces which are more hydrophobic, rougher and coated by surface conditioning films [11]. EPS production, properties of the cell surface, particularly the presence of extracellular elements and the interactions consists of cell–cell communication are required for development and formation of biofilm [10,12]. It is well documented that 99% of all bacteria exist in biofilms, with only 1% living in the planktonic state [2]. In fact, around 65% of microbial infections and diseases are connected to biofilms. In addition, the anatomical characteristics of biofilms may easily protect and support the cells against antimicrobial factors and the host's defense [2]. Even the community and microbial connection inside a biofilm would be protected against tough conditions such as osmotic shock, UV radiation, desiccation, predators and exposure to toxic compounds [2]. One of the reasons for this resistance and strong behavior is the EPS of biofilm in which bacterial polysaccharides is the crucial component. Evidently, it mediates most of the cell-to-cell and cell-to-surface interactions required for biofilm formation and stabilization [13]. In fact, all the cells that encapsulated in the matrix have connected to each other and have a compatible group behavior which is called quorum sensing (QS) [14]. It is addressed that the EPS are high molecular weight biopolymers which consist lipids, polysaccharides, nucleic acids and proteins. It is reported that near 50 to 90% of the whole organic substance in a biofilm is EPS [15,16]. Moreover, it is reported that the main portion of EPS is water (about 97%)

that covers functional and architectonic part of the matrix. There are two parts in the matrix; one is soluble and other is insoluble components. Fimbriae, pili, flagellae [17], cellulose [18] and amyloids are insoluble component [17]. While, proteins, eDNA and gel-forming polysaccharides are mentioned as soluble components [13]. The microdesign of the biofilm would be affected by the EPS exorcise valence of bacteria and this can sufficiently influence the keeping of pathogenic bacteria in the environment [19,20].

Public health faces challenging issues if pathogens grow in the biofilm by means of the EPS influence [21,22]. Apart from the differences of biofilm structural features in the pathogen reserving, there is a pathogen interplay with an endemic biofilm [23,24]. Construction of biofilm, which is made of mediated EPS molecules, is a dynamic and steady process that generates a spatial structure in every cell into the biofilm cluster in microcolonies [25,26]. In addition, it is shown that the ability of biofilm cells to define living situation and environment condition and equip the biofilm mechanical consistency are owned by EPS molecules which are responsible for forming and giving a shape to the cells of the biofilm and give the cells the opportunity to determine their mechanisms [27]. As well as, the blankness in the matrix made by tubes and pores among microcolonies can simplify the portage of fluids into biofilms [28,29].

1.2. THE MERITS AND DEMERITS OF BIOFILM

Biofilm formation has own advantages and disadvantages which are very important for human life. It is well accepted that biofilms play a significant role in bacterial permanence, insistence and antibiotic resistance in chronic infections [2]. It can spoil the food products like sea foods [30], dairy products [9]. Furthermore, it may result in a fatal disease (such as diarrhea and cystic fibrosis), corrupt industrial and medical devices, make an infection by forming in wounds and wound dressings [31]. For instance, biofouling or sedimentation of biological materials can seriously cause lots of damages to the equipment that have a contact with water such as ship hulls, pipelines, reservoirs and fish cages [32,33].While biofilms are commonly pathogenic in the body and damages so many devices composed of metals and plastics, they can be used effectively in treating sewage, contaminated soil, industrial waste [34], fine chemicals construction [35], bioremediation [36], producing of biofuel [37], fermentation [38] and making electricity into the microbial fuel cells [39].

1.3. A BRIEF LITERATURE REVIEW OF BIOFILM

A biofilm is a society of surface-associated microbial cells which is attached to the EPS matrix. The first scientist who described the biofilm was Van Leeuwenhoek in 1684 who used his microscope and observed microorganisms on the plaque of teeth. It can be said that it was the discovery of microbial biofilms. He explained this finding to the Royal Society of London in reportage. In 1940, Heukelekian and Heller announced the progress of colonial growth and bacterial slime which stick to the surfaces [40]. In 1943 Zobell reported the group of bacteria on surfaces which were dramatically more than in the circumfluent medium in seawater [41], while the main discovery of biofilm may wait for the inventing of the electron microscope. Since scientists named biofilm in 1978, biofilm science and engineering turn to be an active field of study all over the world [42]. Indeed, specifying of features and processes that prevent biofilm formation is critical for development and increasing the level of methods which are needed to control diseases and corruptions made by biofilms [43]. On one hand, some of them tried to inhibit biofilm formation or made some antibiotics against biofilm using organic ways and tested herbs and essential oils on them [4,44,45]. On the other hand, researchers used chemical methods such as applying iron particles on biofilm [46]. Moreover, using some enzymes, antibiotics and biomedical methods such as antimicrobial peptides [31,47,48], using antimicrobials to cover surfaces of biofilm [49,50] or even mechanical procedures such as high pressure [51], ice immersion [52] and irradiation [53] are listed among methods of preventing the biofilm formation. Using other biomedical ways like encapsulating surfaces of the biofilm with silver [54,55], or changing the physico-chemical features of the biofilm surfaces are the studies carried out for biofilm inhibition [56].Furthermore, studies on the surfactants showed that surfactants can barricade bacterial attachment and control the growth of biofilm [57]. In another case, antimicrobial products have been used to control biofilm formation, which generally can be seen in dairy production system [9]. (Table 1.1)

Table 1.1. Disinfectants applied to control biofilms in dairy industry [9]

Treatment	**Biofilm type**
Ozone, commercial chlorinated sanitizer	*P. fluorescens*/*Alcaligenes faecalis*
Benzalkonium chloride, hexadecyl trimethyl ammonium bromide, sodium hypochlorite, peracetic acid, hydrogen peroxide, o-cresol, phenol	*E. coli*
Chlorine, peracetic acid, peroctanoic acid	*L. monocytogenes* and *Pseudomonas* sp. mixed biofilms
Chlorine	*E. coli*
Chlorinated-alkaline solution; low-phosphate buffer detergent; dual peracid solution; alkaline solution; hypochlorite	*L. monocytogenes*
Sodium hydroxide; commercial alkaline cleaner	*P. putida*
Chorine; ozone	*P. fluorescens, P. fragi* and *P. putida*
Chlorine, hydrogen peroxide, ozone	*L. monocytogenes*
Glutaraldehyde, ortho-phtalaldehyde, hexadecyltrimethylammonium bromide, sodium dodecyl sulfate, chlorine solution sodium hydroxide	*P. fluorescens*
Sodium hydroxide; nitric acid	Mixed species
Chlorine; chlorine dioxide; commercial detergent	*B. cereus* and *Pseudomonas* spp.
Sodium hypochlorite	*S. typhimurium*
Peroxydes; quaternarium ammonium compounds; chlorine	*L. monocytogenes*
Hydrogen peroxide; sodium dichloroisocyanurate; peracetic acid	*S. aureus*

1.4. RESISTANCE OF BIOFILM AGAINST ANTIBIOTICS AND ANTIMICROBIAL AGENTS

The most important feature of bacterial biofilm formation is the enhanced resistance of the constituent microbes to stressors and antimicrobial agents.This happens due to inherent nature of the biofilm. The characteristics of the unstable cells make resistance to the antibiotics, leading to a protected environment of biofilm against adverse condition and the host's defenses [58].

Various biofilm factors, which affect antibiotic resistance, have been stated. Some of these factors are explained below.

- Antimicrobial agents must spread between the EPS matrix to join and inactivate the organisms within the biofilm. Originally, matrix behaves like a diffusion obstacle. EPSs retard diffusion and chemically reacting with the antibiotic agent or by reducing their amount of transport [59].

- The organisms that formed biofilm have decreased the speed of biofilm formation.As a consequence, reducing the rate that antimicrobial agents are taken into the cell and so affecting inactivation dynamic of biofilm [60].

- The environment immediately surrounding the cells within a biofilm may provide conditions that further protect the organism. In other words, micro environments can be made into the biofilm [61].

- Increased oxidative stress may reshape the physiology of bacteria [42].

It is obvious that antibiotics have the ability of penetration into the thick combination of DNA, protein and polysaccharide to reach its target [62]. It can be seen that decreasing of oxygen and nutrients inside biofilms may lead to change the metabolic activity of biofilm result in the slow growth of the microorganism. Therefore, slow growth may cause the biofilm to be killed or inhibited [62].

1.5. PERSISTER CELLS

The first scientist mentioned the persister cells was Bigger [63], who wrote that it is impossible to sterilize the culture of growing bacteria by penicillin and named them‘persisters’ [64]. Persister cells are mentioned as cells which are slow-growing or even considered as non-growing cells. These cells have a great potential to limit the sensitivity to antimicrobial agents and antibiotics [65]. Persister cells are formed by bacterial populations and never get killed in attendance of microbicides [64]. Nowadays, the importance of persisters comes after considering the biofilm tolerant to antibiotics and it has been thought that persisters are responsible for biofilm resistance [66,67]. The studies show that biofilms are very patient and strong *in vivo* studies compared with planktonic cells. However, antibiotic therapy *in vivo* reduces the planktonic cells and majority of biofilm except for persisters. Therefore, the persisters resurrect the biofilm formation [65,68]. As shown in Figure 1.3, in a first apply of antibiotic therapy on the bulk

of biofilm and whole planktonic cells were destroyed. The planktonic persisters get eliminated by the immune system, but the biofilm persister cells are survived from host defenses by the EPS matrix. After the concentration of antibiotic fallen off, the cells vitalize the biofilm and the infection reoccurs [65]. It is found that persisters are the expert protector and survivor cells [64].

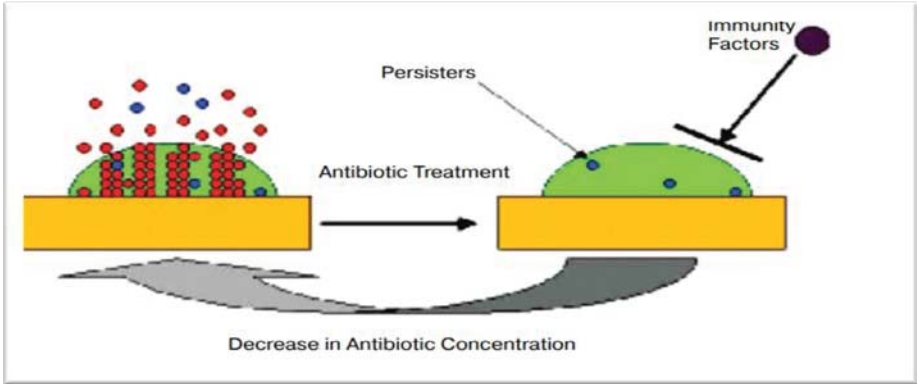


Figure 1.3. An example of biofilm resistance relies on persister cells protection [65].

1.6. ATTEMPTS TOWARD BIOFILM DEGRADATION

It is a hard work to destroy biofilms due to their resistant phenotype [9]. According to the literature, it is clear that treating the mature biofilm is always harder than controlling the formation. Moreover, there is no recognized method to cure biofilm completely without undesirable effect for a human being or the environment. One of the important and original tactics for inhibition of biofilm formation is to erase, scrub and disinfect the areas that have potential for biofilm formation [57,69].

To inhibit harmful biofilm formation, some main strategies are listed below.

- Controlling biofilm by prophylaxis and attacking the molecules of the surface [70].
- Using QS and applying inhibitor molecules to affect proper signaling and prevent biofilm formation by quorum sensing passages [71,72].
- Killing by attacking the bacterial membrane or subpopulations with various types of antibiotics or substances [70].

- Designing new anti-biofilm peptides or, in other words, biofilm-specific treatment detection [73,74].
- Interrupting or cutting off by some mechanical ways [51,52,70]. Focusing on intracellular and extracellular signaling molecules or biological interruption can be performed by enzymes [70].
- Preventing of stream pumps of the matrix, diminishing by the depreciation of extracellular matrix or targeting of extracellular and intracellular signaling molecules [70].

1.7. *Pseudomonas aeruginosa* BIOFILM

*Pseudomonas aeruginosa*is a Gram-negative bacterium and in the same time opportunistic pathogen. *P.aeruginosa* have the capacity to cause a wide variety of acute as well as chronic infections. Additionally, it is the main common nosocomial pathogen and life-threatening that leads human to get serious sicknesses such as an intro-abdominal wound, pneumonia, urinary tract infections (UTIs) and cystic fibrosis (CF). Patients that have UTIs, bronchiectasis, acquired-immune deficiency syndrome (AIDS), burn wounds, cancer and CF are more assailable to get *P. aeruginosa* infections [75,76]. Furthermore, it has great inherent and acquired antibiotic resistance rate [47,77]. Therefore, curing patients with antibiotic therapy is more demanding [78]. *P. aeruginosa* is one of the major model bacteria for the biofilm studies due to the simplicity of stable growth *in vitro* conditions and making multiple virulence factors [77]. This organism gets strong during biofilm formation and high resistant to any kinds of antibiotic therapy and reactions of the patient immune system [77]. Thus, this is acutely tough to cure biofilm-associated infections. On the other words, *P. aeruginosa* cells are more resistant to any kinds of antibiotic agents in a biofilm stage of growth than in the planktonic phase [79].

1.7.1. Progress of *Pseudomonas aeruginosa* biofilm

It is extensively reported that Psl, Pel polysaccharides and alginate have a basic role in *P.aeruginosa* biofilm maturity and also combination and architecture of that[3,80,81].

Although, eDNA have a significant task in the first stage of biofilm formation to retain the communication of the biofilm cells. Additionally, eDNA have the main role in antibiotic resistance as well [3,82,83]. Furthermore, in all gram-negative bacteria including *P.aeruginosa* there is a main class, acyl homoserine lactones (AHLs), which is a tiny molecule helps to send proper quorum sensing signals in a developing stage of biofilm [3,84]. Fimbriae or pili exist in most of the gram-negative bacteria and also *P.aeruginosa* species. They are made by filamentary attachments and have 4-35 nm wide with a several micrometers length. Generally, fimbriae structures are straight and do not move. They only have an operation and function to stick to the inorganic particles and other bacterial cells and make cells viscid to cling strongly [9,85].

1.8. *Escherichia coli* BIOFILM

Escherichia coli is a gram-negative and dominant organism between several facultative anaerobic bacteria of the gastrointestinal tract [86]. *Escherichia coli* normally migrates into the gastrointestinal tract of the newborn child of human in the first hours of birth and coexists with its host for many years as well as double-faced profit. *E.coli* is one of the well-known and most studied bacteria for its cellular processes among whole organisms owing to its great appointed and genome sequence. This bacteria is a pathogen with specific virulence factors that can cause a large variety of diseases and put human health at the serious risk all over the world [87,88]. It has more than 250 species, found everywhere from aquatic environments to the human body. As an example, it is the cause of regressive urogenital infections, diarrhea, gastroenteritis and colonizers of medical devices [86]. It can be called inoffensive gut commensal and at the same time extra- or intra-intestinal pathogens [86]. *E. coli* can form biofilm quickly in a water reservoir and one of the most seen bacteria in aquatic environments with high virulence potential [89]. It has a very complex biofilm formation process (Figure 1.4) [90].

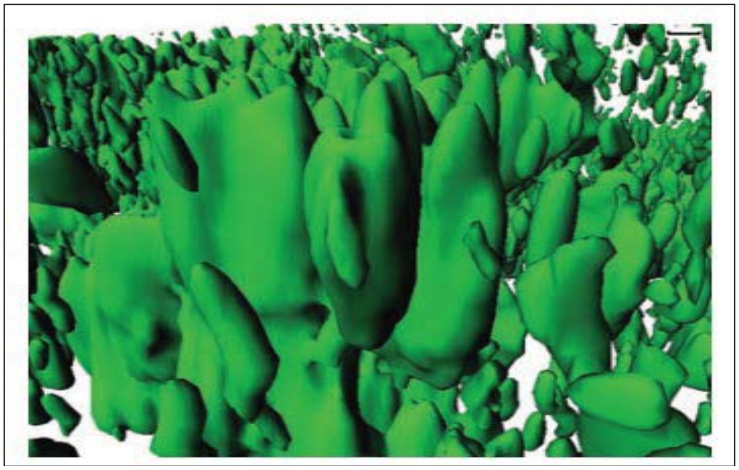


Figure 1.4. Biofilm of *Escherichia coli* type BW25113 under confocal microscopy and IMARIS software, with applying green-fluorescent-protein [90].

Scientists have used DNA microarray technology to analyze transcriptome profiling and genetic basis of *E. coli* to get a good comprehension of how biofilm works and develops [91,92]. Moreover, proteins which are related to the formation of biofilm have been observed (Figure 1.5) [90,93].

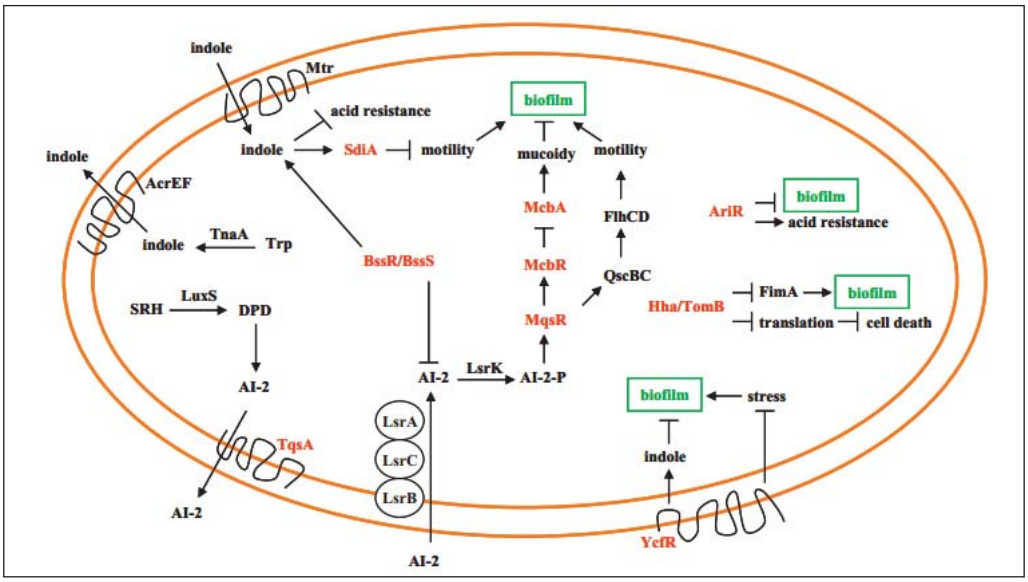


Figure 1.5. Schematic of explored proteins in the formation of *E. coli* biofilm [90].

1.9. BORON

Boron element is a semiconductor metalloid that has atomic number 5 [94,95]. It is found in nature as two isotopes ^{10}B and ^{11}B with an 80/20 ratio [95]. Boron is found highly

reactive with oxygen (O_2) and connected with that in a form of B-O in normal situations [96]. The first discovery of boron goes back to 1923 which is recognized in vascular plants as an essential mineral [97]. It is documented on a number of articles that plants need boron for their enzyme metabolism and arranges the activity of that [98], pollination [99] and cell wall structure and stability [100,101]. A long time after recognizing boron efficacy on plants, the importance of it determined in animals [102] due to rare boron deficiency in humans because of the existence of boron in seeds and fruits [94]. It has been proven that other organisms such as zebrafish, mouse, frog, trout and buffalo need boron for their function as well [103,104]. Other than that, the importance of boron for human metabolism has been identified [105]. The first studies on boron requirement for human health related to work on the bone which was the bone of rat and pig that did not grow well in a lack of boron in their diet [106]. Boron exists in the human body, as in tissues or fluids, which are approximately between 3-20 mg. The highest concentration of boron can be found in bones, nails and hairs, which is 4.3-17.9 ppm. In addition, health condition can extremely affect the boron concentration [102]. From the investigations, it can be found that boron is the critical substance in natural waters, rocks, soils and everywhere on the planet [96]. Turkey has the most of the world' reserves of Boron (about 70%) [96]. The effect of boron has discovered on prostate cancer and scientists have studied on boric acid exposure on cell lines of prostate cancer and got that boric acid inhibited PC-3, LNCaP and DU-145 cell reproduction [107]. Apart from this, it is documented boron treatment can inhibit lung, cervical and breast cancer development[108-110]. Also, some investigations have proved the effect of boron on wound healing due to the anti-inflammatory effect of that [111]. The other remarkable feature of boron derivatives is biocide effect on wide range microorganisms such as *E. coli*, *Klebsiella* spp, *Acinetobacter calcoaceticus*, *Enterobacter* spp, *Staphylococcus* spp, *Morganella* spp, *Proteus* spp, *P. aeruginosa* ,*Citrobacter* spp and etc and other than that have an anticandidal effect as well [112]. Additionally, boron-containing antibiotics were started to use such as boromycin [113], tartralon B [114] and aplasmomycin [115] (Figure 1.6).

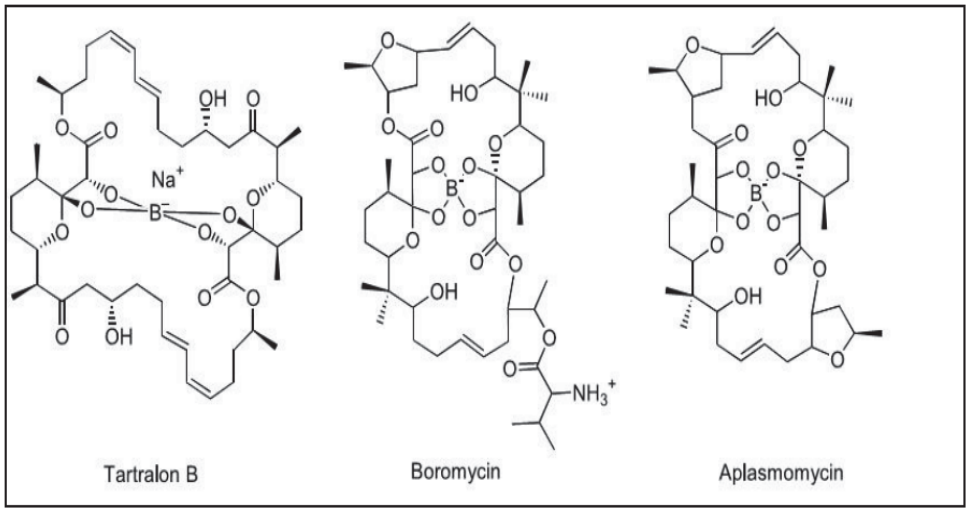


Figure 1.6. Chemical formulas of boron-containing antibiotics [116].

1.9.1. Boric Acid (BA)

Boron can be found in nature as BA [96]. Also, reacts with hydroxyl group-bearing compounds and alcohols to create esters [117]. In several cases, Boric acid can be used such as cleansing compounds for cleaning of animal skin and ear [118], treatment of *Candida* sp. Infection [119], yeast infections [120-122] and antibacterial activity [123]. In fact, first bacteriostatic effects of boric acid reported in 1969 [124]. Hereby, it can be found that boric acid is a promising antibacterial factor to treat bacterial sicknesses, disinfectants and pesticides.

1.9.2. Disodium Octaborate Tetrahydrate (DOT)

Disodium octaborate tetrahydrate with a chemical formula $Na_2O.4B_2O_3.4H_2O$ or $Na_2B_8O_{13}.4H_2O$ is a boron derivative and white powder with high solubility in water. It is used in many cases like industrial cleaning [125,126], bactericidal and insecticide [127].

1.9.3. Sodium Tetraborate Decahydrate (STD)

This substance with a chemical formula $Na_2O.2B_2O_3$or $Na_2B_4O_7$is recognized as borax as well. STD is accessible in forms of glass and crystalline [128]. Researchers use crystal

form for their researches and experiments [116]. This compound has an antibacterial [129] and anticandidal [130] features same as BA and DOT. Additionally, it is used in soap industry for hand cleaning due to its alkaline structure [130]. The solubility of STD is shown in Figure 1.7 [119].

Solvent	Temperature, °C	Solubility, wt %		
		$B(OH)_3$	$Na_2B_4O_7 \cdot 5H_2O$	$Na_2B_4O_7 \cdot 10H_2O$
glycerol, 86.5%	20	21.1	47.1	
glycerol, 98.5%	20	19.9	52.6	
glycerol	25	17.5		
ethylene glycol	25	18.5	41.6	31.2
propylene glycol	25	15.1		21.9
diethylene glycol	25	13.6	18.6	10.0
mannitol, 10%	25	6.62		
methanol	25	173.9[a]	19.9	16.9
ethanol	25	94.4[a]		
n-propanol	25	59.4[a]		
n-butanol	25	42.8[a]		
2-methylbutanol	25	35.3[a]		
isoamyl alcohol	25	2.39		
acetone	25	0.6	0.60	
methyl ethyl ketone	20	0.7		
ethyl acetate	25	1.5	0.14	
diethyl ether	20	0.008		
dioxane	25	~14.6[a]		
pyridine	25	~70[a]		
aniline	20	0.15		
acetic acid, 100%	30	6.3		

[a]Solubility values are in g/L.

Figure 1.7. Solubility of boric acid, sodium tetraborate decahydrate in natural solvents [26].

1.9.4. Boron Toxicity

At some concentrations, the way of usage and period of exposure time can give toxicity effect in micronutrient elements [131]. Boron derivatives toxicity has been widely studied in the laboratory. In the evaluation of long exposure time, no sign of cancerogenic effects or genetic mutation were observed [132]. Reversible inhibition of spermiation was observed by exposure of boron only at low doses (175 mg/kg). However, single-dose exposure (2000 mg/kg boric acid) did not have any influence. Furthermore, a study performed on a group of dogs showed that adjusting their dietary to intake STD or BA at doses up to 10.2 mg B/kg bw/day (62.4 mg boric acid/kg bw/day and 84.7 mg STD/kg bw/day), caused no negative effect [132]. An experimental investigation revealed that

more than 6150 and 3980 mg of sodium tetraborate decahydrate and boric acid/kg, respectively, were measured for oral LD50 in a dog. Other studies did not show any other adverse impacts [133]. In mice and rats, intake of boron exceeds 4000 mg boric acid kg^{-1}can result in testicular atrophy and cell damage [134]. Nausea, diarrhea, lethargy and vomiting are the symptoms of acute toxicity in humans with extreme boron exposure [134]. Moreover, weight loss, nausea, poor appetite and decreased sexual activity and sperm number were observed in chronic boron toxicity [134]. In another study, scientists show that unexpected poisonings with an acutemortal amount of boric acid are 15,000-20,000 mg for adults and 3000-6000 mg for infants. Clinical effects of boric acid toxification are congestion, gastrointestinal problems, irritability, exfoliation of the mucosa, seizures and inflammation. Additionally, in dose area of 100 to 55,500 mg have been described for boron toxicity which depends on body weight and age of humankind [135]. No special cure or antidote is available for boric acid poisonous but using activated charcoal has been proposed in some cases considering the low quantity of boric acid absorption by that. Riboflavin is one of the suggested drugs to boron toxicity treatment [136].

1.10. THE AIM OF THE THESIS

The aim of this research was to analyze and estimate the disruptive and inhibitory potentials of some compounds and chemicals in order to find some essential ways and methods to inhibit or disrupt the formation of biofilm in places such as swimming pools, bathrooms, water tanks and bathtubs which have ceramic in their constructions and have high potential to gather microorganisms and make a biofilm. It is aimed to fulfill this demand with effective biocidal, safe to use and non-toxic substances such as boron derivatives like Sodium tetraborate decahydrate, Disodium octaborate tetrahydrate and Boric acid.

2. MATERIALS

2.1. CHEMICALS

- Distilled water
- Pure water
- Boric acid (Biobasic, 10043-35- 3)
- Sodium tetraborate decahydrate (Sigma-Aldrich, 31457-1KG)
- Disodium octaborate tetrahydrate(Sigma)
- Crystal violet indicator (Riedel-dehaen, 32675)
- Methanol 99.9% (Sigma-Aldrich, 34885-2.5L-R)
- Ethanol 99.8% (Sigma-Aldrich, 34870-2.5L)
- Phosphate-buffered saline (PBS) (Biotech Gmbh, P04-53500)
- Tryptone soy broth (TSB) (Himedia, M011-500G)
- Tryptone soy agar (TSA) (Lab011)
- Glucose monohydrate (Sigma-aldrich 16301-1kg)
- Luria–Bertani (LB) (acumedia 7279A)

2.2. EQUIPMENT

- Laminar flow hood (ESCO Class II, Type A2)
- Erlenmeyer flasks, 250mL (Isolab)
- Autoclave (Tuttnauer 5050 ELV)
- Inoculation loops (Isolab)

- Cell scraper (Isolab)
- Petri plates (Isolab)
- Incubator (memmert)
- Shaker (Sartoriusstedim Biotech)
- Spectrophotometer Cuvettes 1.5 to 3.0 ml (Isolab)
- Vortex (Stuart SA8)
- -80°C freezer (Thermo Forma -86C ULT Freezer)
- 96well Plate Reader (Multiskan spectrum) (Thermo Lab system)
- Spectophotometer (Genesis 10S UV-VIS/ Thermo fisher scientific)
- 96 well Cell culturetestplate (Costar, 3599)
- CentrifugeEppendorfs 2-1,5 ml (Isolab)
- Centrifuge tubes 50 ml (Isolab)
- Serological Pipettes 50-25- 10-5ml (Biofilm)
- Micro pipettes 1000-200- 10µl (Eppendorf Research Plus)
- Tips (ExpellPlus)
- Sterile Disposable Syringe 10,50 mL (Set Medikal, Istanbul-Turkey)
- Sterile and endotoxin Disposable PES Filter media units 0.45µm (Sartorius)
- Magnetic stirrer (Benchmark)
- Aluminium foil
- Parafilm

3. METHODS

3.1. PREPARATION OF CHEMICAL AGENTS

In this part, for inhibition assay experiment, 250 mg of Boric acid (BA), Sodium tetraborate decahydrate (STD) and Disodium octaborate tetrahydrate (DOT) were dissolved in 10 ml of pure water in separate centrifugation tubes. Then, to solve whole powder, they were mixed by vortexing for 15 minutes. Final volumes of solutions were 25mg/ml for each agent. The steps were repeated with the same amount of water for all mentioned chemicals with the following concentration of 18.5, 12.5, 10, 9, 8, 7, 6, 5, 4 and 3mg/ml, respectively. At the end, chemical agents were sterilized with 0.45µm disposable filter and stored. Then, 100 µl of each solution was inoculated to TSA and no bacterial growth was observed. For disruption assay, the concentrations used were 40, 30, 25, 18.5, 12.5 mg/ml.

3.2. STRAINS AND BASIC CULTURE CONDITIONS

All strains which were selected for this study are *Pseudomonas aeruginosa* type strain (ATCC-27853) and *Escherichia coli* strain (MG1655/K12, ATCC-700926). These bacteria were kept in glycerol stock at -80 °C. Then, they were streaked onto Tryptone soy agar (TSA) medium plate and incubated at 37°C for 24 hours. Next, to make more colonies from the plates, *Pseudomonas aeruginosa* and *Escherichia coli* were grown overnight in 50 ml of Tryptone soy broth (TSB) medium and LB, respectively, in falcon tubes separately and were incubated on the shaker at 37 °C and then stored at 4 °C.

3.3. BIOFILM FORMATION ASSAY

Biofilm formation assay was performed using a previously characterized protocol with some modifications [4,47]. First, the strains which were under study were cultured in 5ml TSB and LB for 24 hrs at 37°C with 50rpm. Second, they were diluted 1:50 into the new TSB. The final concentration was approximately 1×10^8 cfu/100µl for *Pseudomonas*

aeruginosa and 5×10^6 cfu/100µl for *Escherichia coli*. Then, the mentioned suspension was inoculated into each 96-well culture plate. Next, it was incubated at 37°C for 24, 48 hrs (Figure 3.1). As a negative control, TSB has been used. After the incubation, the planktonic cells were aspirated smoothly from each well and were washed three times with 200µl physiological buffered saline (PBS) solutions and were left to dry. To fix the attached bacteria, 200µl of 99% methanol was added (Figure 3.2) gently to each well and aspirated after 15 minutes (min) and were air dried again. In addition, wells were stained for 10min with 200µl of 0.1% crystal violet (CV) (Figure 3.3). Stained biofilms were mildly rinsed off with distilled water and air dried (Figure 3.4). 200µl of 95% ethanol was added to dissolve the stains which were fixed in each well within 20 min (Figure 3.5). Aliquots of the ethanol and crystal violet solution were transferred from each well (150µl) to the new clear flat bottom 96-well cultured microtiter plate and the absorbance (OD) were measured at 595 nm (Figure 3.6).

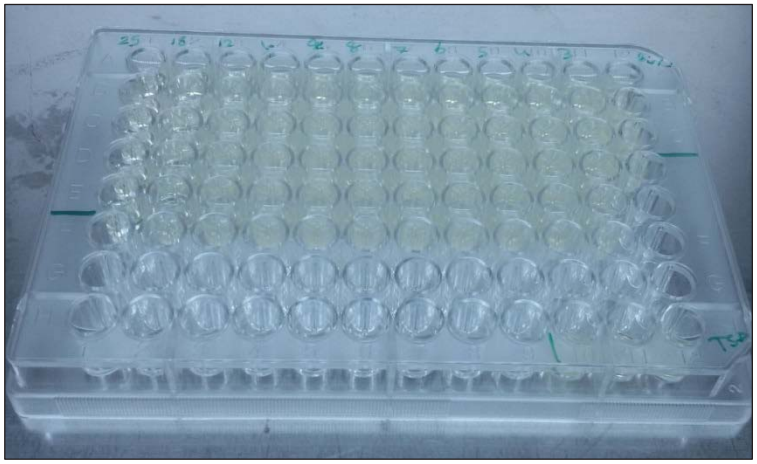


Figure 3.1. The biofilm formation after 24,48 hours incubation in 37°C.

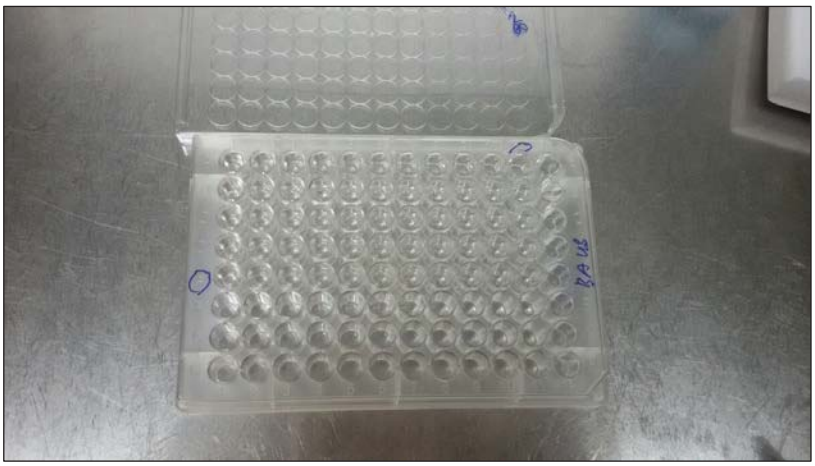


Figure 3.2. Adding methanol to fix the biofilm.

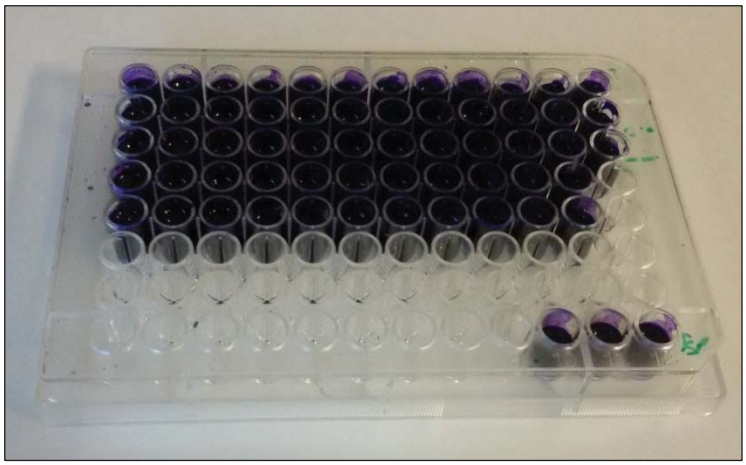


Figure 3.3. Adding crystal violet to the plate to bind with biofilm.

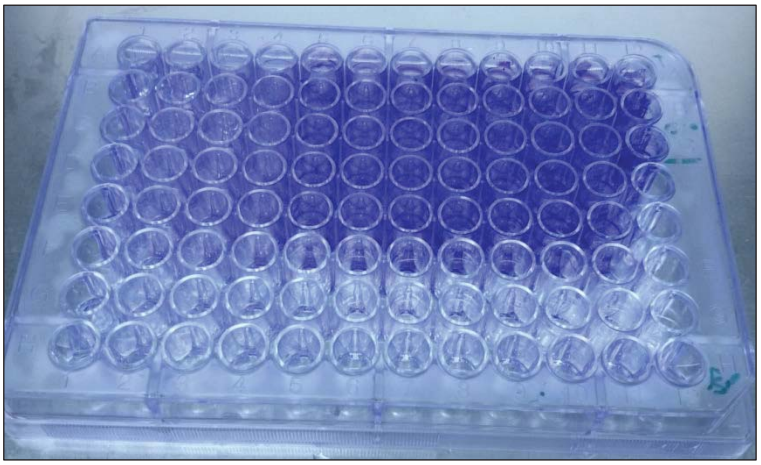


Figure 3.4. Washing with tap water or distilled water

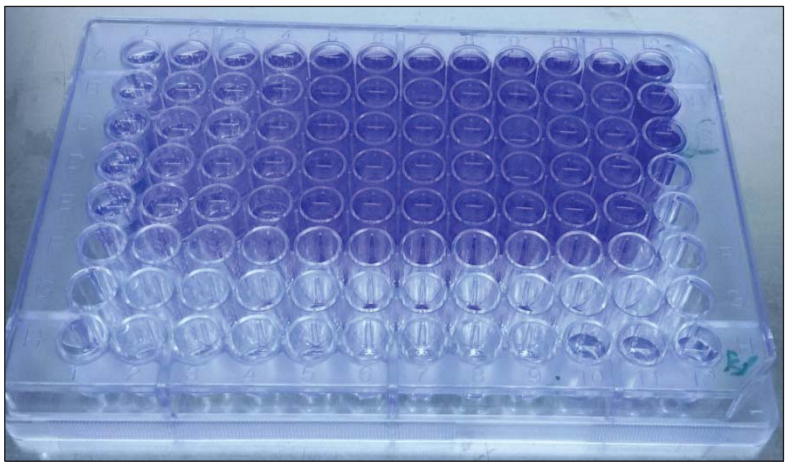


Figure 3.5. Applying the ethanol for resolubilizing the adherent cell bound

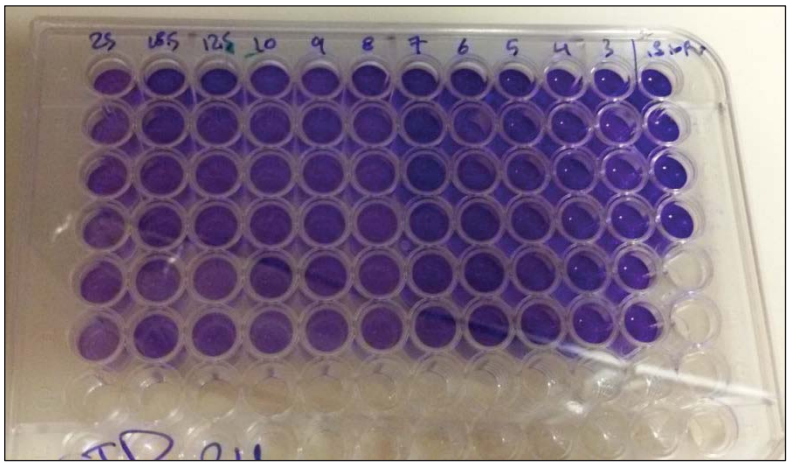


Figure 3.6. The re-solubilized liquid which measured at OD 595nm

3.4. BIOFILM INHIBITION ASSAY

The strains (1×10^8cfu/100µl) for *Pseudomonas aeruginosa* and (5×10^6cfu/100µl) for *Escherichia coli* in TSB and LB were put into each 96-well microtiter plate with 100µl of BA, DOT and STD agents with 25, 18.5, 12.5, 10, 9, 8, 7, 6, 5, 4 and 3mg/ml concentrations. Then, incubated at 37°C for 24 and 48 hrs. The positive controls were *Pseudomonas aeruginosa* (ATCC-27853) in TSB and *Escherichia coli* in LB without any other chemicals or antibiotics. The negative control was TSB and LB. After 24, 48hrs of incubation, wells were washed twice with PBS and the rest of experiment was done exactly same as biofilm formation assay part after rinsed off with PBS respectively. At the end,

each well plate was measured at 595 nm. For each agent, a separate 96-well microtiter plate was used separately. Each assay was done three times [45].

3.5. BIOFILM DISRUPTION ASSAY

Strains were plated into a 96-well microtiter plate without the chemicals (BA, STD, and DOT). The negative control was TSB and LB and the positive was bacteria strain which was used for study separately. After 24h incubation at 37°C, 100µl of agents that described above were added to determined concentration (40, 30, 25, 18.5 and 12.5) to each plate and incubated further for 24,48hrs. Next, the procedures of inhibition and formation assay were performed for this assay as well and the same techniques were done after washing with PBS and OD measured at 595nm.

4. RESULTS

4.1. BIOFILM INHIBITION ASSAY FOR *Pseudomonas aeruginosa* (ATCC-27853) USING BA CONCENTRATIONS

A significant inhibition was observed for the dosages of 18.5 and 25 mg/ml both for 24 and 48 hrs (Figure 4.1, Figure 4.2 and Figure 4.3). The growth of microorganisms at these high dosages for first and second trial is almost less than 20%. In the third trial, however, the growth at these two dosages is around 30%, which is debatable, can lead the microorganism to grow more and make a strong wall not to let any chemicals in and inhibit. The other concentrations resulted in no blockage in BA inhibition assay. Additionally, more variation in biofilm formation is observed in the first trial compared to the others. While in the second and third trial, the biofilm formation percentage is rather constant for dosages less than 12.5 mg/ml. According to the results, when more than 12.5 mg/ml BA is used, the rate of biofilm formation decreases suddenly.

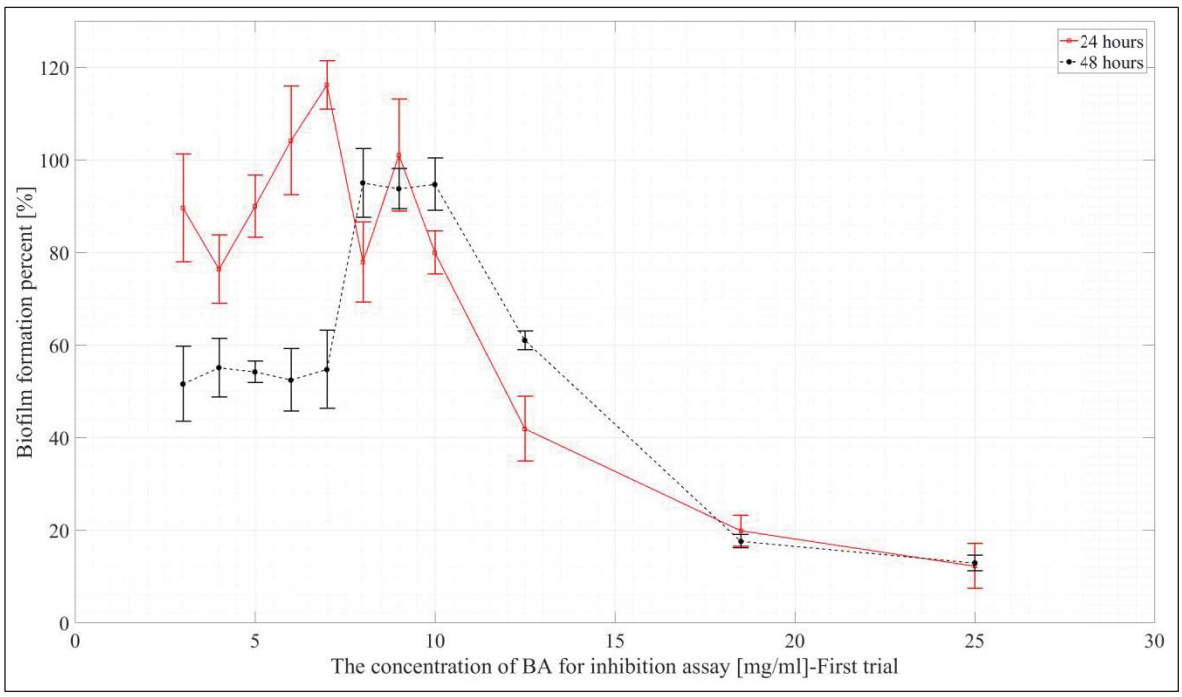


Figure 4.1. The experiment of BA for inhibition assay-First trial

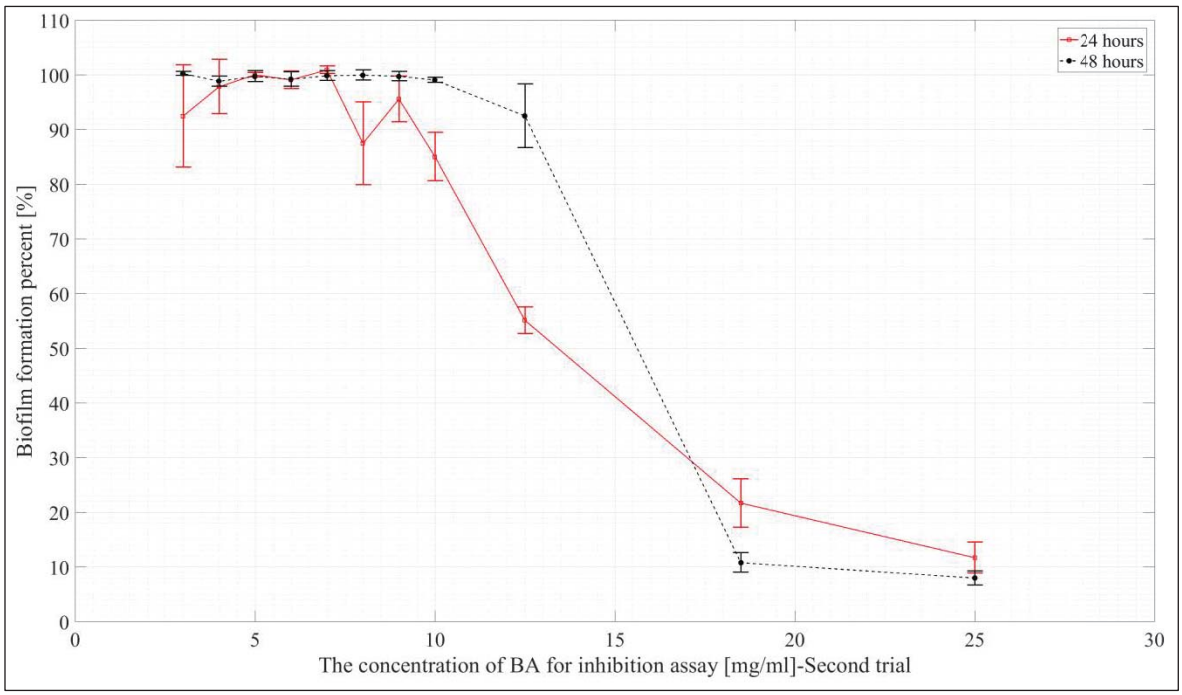


Figure 4.2. The experiment of BA for inhibition assay-Second trial.

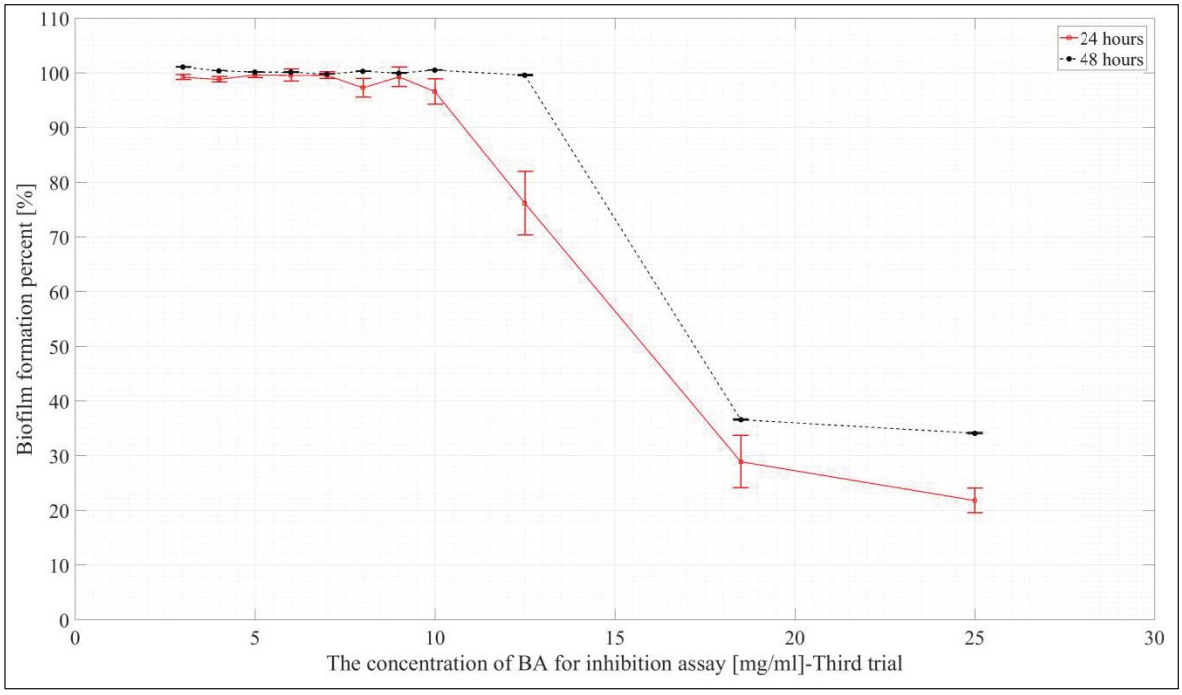


Figure 4.3. The experiment of BA for inhibition assay-Third trial.

4.2. BIOFILM INHIBITION ASSAY FOR *Pseudomonas aeruginosa* (ATCC-27853) USING DOT CONCENTRATIONS

Figure 4.4, Figure 4.5 and Figure 4.6 present the biofilm formation percent for various dosages of DOT. The biofilm percent reduction rate at the experiment performed for 24 hrs is larger than for 48 hrs with the same dosages. Similar to the previous experiment, the first trial has chaos behavior for lower concentration values. The results of the second and third trial for the lower concentration of DOT show a stable pattern. By increasing the concentration value for all the trials, the biofilm formation percent decreases to 20%. However, reaching a lower value would not be possible for the concentrations tested.

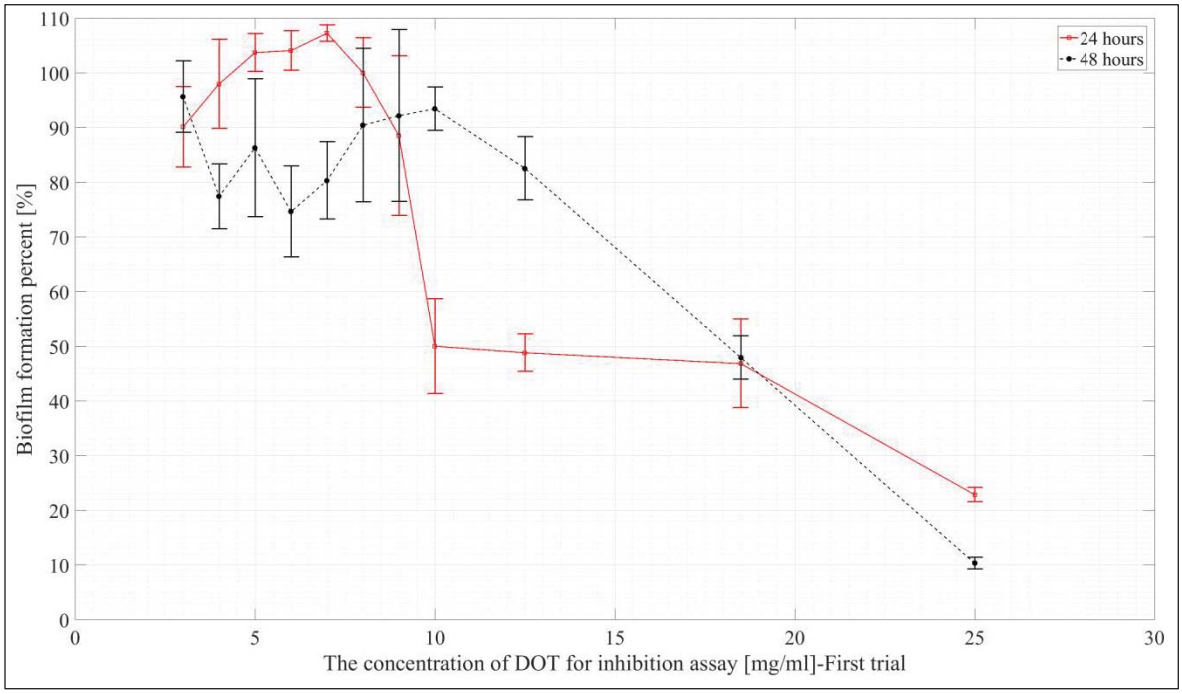


Figure 4.4. The experiment of DOT for inhibition assay-First trial.

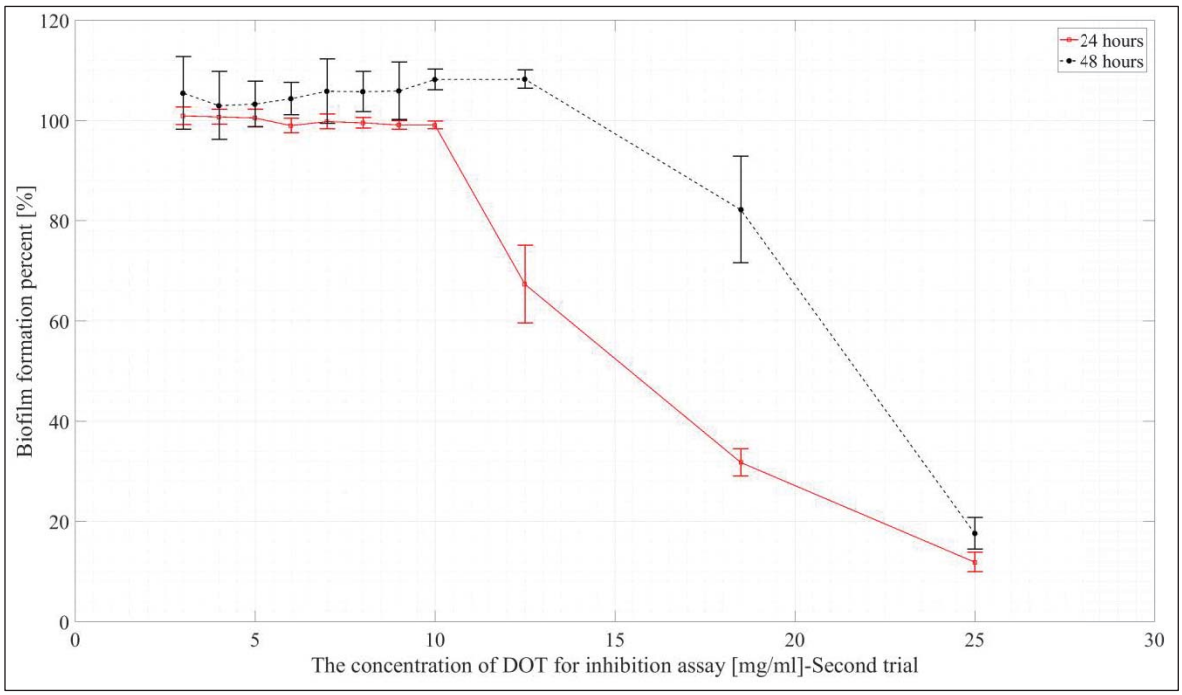


Figure 4.5. The experiment of DOT for inhibition assay-Second trial.

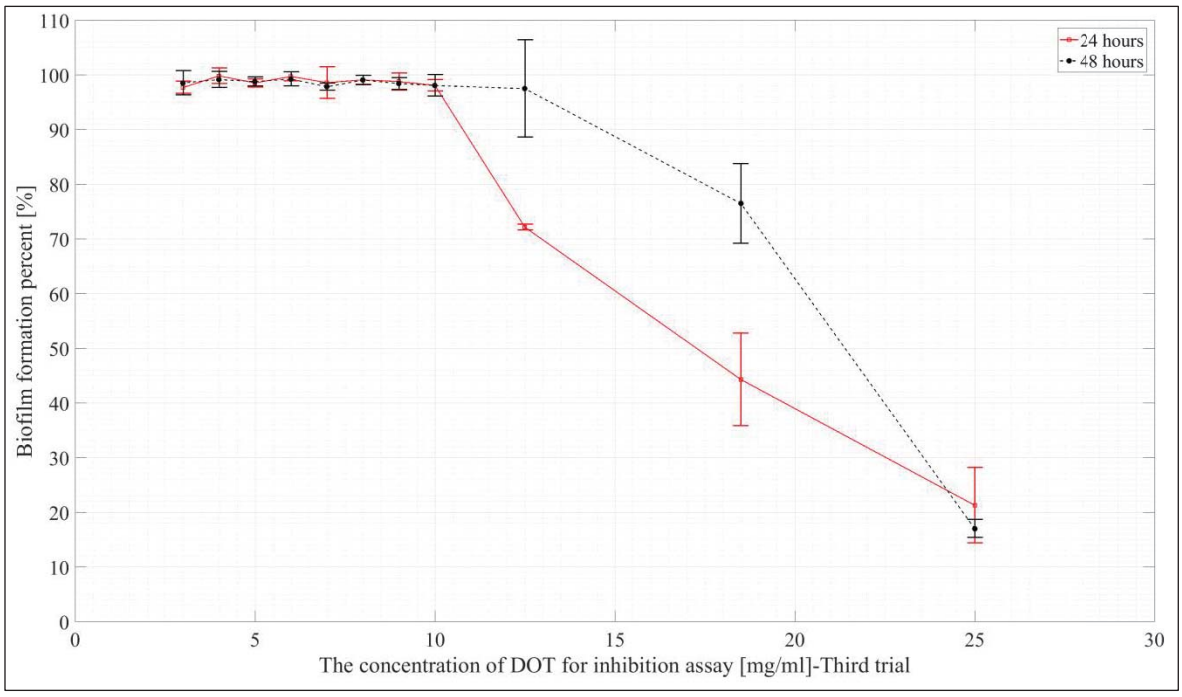


Figure 4.6. The experiment of DOT for inhibition assay-Third trial.

4.3. BIOFILM INHIBITION ASSAY FOR *Pseudomonas aeruginosa* (ATCC-27853) USING STD CONCENTRATIONS

The results of STD experiment are shown in Figure 4.7, Figure 4.8 and Figure 4.9. The biofilm inhibition experiment by STD gave different results than the one by other compounds. Firstly, the rate of biofilm inhibition in both 24 and 48 hrs was almost around 90 to 95% for two high concentration. In addition, the concentration of 12.5mg/ml results in inhibition of 80 to 85% and for 10 mg/ml is around 75%. Moreover, it can be seen that the biofilm formation for the concentrations less than 8 mg/ml increases suddenly.

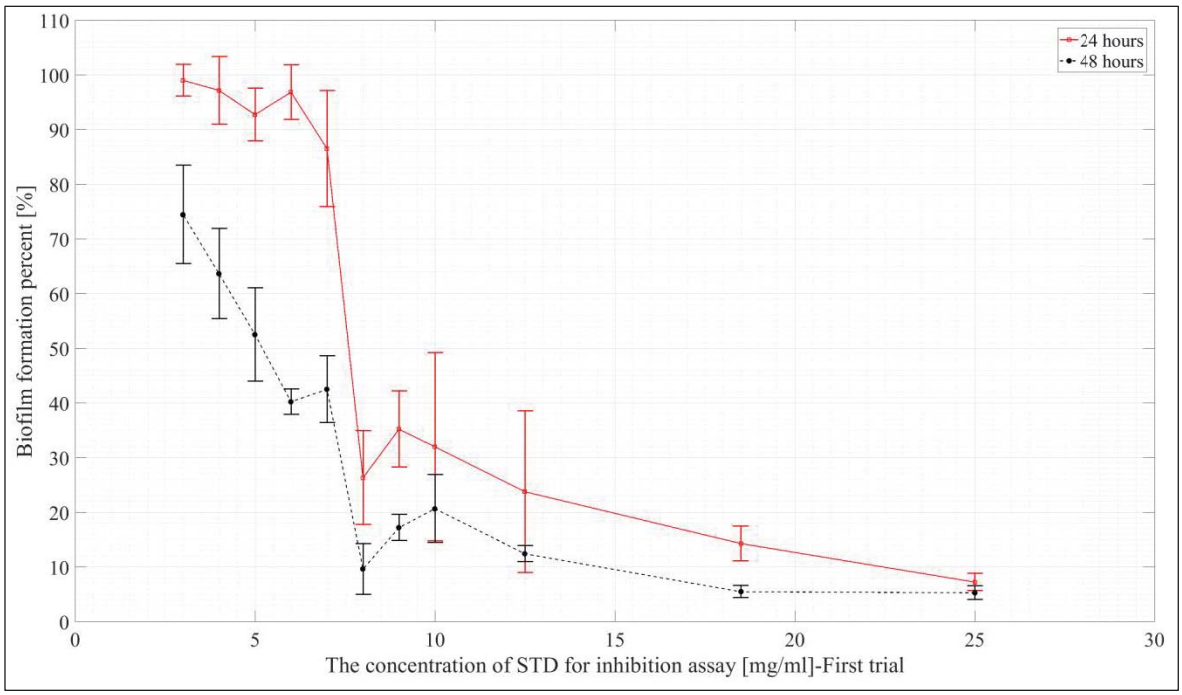


Figure 4.7. The experiment of STD for inhibition assay-First trial.

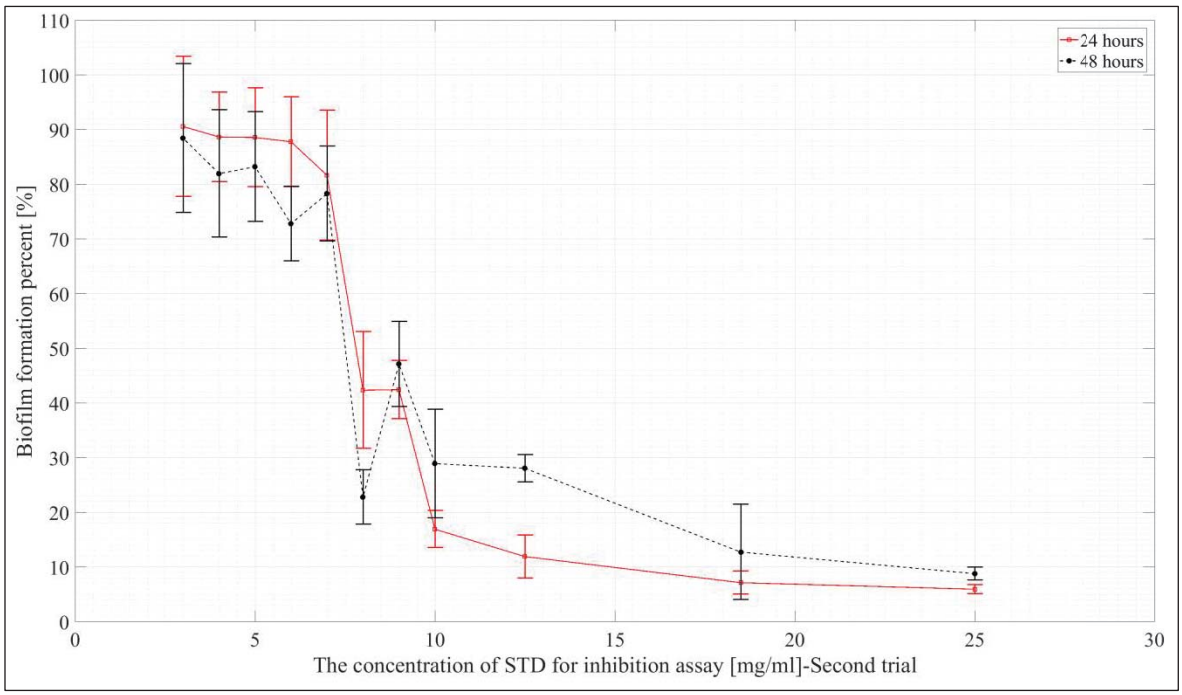


Figure 4.8. The experiment of STD for inhibition assay-Second trial.

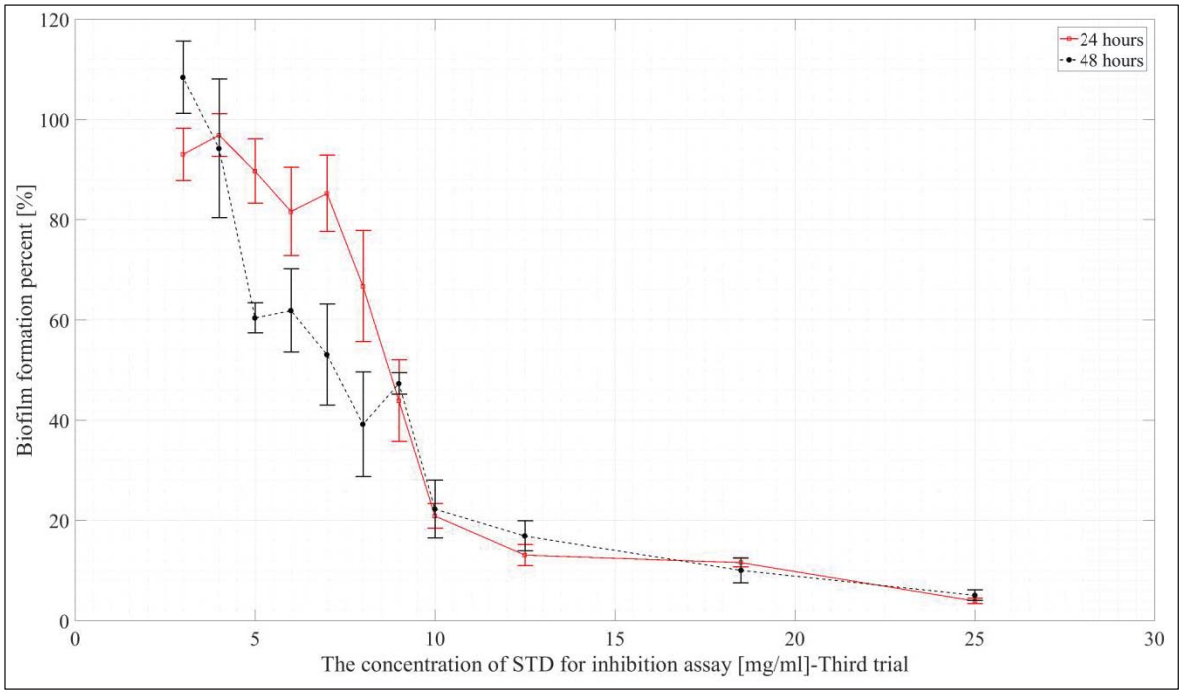


Figure 4.9. The experiment of STD for inhibition assay-Third trial.

4.4. BIOFILM DISRUPTION ASSAY FOR *Pseudomonas aeruginosa* (ATCC-27853) USING BA CONCENTRATIONS

The results of disruption assay performed by BA are presented in Figure 4.10, Figure 4.11 and Figure 4.12. According to the results of experiments which were conducted in 24 hrs, there is no significant effect of the substance on the biofilm formation. However, a slight reduction of biofilm can be detected after longer treatment. Especially, the highest concentration shows better performance compared to others.

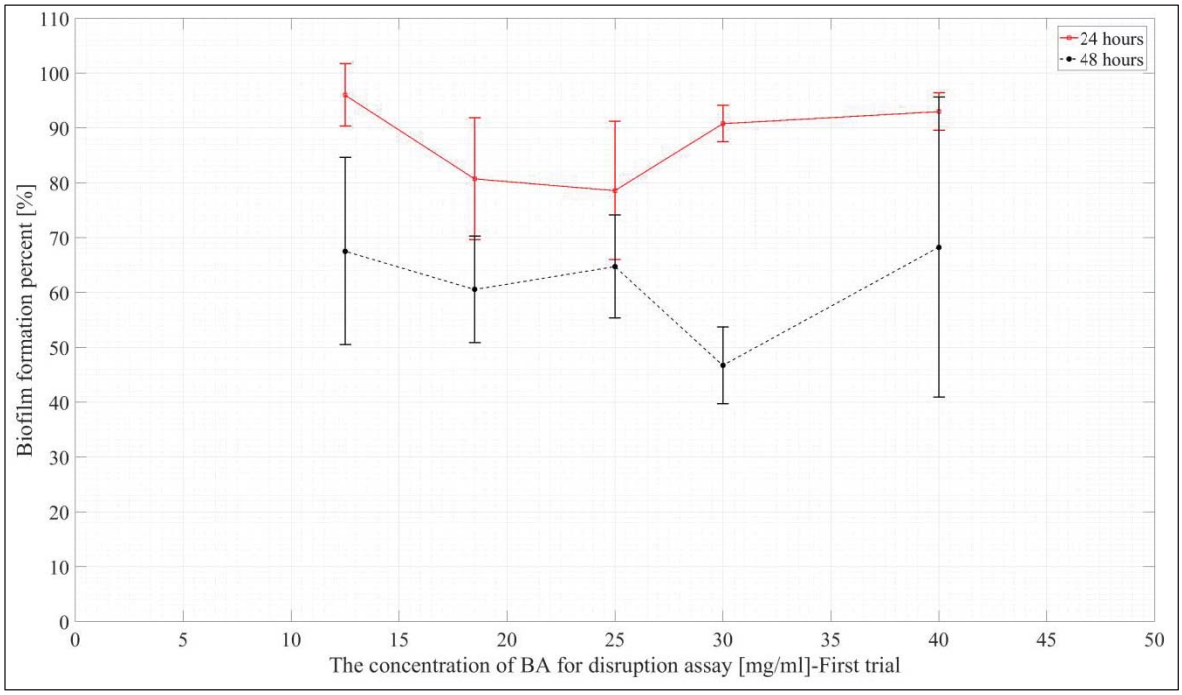


Figure 4.10. The experiment of BA for disruption assay-First trial.

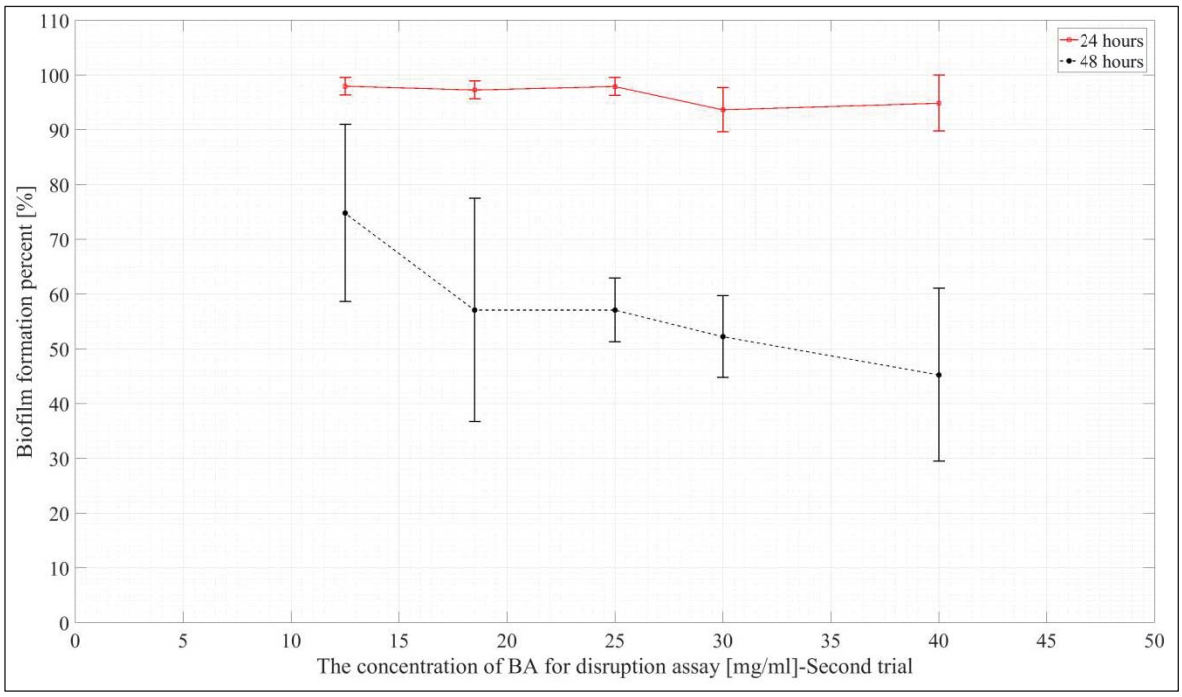


Figure 4.11. The experiment of BA for disruption assay-Second trial.

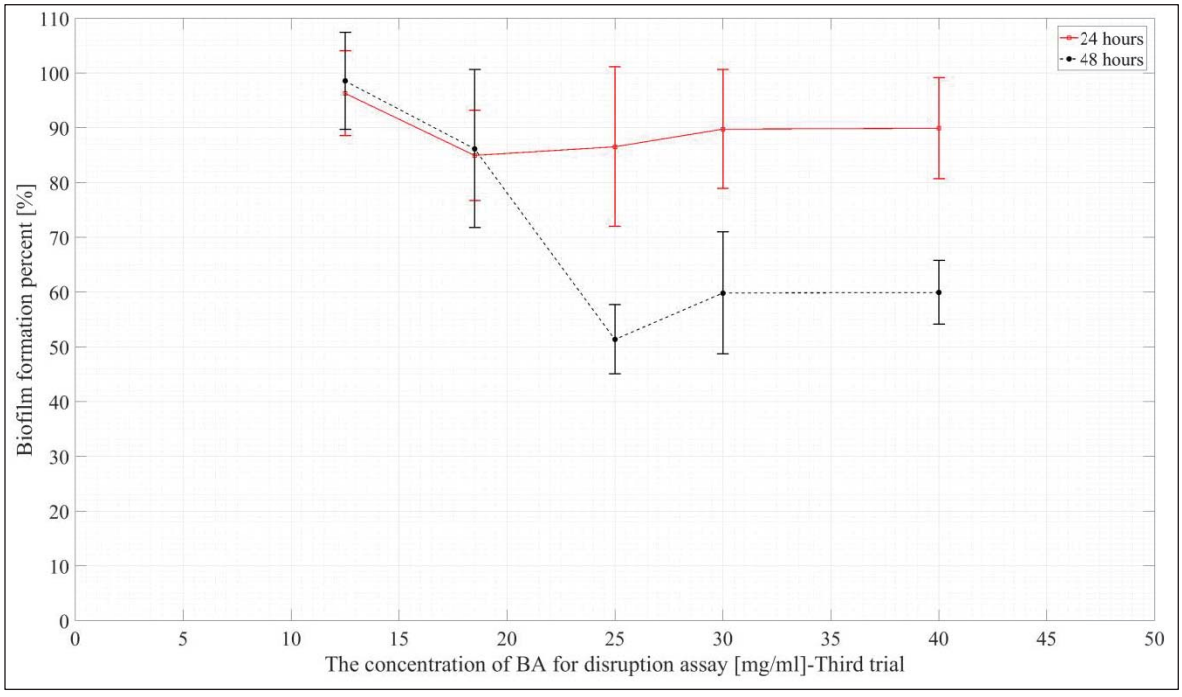


Figure 4.12. The experiment of BA for disruption assay-Third trial.

4.5. BIOFILM DISRUPTION ASSAY FOR *Pseudomonas aeruginosa* (ATCC-27853) USING DOT CONCENTRATIONS

Figure 4.13, Figure 4.14 and Figure 4.15 demonstrate the disruption assay for *Pseudomonas aeruginosa* (ATCC-27853) using DOT various concentrations. In this part, interesting results were obtained. After treatment for 24 hrs, the disruption is lower than 20% which can not be accepted. However, a considerable maximum disruption of 45% was obtained for 48 hrs experiments. In addition, it is observed that by increasing the concentration of DOT the biofilm formation increases.

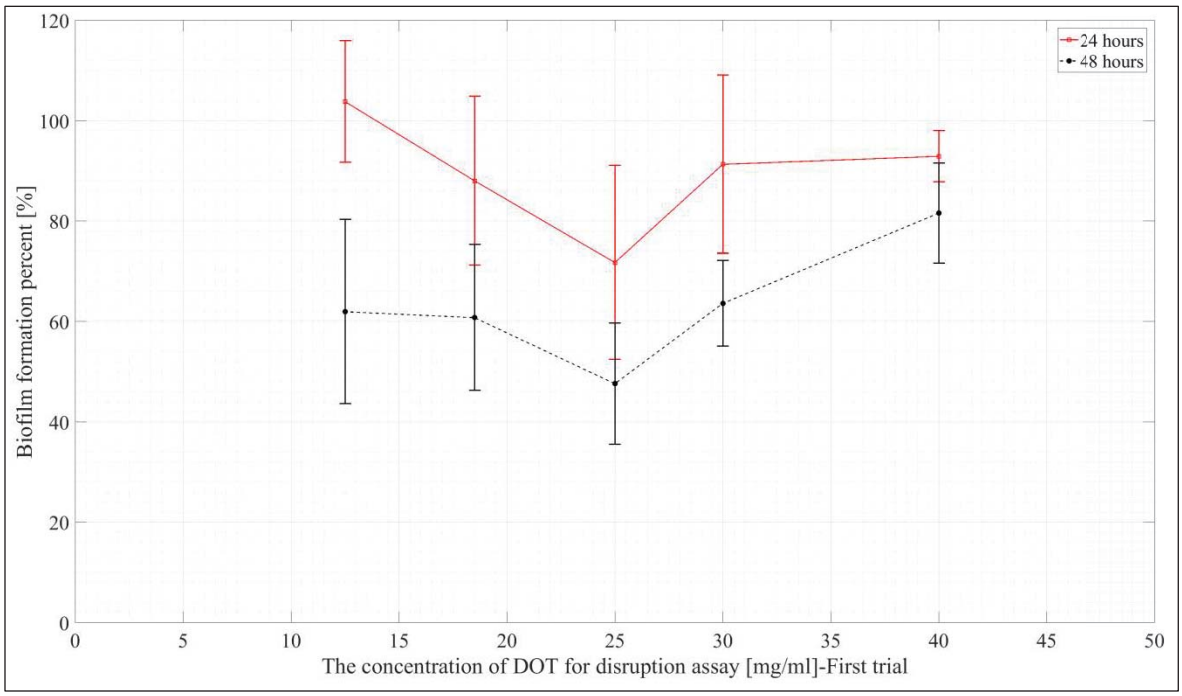


Figure 4.13. The experiment of DOT for disruption assay-First trial.

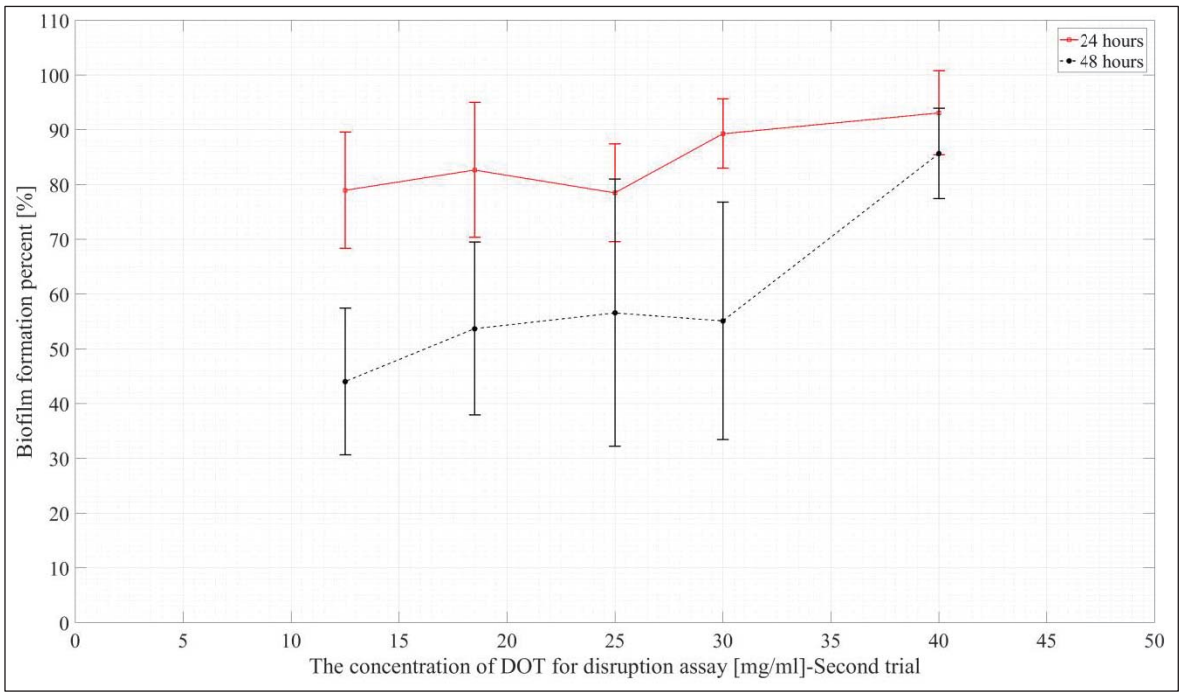


Figure 4.14. The experiment of DOT for disruption assay-Second trial.

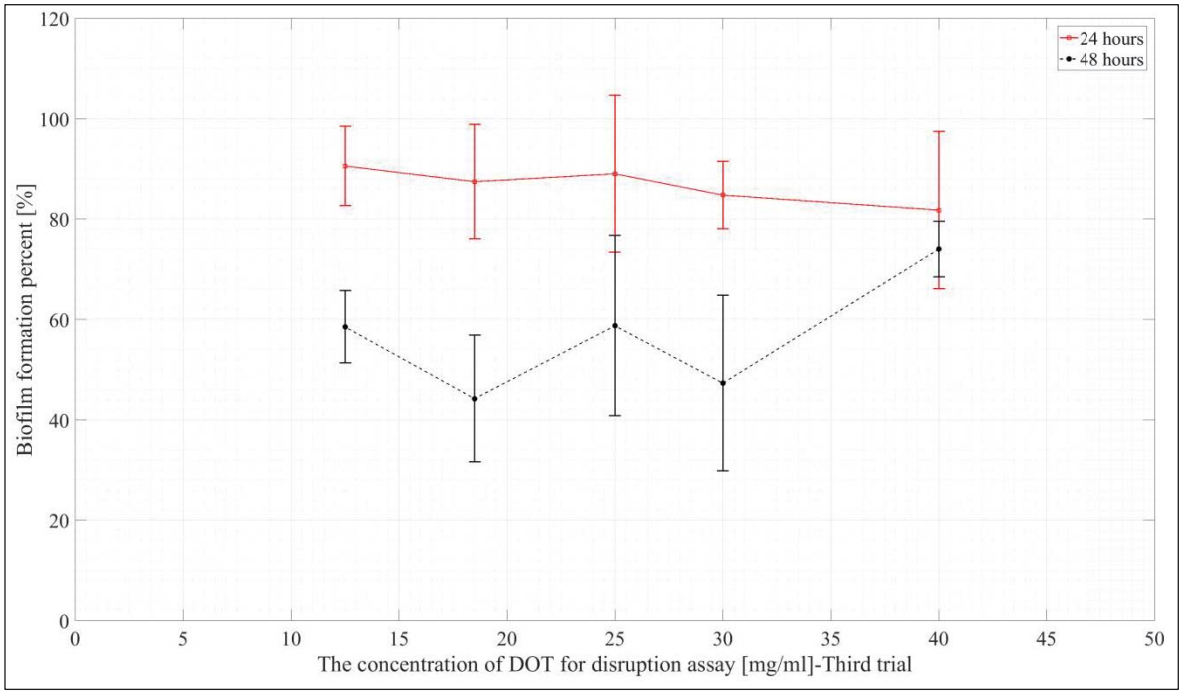


Figure 4.15. The experiment of DOT for disruption assay-Third trial.

4.6. BIOFILM DISRUPTION ASSAY FOR *Pseudomonas aeruginosa* (ATCC-27853) USING STD CONCENTRATIONS

The results for this part are presented in Figure 4.16, Figure 4.17 and Figure 4.18. A broadly similar pattern for 48 hrs experiments can be seen in the results. The maximum biofilm disruption occurs for 30 mg/ml in all the trials for 48 hrs experiments. While a unique pattern does not exist for 24hrs experiments. It should be mentioned that no remarkable result was obtained by this experiment.

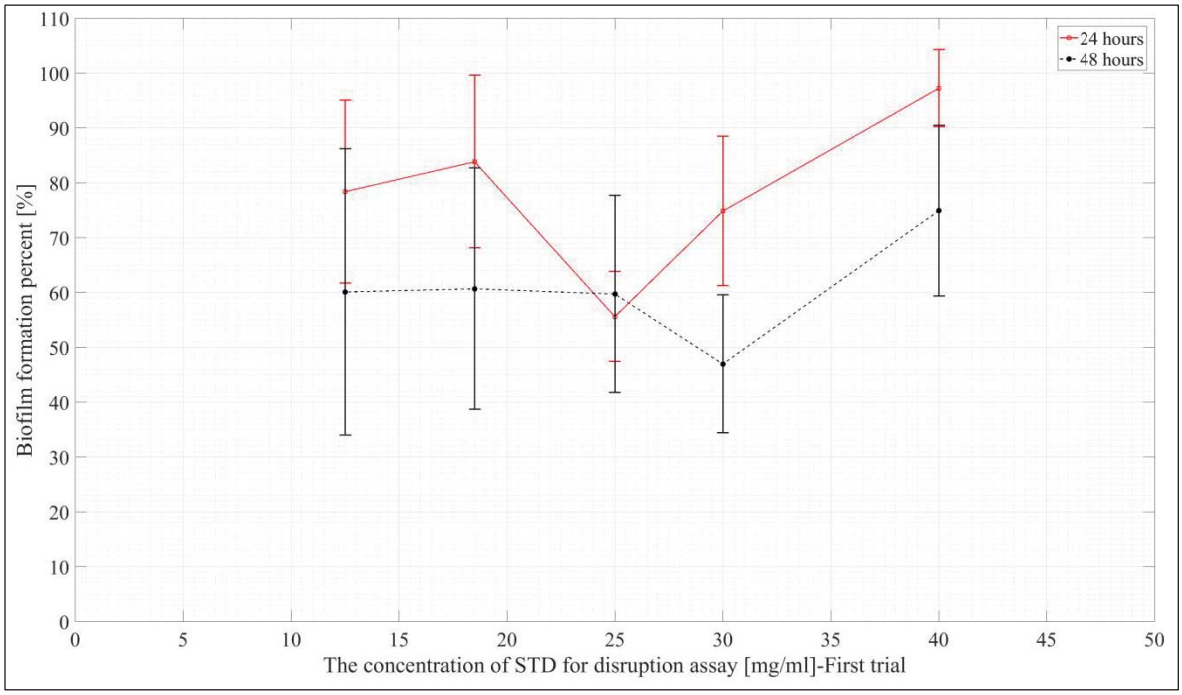


Figure 4.16. The experiment of STD for disruption assay-First trial.

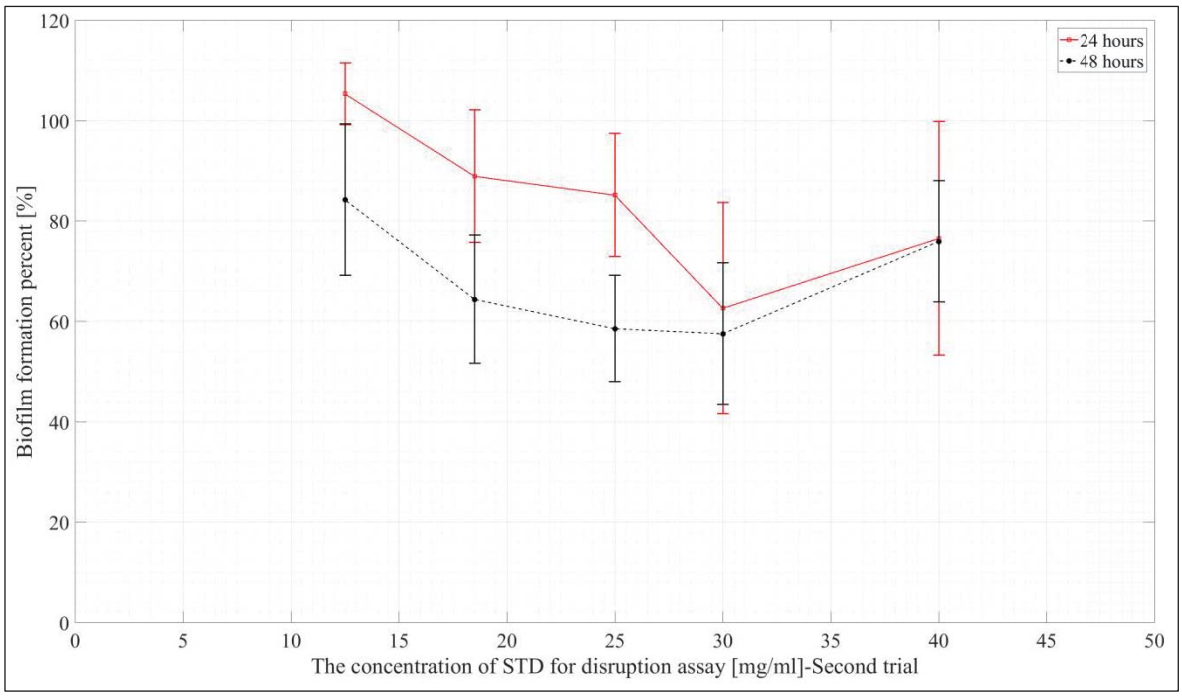


Figure 4.17. The experiment of STD for disruption assay-Second trial.

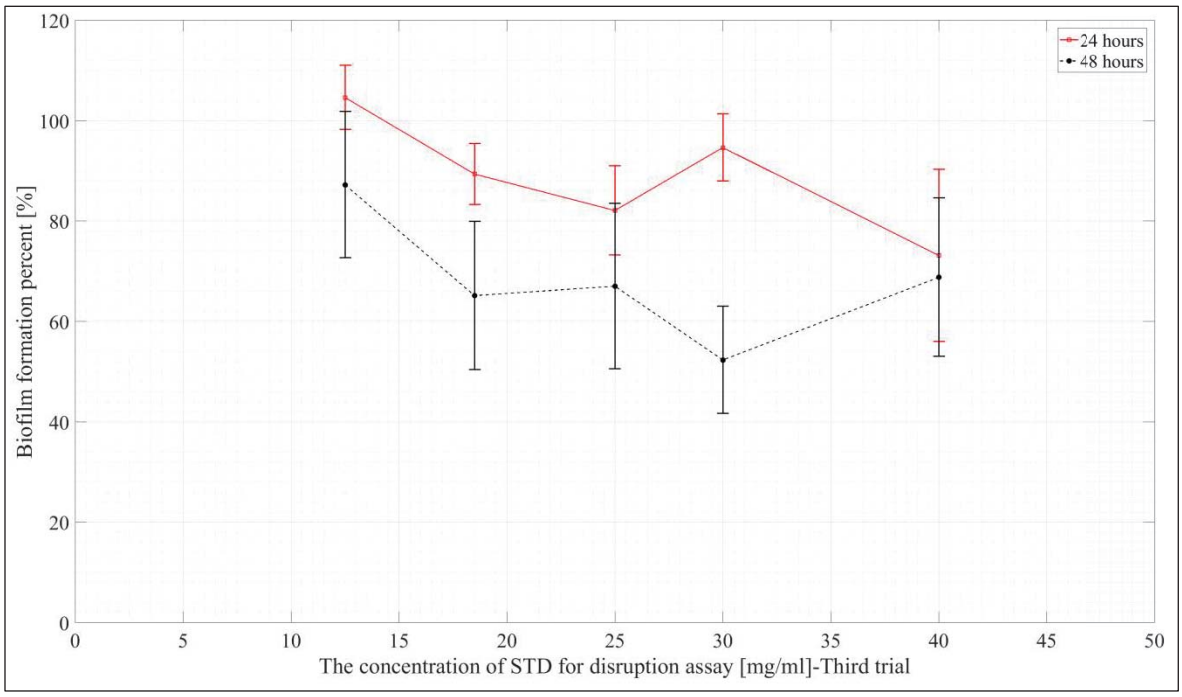


Figure 4.18. The experiment of STD for disruption assay-Third trial.

4.7. BIOFILM INHIBITION ASSAY FOR *Escherichia coli* (MG1655-K12) USING BA CONCENTRATIONS

Various BA concentrations were tested for biofilm inhibition assay of *E. coli*. As it can be seen in Figure 4.19, Figure 4.20 and Figure 4.21, the highest concentration of BA, 25 mg.ml, showed a reduction of 50 to 60% of the biofilm formation. Despite the random behavior of biofilm formation for the lower concentrations of BA in 24 and 48 hrs, an overall reduction of biofilm formation can be detected for most of the cases. However, the most effective concentration for both 24 and 48 hrs is 25mg/ml of BA.

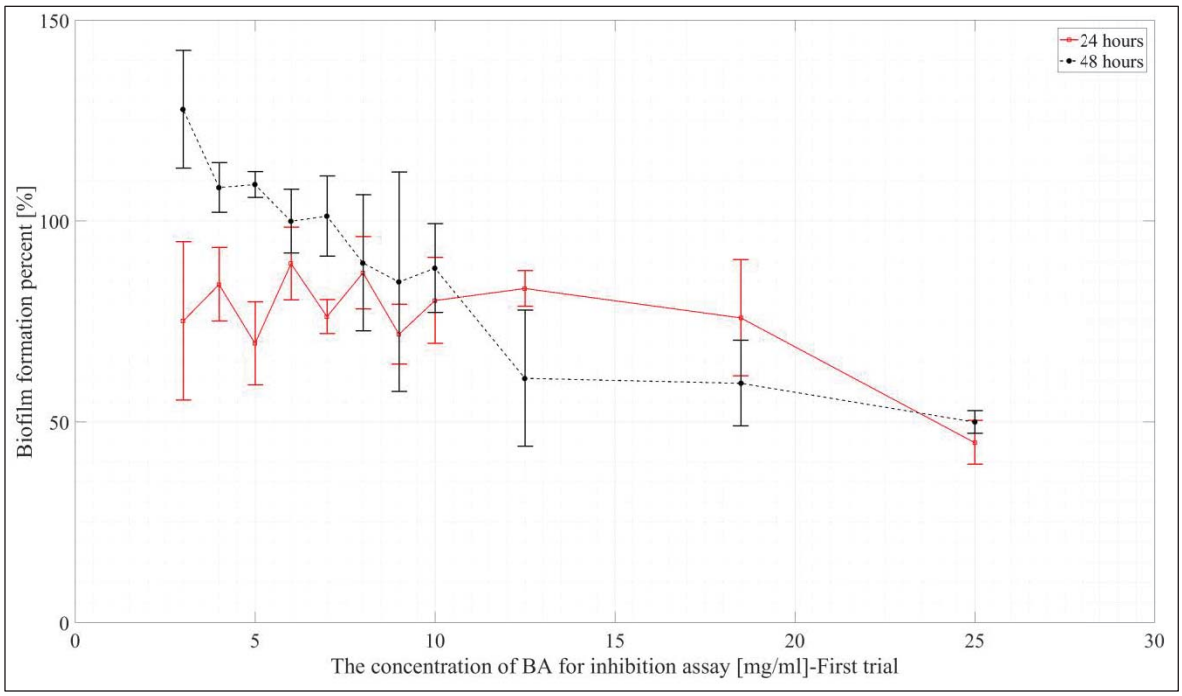


Figure 4.19. The experiment of BA for inhibition assay-First trial.

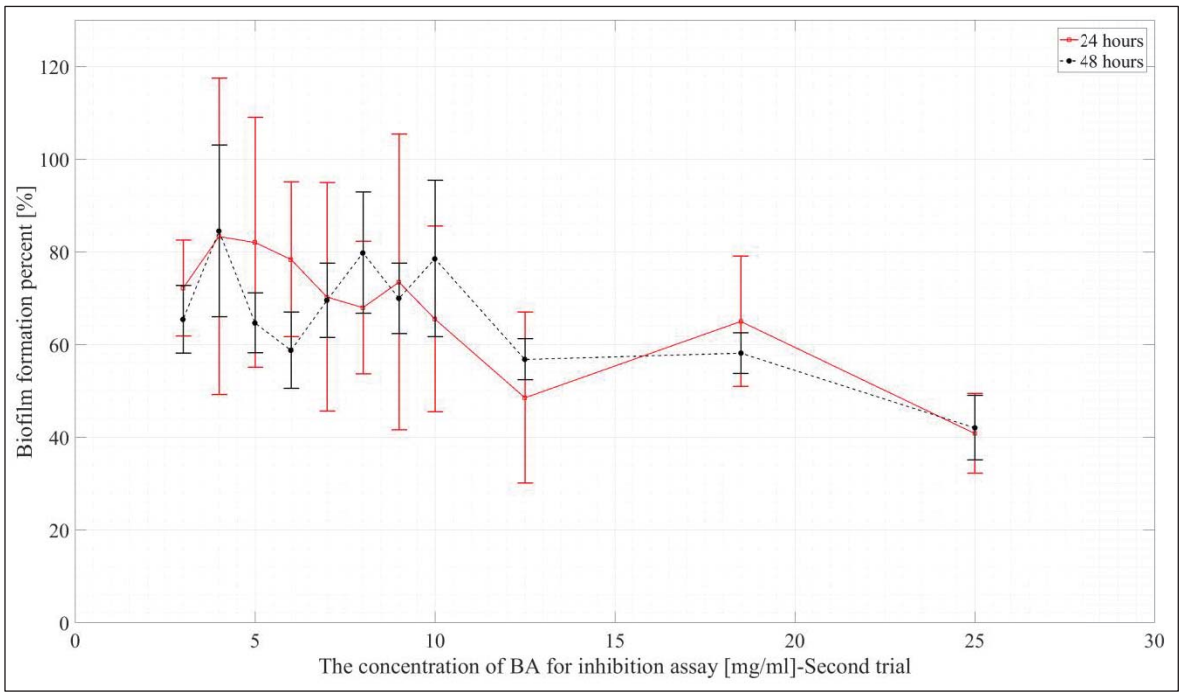


Figure 4.20. The experiment of BA for inhibition assay-Second trial.

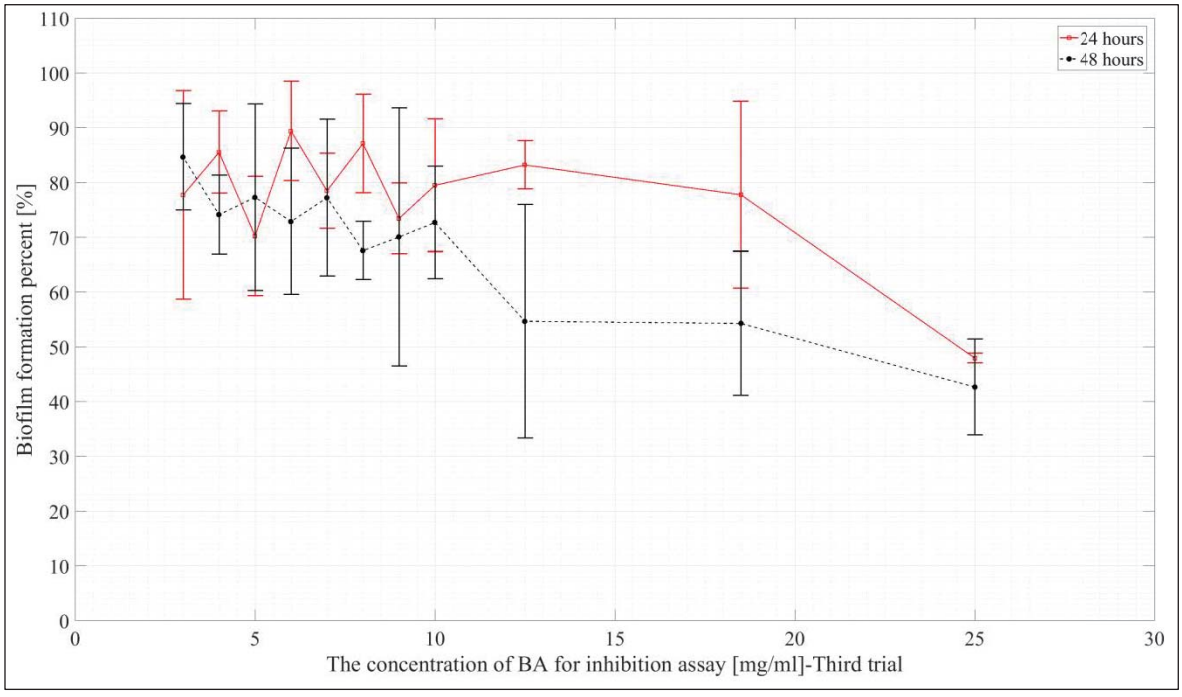


Figure 4.21. The experiment of BA for inhibition assay-Third trial.

4.8. BIOFILM INHIBITION ASSAY FOR *Escherichia coli* (MG1655-K12) USING DOT CONCENTRATIONS

Figure 4.22, Figure 4.23 and Figure 4.24 represent the biofilm inhibition assay for *E. coli* using DOT concentration. A significant decline of biofilm formation can be observed for 18.5 and 25 mg/ml of DOT in all the cases. Compared to BA, high dosages of DOT are highly capable of preventing biofilm formation. As shown before, BA only was able to decrease the biofilm formation till 40%. While DOT can reduce it to 20%. A strange rise found for dosages 5-12.5 mg/ml for all of the 48hrs trials.

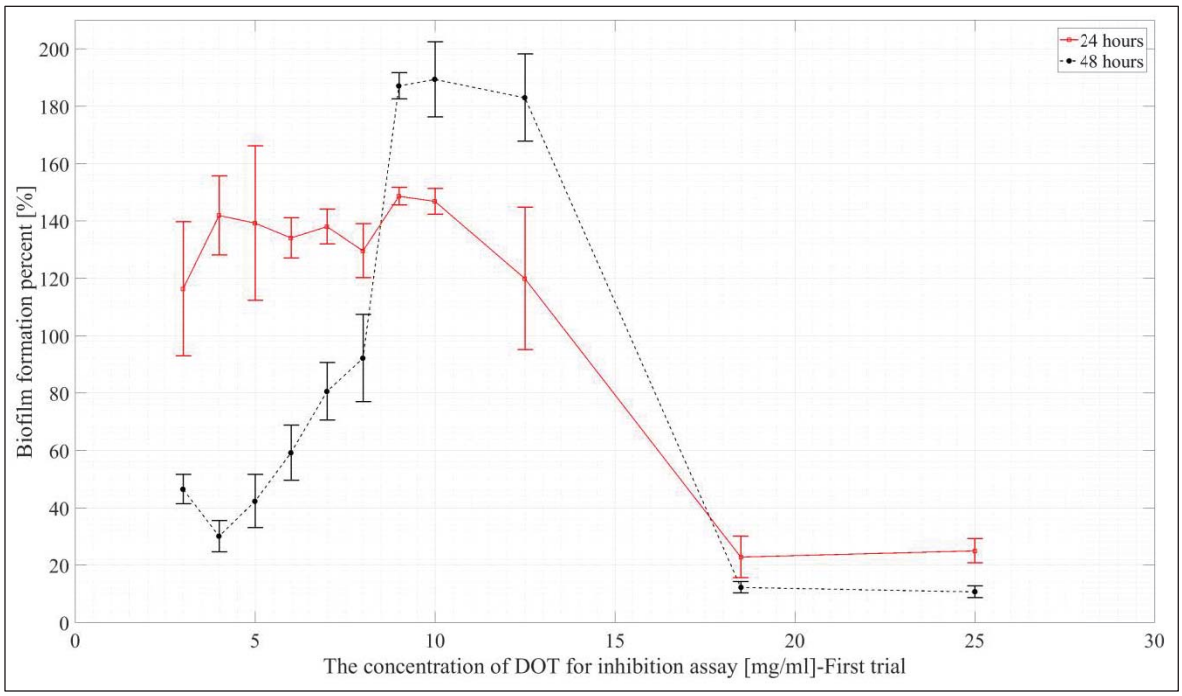


Figure 4.22. The experiment of DOT for inhibition assay-First trial.

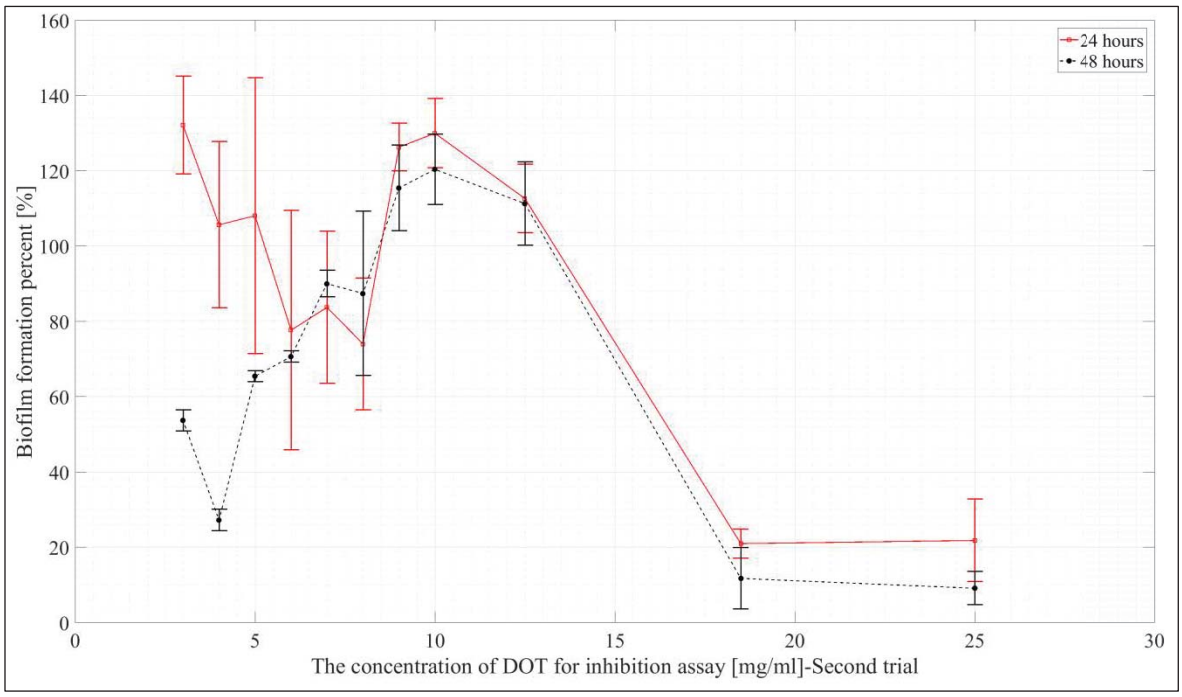


Figure 4.23. The experiment of DOT for inhibition assay-Second trial.

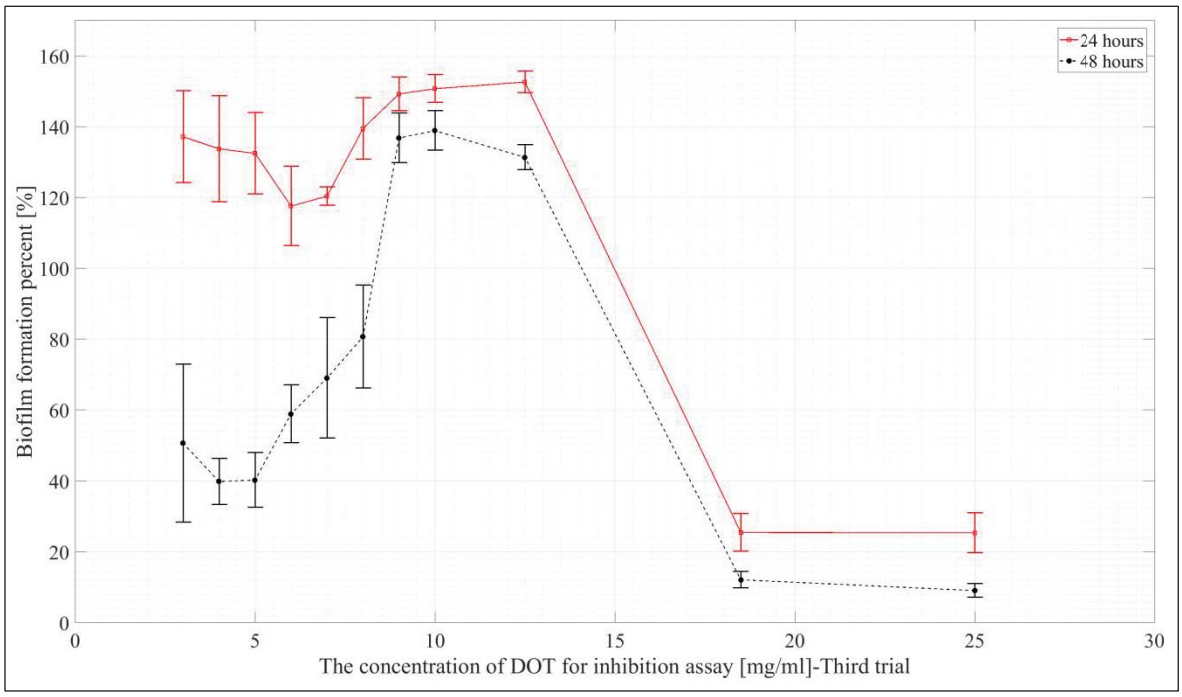


Figure 4.24. The experiment of DOT for inhibition assay-Third trial.

4.9. BIOFILM INHIBITION ASSAY FOR *Escherichia coli* (MG1655-K12) USING STD CONCENTRATIONS

Biofilm inhibition assay for *E. coli* was performed using STD concentrations. Similar to DOT, the highest concentration of STD shows an acceptable decrease of biofilm formation (Figure 4.25, Figure 4.26 and Figure 4.27). A reduction of more than 80% of biofilm formation for all the cases of both 24 and 48 hrs is particularly impressive. Furthermore, it should be noticed that 48 hrs trails give more effective results. Nevertheless, the standard deviation of some concentrations is rather high.

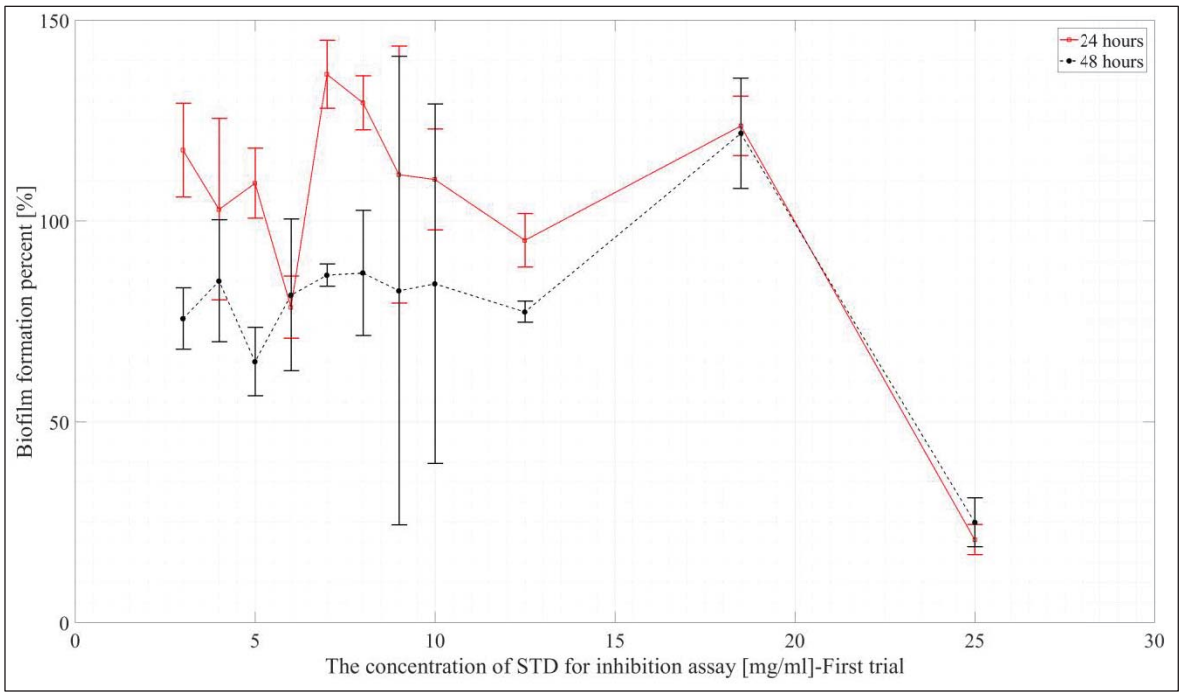


Figure 4.25. The experiment of STD for inhibition assay-First trial.

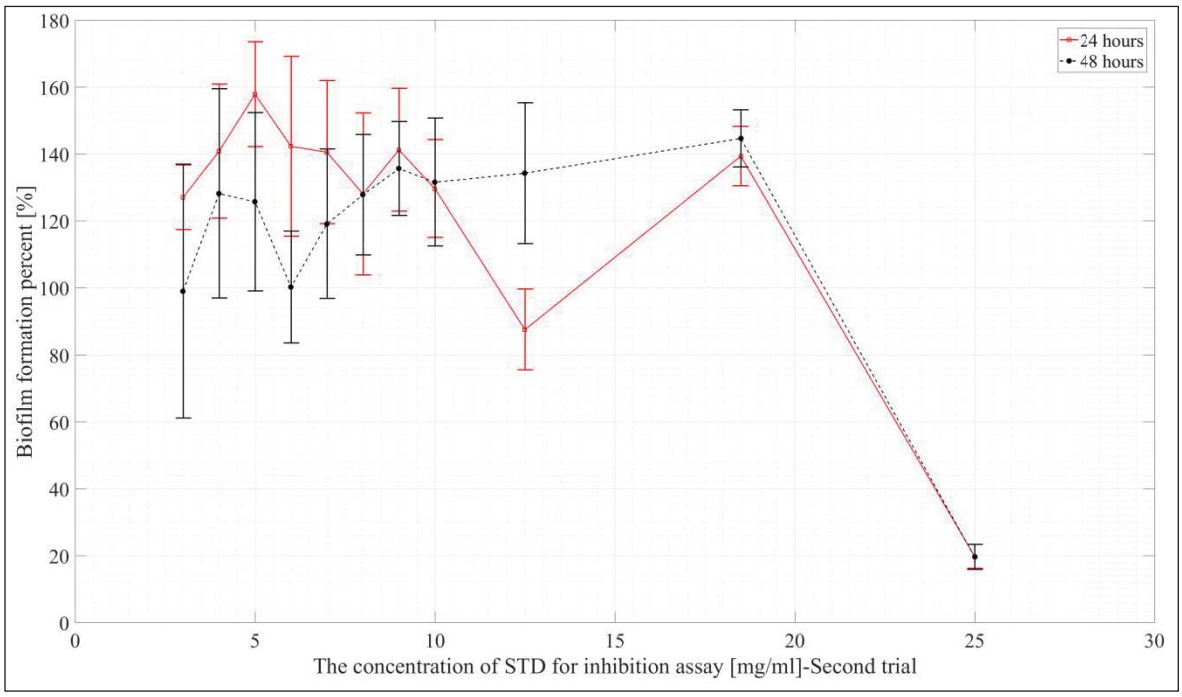


Figure 4.26. The experiment of STD for inhibition assay-Second trial.

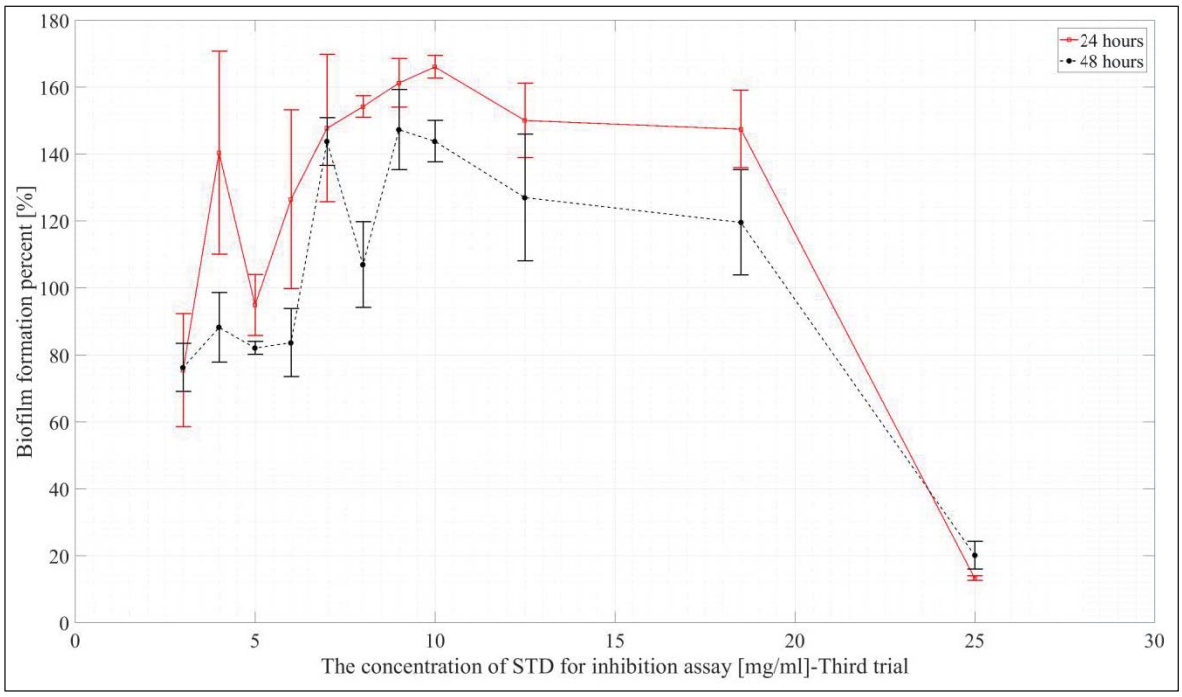


Figure 4.27. The experiment of STD for inhibition assay-Third trial.

4.10. BIOFILM DISRUPTION ASSAY FOR *Escherichia coli* (MG1655-K12) USING BA CONCENTRATIONS

The disruption assay for *E. coli* using BA found ineffective. No distinguishable pattern is observed for disruption assay using BA. As presented in Figure 4.28, Figure 4.29 and Figure 4.30, the biofilm formation can not be controlled or avoided by using BA.

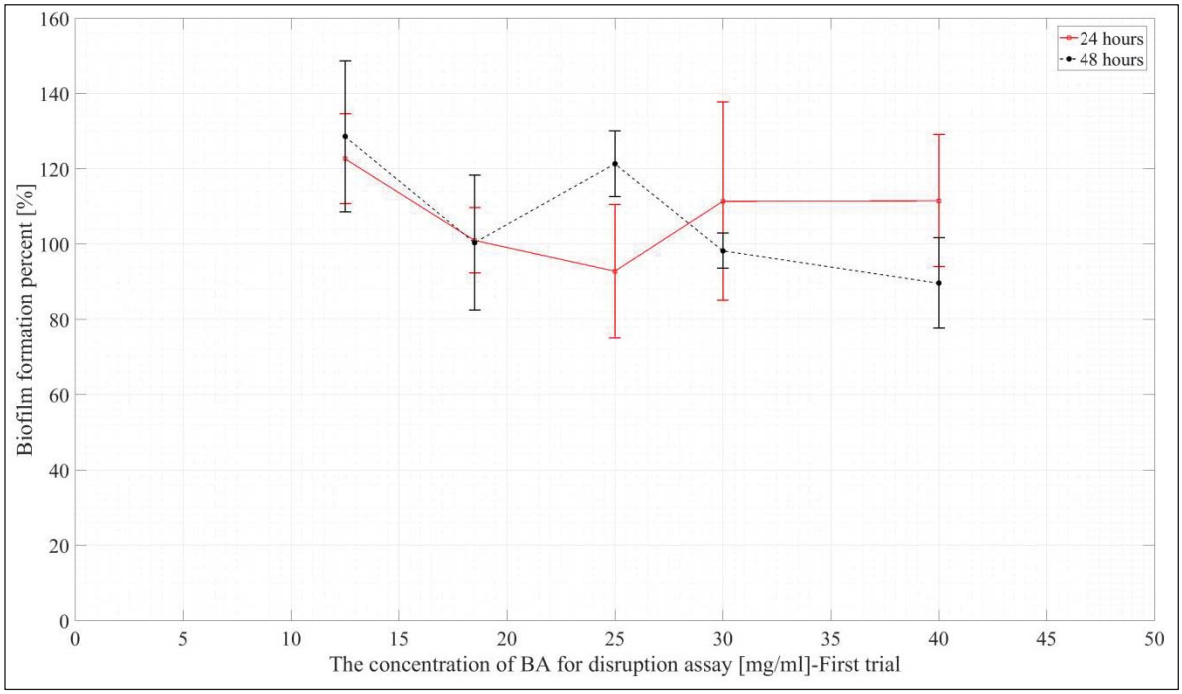


Figure 4.28. The experiment of BA for disruption assay-First trial.

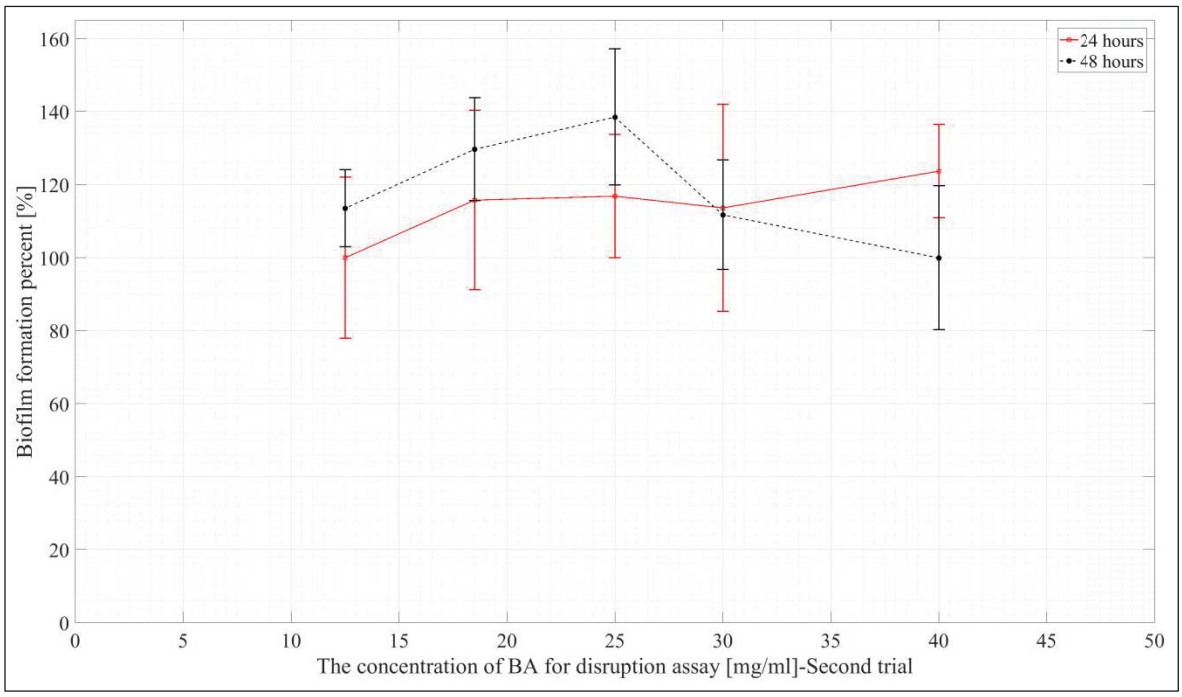


Figure 4.29. The experiment of BA for disruption assay-Second trial.

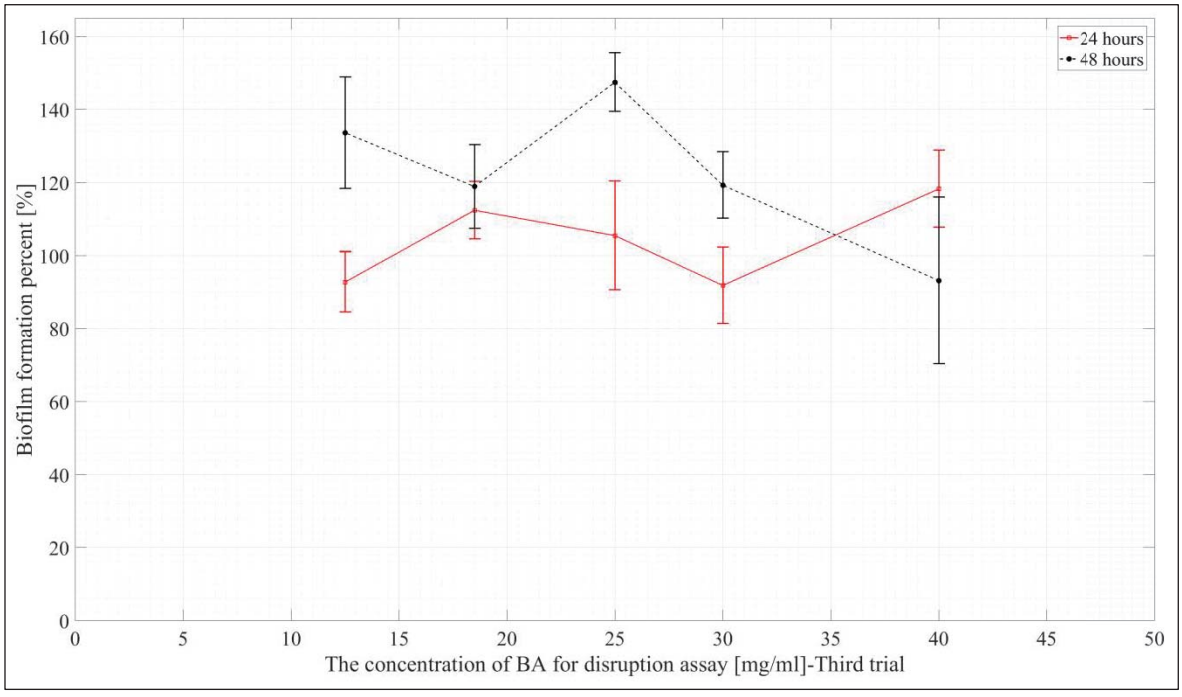


Figure 4.30. The experiment of BA for disruption assay-Third trial.

4.11. BIOFILM DISRUPTION ASSAY FOR *Escherichia coli* (MG1655-K12) USING DOT CONCENTRATIONS

The results of three different trials of disruption assay using DOT at 24 and 48 hrs look almost similar qualitatively (Figure 4.31, Figure 4.32 and Figure 4.33). A steady decrease of biofilm formation to an average of 40% for 30mg/ml is found at 48 hrs experiments. Then by increasing the dosage to 40 mg/ml, a quick growth of biofilm is measured. The same behavior can be seen for 24 hrs measurement with less increase. Above all, the result of experiments at 48 hrs is reasonably superior.

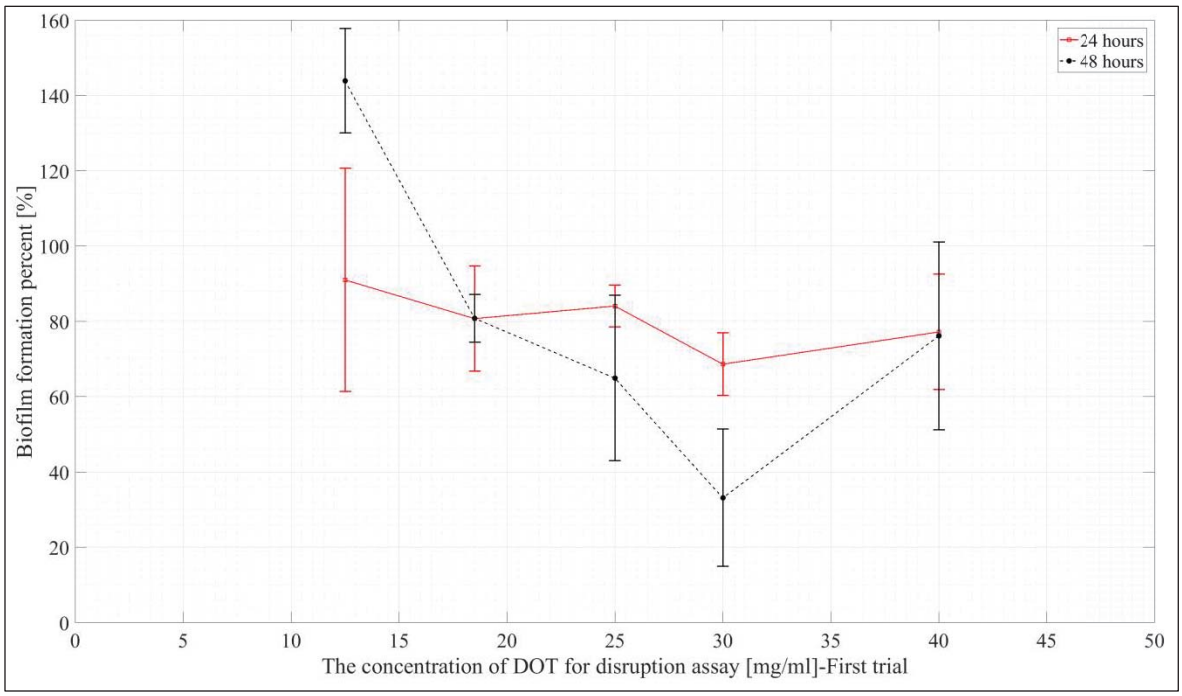


Figure 4.31. The experiment of DOT for disruption assay-First trial.

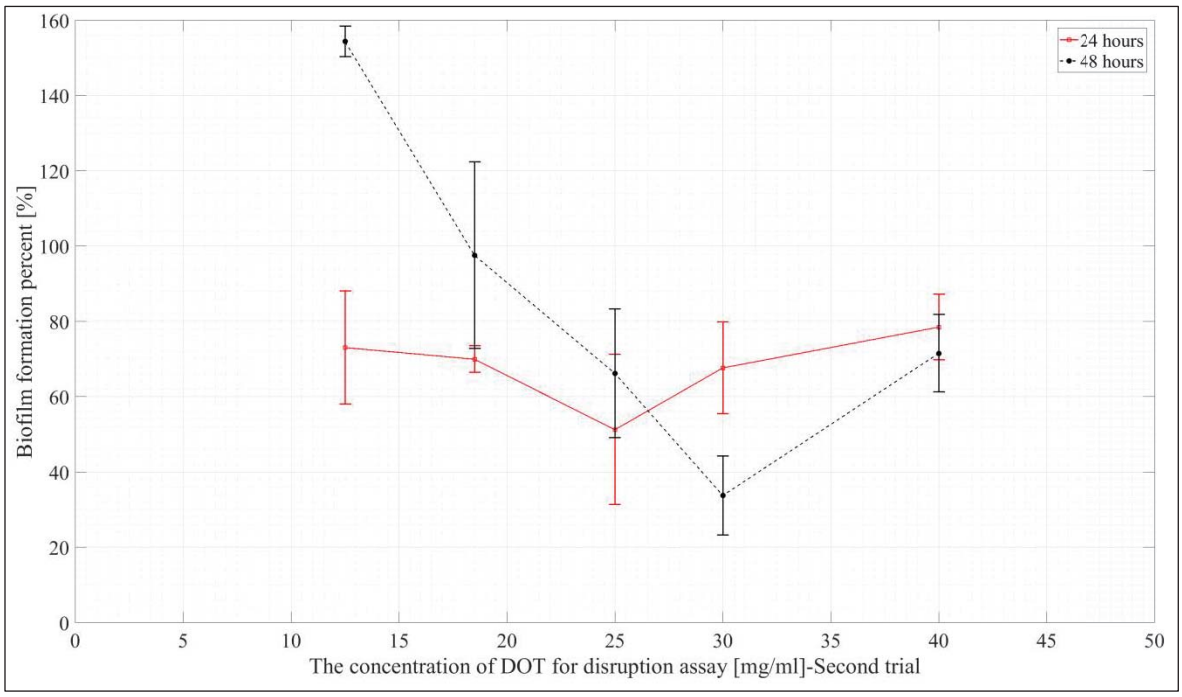


Figure 4.32. The experiment of DOT for disruption assay-Second trial.

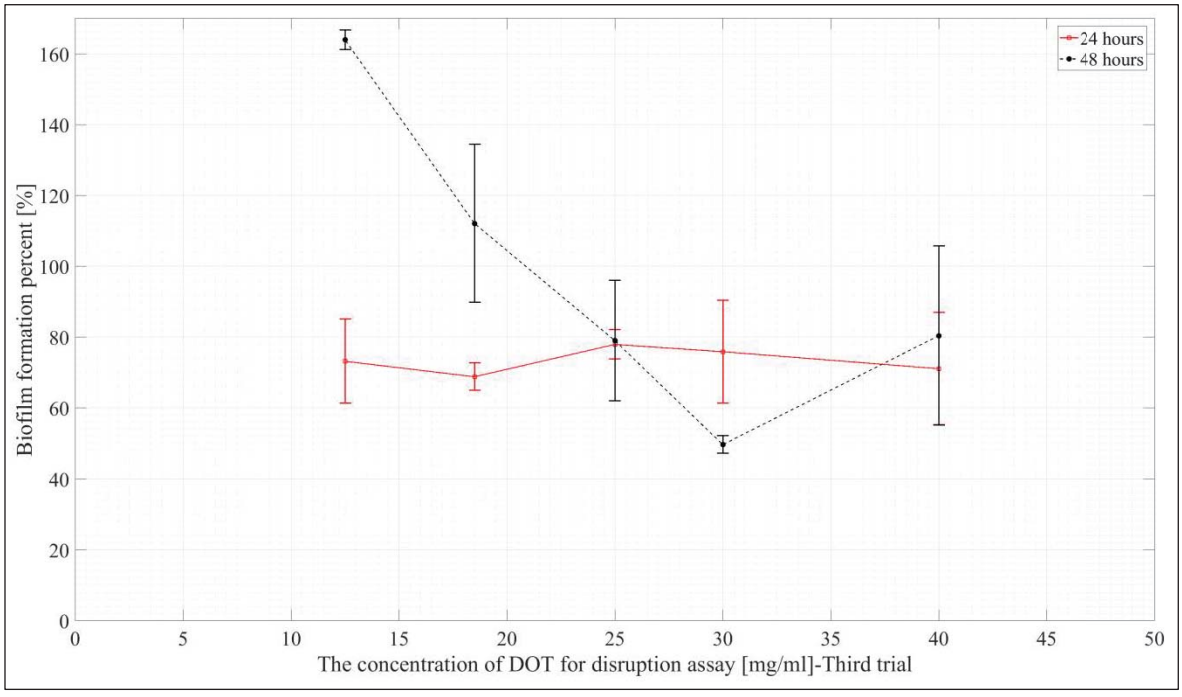


Figure 4.33. The experiment of DOT for disruption assay-Third trial.

4.12. BIOFILM DISRUPTION ASSAY FOR *Escherichia coli* (MG1655-K12) USING STD CONCENTRATIONS

Biofilm disruption assay using STD was performed for *E. coli* at 24 and 48 hrs. A gradual decline in biofilm formation is observed for all the trials including 24 and 48 hrs, as presented in Figure 4.34, Figure 4.35 andFigure 4.36. In general, improved results are obtained with experiments conducted at 24 hrs. Furthermore, the highest dosage, 40 mg/ml gives lowest biofilm formation which is around 40%.

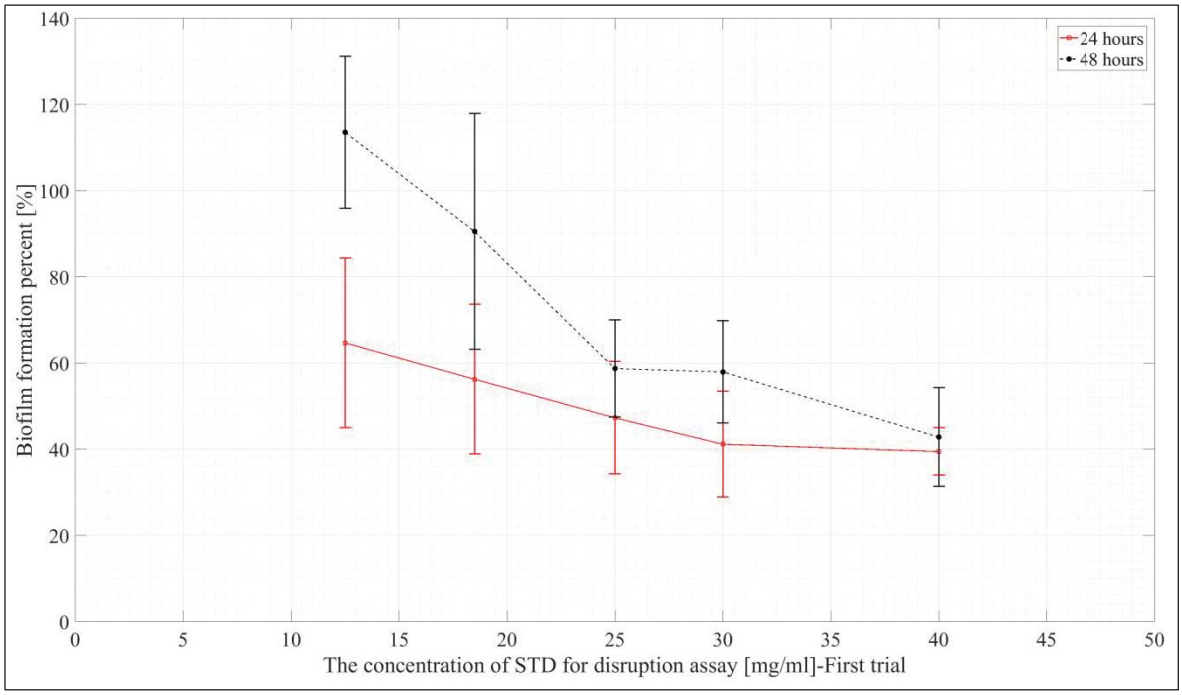


Figure 4.34. The experiment of STD for disruption assay-First trial.

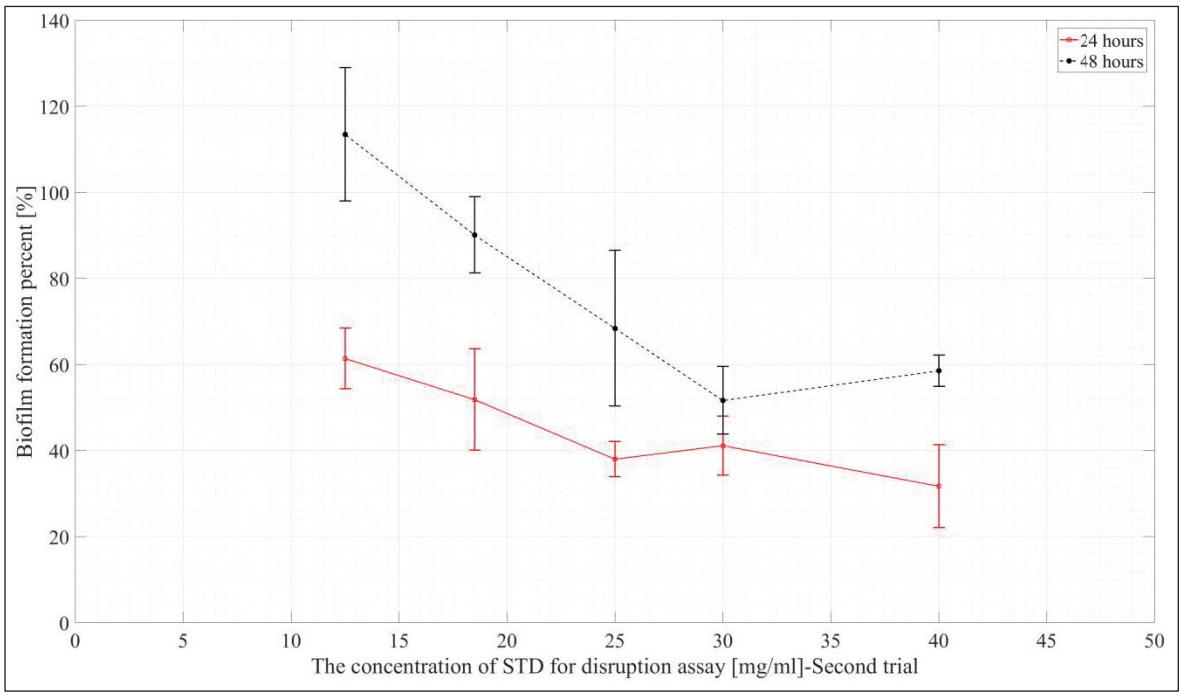


Figure 4.35. The experiment of STD for disruption assay-Second trial.

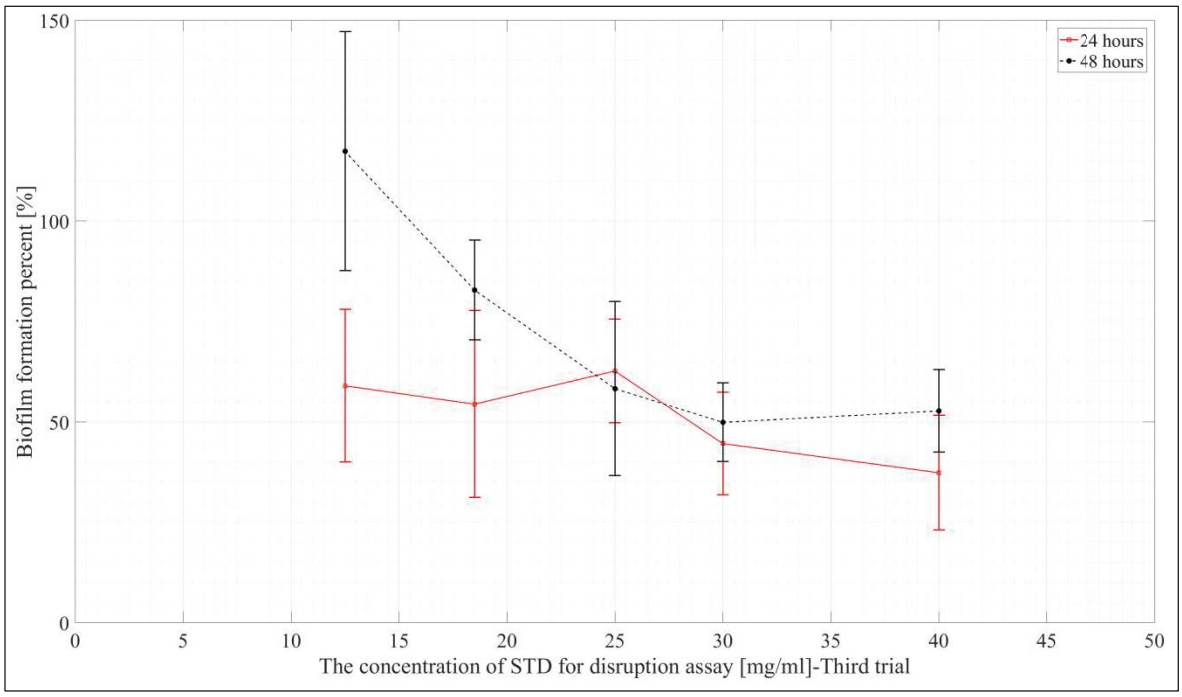


Figure 4.36. The experiment of STD for disruption assay-Third trial.

5. DISCUSSION

The importance of biofilm formation has got attention since the 1970s. Biofilms widely occur on surfaces which have a direct contact with or even in the vicinity of water [137]. As described in the introduction, swimming pools are one of the suitable places that biofilm can be found. Many people get serious infections due to using swimming pools or bathrooms all over the world. Various approaches and methods, including theoretical and experimental, are required to overcome the biofilm resistance problem. The use of a proper approach and anti-biofilm agents is an essential element to acquire satisfactory and easy-to-reproduce results which can lead the main goal of the research.

Despite the fact that many microorganisms are involved in biofilm formation in mentioned places (swimming pools and bathrooms etc.), two major bacteria are chosen in the current study; *Escherichia coli* and *Pseudomonas aeruginosa*. Inhibition and disruption assay as used methods were performed on these two bacterial strains.

According to the obtained results, the various behaviors of biofilm formation by *Pseudomonas aeruginosa* and *Escherichia coli* in a certain treatment period using different anti-biofilm agents were observed. In all the cases for inhibition assay, the higher concentration of antimicrobial agents (BA, DOT and STD) the lower biofilm formation. The minimum biofilm formation occurred in the concentration of 25 mg/ml. In addition, the lower concentrations of BA and DOT could not be effective to inhibit the biofilm formation for *Pseudomonas aeruginosa*.

Regarding the interesting results of inhibition assay by STD for *Pseudomonas aeruginosa*, it was observed that the concentrations higher than 10mg/ml can highly affect the biofilm formation. In contrast to the higher dosages, no significant inhibition was noticed for lower concentrations. Both experiments in 24 and 48 hrs demonstrated fairly similar behavior for all the concentrations which shows the time of treatment did not affect the final result.

Compared to the inhibition assay, no significant decrease in biofilm formation was obtained by disruption assay for *Pseudomonas aeruginosa*. However, biofilm formation can decrease to 50% in some cases. This result was expected due to the biofilm features which may prevent antibacterial agents from destroying it. The results at 48 hrs showed improvement in biofilm disruption compared to 24 hrs for all the cases. A possible

explanation for this behavior may be the lack of nutrition for biofilm after a certain time period.

The nutrition factor is essential for the growth of biofilm [138]. TSB was the first media tried to grow *E. coli* which led any result. However, adding rich broth such as Luria–Bertani (LB) with additional glucose helped the biofilm to form.

The inhibition assay for *E. coli* revealed that DOT and STD can provide more efficient result than BA. BA only could reduce the biofilm formation to 40%. Whereas DOT and STD had remarkably similar results, more than 80%, for the highest concentration of DOT and STD 25 mg/ml. Moreover, the biofilm growth for 18.5 and 25 mg/ml of DOT was close. Therefore, using DOT would be beneficial if the concentration value is of importance.

Similar to inhibition assay for *E. coli*, using DOT and STD in disruption assay yielded satisfactory results compared to BA. BA was not capable of disrupting the biofilm even at high concentration. Whereas, 30 mg/ml of DOT could decline the biofilm formation by an average 60% at 48 hrs treatment. It is noteworthy to mention that for all the trails of DOT, longer treatment was more effective. Furthermore, by increasing the DOT concentration an adverse effect was observed at 48 hrs treatment. A sudden increase of biofilm formation occurred at 40 mg/ml may result from the microorganism behavior which can consider the antibiofilm agent as a nutrition.Unlike DOT results, 24 hrs treatment with STD showed a better effect compared to longer treatment and a reduction of 50% was noticed.

According to the obtained results of this research inhibition assay for *Pseudomonas aeruginosa* using three antibiofilm agents was successful and a significant effect of those agents was observed for the highest dosages at the both treatment times; more than 80%, 70% and 90% biofilm inhibition for BA, DOT and STD, respectively. Nevertheless, the agents were not capable of disrupting the biofilm and no influence was seen.

It can be concluded that the highest dosage of DOT and STD could inhibit *E. coli* at both 24 and 48 hrs treatment. BA also had the same behavior with a lower decline of biofilm formation. Particularly, the concentration of 25 mg/ml of STD could inhibit the biofilm by more than 75%. The disruption assay using DOT and STD led a fairly decrease in biofilm, while BA acted strangely.

6. CONCLUSION

With novel strategies and technologies, the efforts are based on clean and free of pathogenic microorganisms in swimming pools and water tanks. Biofilm can be long-term reservoirs depends on ecological and epidemiological features of the aquatic places. However, it has become clear that, according to this study, boron derivatives in some individual concentrations are a promising factor to inhibit and in some cases disrupt the biofilm.

Disruption and inhibitory effects of different boron derivatives including boric acid (BA), sodium tetraborate decahydrate (STD) and disodium octaborate tetrahydrate (DOT) on biofilm formation by two bacterial species, *Pseudomonas aeruginosa* and *Escherichia coli*, were investigated *in vitro* micro-well bioassay in this study. The results proposed that DOT and STD are potential antimicrobial agents for *E. coli* inhibition. Additionally, three antimicrobial agents used in this study have an antimicrobial effect against biofilm formation in *Pseudomonas aeruginosa*. Further studies are demanded to recognize in what dose of boron biofilm starts to mechanistically inhibit the microorganisms.

In addition, further studies are required to confirm that boron derivatives may be beneficial to use in the drug industry and construction material such as ceramic in order to control biofilm formation and related health problems in the future. For instance, with considering the roughness and thickness of ceramic to well plate, experiments can be performed on ceramic to prove the anti-biofilm effects of boron derivatives.

REFERENCES

1. J. W. Costerton, Z. Lewandowski, D. E. Caldwell, D. R. Korber and H. M. Lappin-Scott. Microbial Biofilms. *Annual Review of Microbiology*, 49:711-745, 1995.

2. S. M. Abdel-aziz and A. Aeron. Bacterial Biofilm: Dispersal and Inhibition Strategies. *Scholarena Journal of Biotechnology*, 1:1-10, 2014.

3. P. K. Taylor, A. T. Y. Yeung and R. E. W. Hancock. Antibiotic Resistance in *Pseudomonas aeruginosa* Biofilms: Towards the Development of Novel Anti-Biofilm Therapies. *Journal of Biotechnology*, 191:121-130, 2014.

4. J. Zhang, X. Rui, L. Wang, Y. Guan, X. Sun and M. Dong. Polyphenolic Extract from Rosa Rugosa Tea Inhibits Bacterial Quorum Sensing and Biofilm Formation. *Food Control*, 42:125-131, 2014.

5. R. M. Donlan. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases*, 33:1387-1392, 2001.

6. P. Brouqui, M. C. Rousseau, A. Stein, M. Drancourt and D. Raoult. Treatment of *Pseudomonas aeruginosa*-Infected Orthopedic Prostheses with Ceftazidime-Ciprofloxacin Antibiotic Combination. *Antimicrobial Agents and Chemotherapy*, 39:2423-2425, 1995.

7. H. Sun, B. Shi, D. A. Lytle, Y. Bai and D. Wang. Formation and Release Behavior of Iron Corrosion Products under the Influence of Bacterial Communities in a Simulated Water Distribution System. *Environmental Science: Processes and Impacts*, 16:576-585, 2014.

8. J. Bryers and B. Ratner. Bioinspired Implant Materials Befuddle Bacteria. *ASM News*, 70:232-237, 2004.

9. M. Simões, L. C. Simões and M. J. Vieira. A Review of Current and Emergent Biofilm Control Strategies. *LWT - Food Science and Technology*, 43:573-583, 2010.

10. R. M. Donlan. Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases*, 8:881-890, 2002.

11. N. Raman, M. R. Lee, S. P. Palecek and D. M. Lynn. Polymer Multilayers Loaded with Antifungal B-Peptides Kill Planktonic *Candida albicans* and Reduce Formation of Fungal Biofilms on the Surfaces of Flexible Catheter Tubes. *Journal of Controlled Release*, 191:54-62, 2014.

12. D. Allison. The Biofilm Matrix. *Biofouling*, 2003.

13. H. C. Flemming and J. Wingender. The Biofilm Matrix. *Nature Reviews. Microbiology*, 8:623-633, 2010.

14. L. H. Zhang and Y. H. Dong. Quorum Sensing and Signal Interference: Diverse Implications. *Molecular Microbiology*, 53:1563-1571, 2004.

15. L. Yang, Y. Hu, Y. Liu, J. Zhang, J. Ulstrup and S. Molin. Distinct Roles of Extracellular Polymeric Substances in *Pseudomonas aeruginosa* Biofilm Development. *Environmental Microbiology*, 13:1705-1717, 2011.

16. B. Vu, M. Chen, R. Crawford and E. Ivanova. Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. *Molecules*, 14:2535, 2009.

17. L. Hobley, C. Harkins, C. E. MacPhee and N. R. Stanley-Wall. Giving Structure to the Biofilm Matrix: An Overview of Individual Strategies and Emerging Common Themes. *FEMS Microbiology Reviews*, 39:649-669, 2015.

18. D. O. Serra, A. M. Richter and R. Hengge. Cellulose as an Architectural Element in Spatially Structured *Escherichia coli* Biofilms. *Journal of Bacteriology*, 195:5540-5554, 2013.

19. P. Vandevivere and D. L. Kirchman. Attachment Stimulates Exopolysaccharide Synthesis by a Bacterium. *Applied and Environmental Microbiology*, 59:3280-3286, 1993.

20. D. G. Allison and I. W. Sutherland. The Role of Exopolysaccharides in Adhesion of Freshwater Bacteria. *Microbiology*, 133:1319-1327, 1987.

21. Y. Liu, C. H. Yang and J. Li. Adhesion and Retention of a Bacterial Phytopathogen *Erwinia chrysanthemi* in Biofilm-Coated Porous Media. *Environmental Science and Technology*, 42:159-165, 2007.

22. S. Park, W. Mackay and D. Reid. *Helicobacter* sp. Recovered from Drinking Water Biofilm Sampled from a Water Distribution System. *Water Research*, 35:1624-1626, 2001.

23. K. E. Searcy, A. I. Packman, E. R. Atwill and T. Harter. Capture and Retention of *Cryptosporidium parvum* Oocysts by *Pseudomonas aeruginosa* Biofilms. *Applied and Environmental Microbiology*, 72:6242-6247, 2006.

24. E. A. Wolyniak, B. R. Hargreaves and K. L. Jellison. Retention and Release of *Cryptosporidium parvum* Oocysts by Experimental Biofilms Composed of a Natural Stream Microbial Community. *Applied and Environmental Microbiology*, 75:4624-4626, 2009.

25. H. C. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S. A. Rice and S. Kjelleberg. Biofilms: An Emergent Form of Bacterial Life. *Nature Reviews Microbiology*, 14(9):563-75

26. T. R. Neu and J. R. Lawrence. Innovative Techniques, Sensors, and Approaches for Imaging Biofilms at Different Scales. *Trends in Microbiology*, 23:233-242, 2015.

27. A. Persat, C. D. Nadell, M. K. Kim, F. Ingremeau, A. Siryaporn, K. Drescher, N. S. Wingreen, B. L. Bassler, Z. Gitai and H. A. Stone. The Mechanical World of Bacteria. *Cell*, 161:988-997, 2015.

28. J. N. Wilking, V. Zaburdaev, M. De Volder, R. Losick, M. P. Brenner and D. A. Weitz. Liquid Transport Facilitated by Channels in *Bacillus subtilis* Biofilms. *Proceedings of the National Academy of Sciences of the United States of America*, 110:848-852, 2013.

29. A. Karimi, D. Karig, A. Kumar and A. M. Ardekani. Interplay of Physical Mechanisms and Biofilm Processes: Review of Microfluidic Methods. *Lab Chip*, 15:23-42, 2015.

30. M. F. Mizan, I. K. Jahid and S. D. Ha. Microbial Biofilms in Seafood: A Food-Hygiene Challenge. *Food Microbiology*, 49:41-55, 2015.

31. P. L. Phillips, R. D. Wolcott, L. J. Cowan and G. S. Schultz. 3 - Biofilms in Wounds and Wound Dressing. *Wound Healing Biomaterials*, pages 55-78, 2016.

32. S. E. Coetser and T. E. Cloete. Biofouling and Biocorrosion in Industrial Water Systems. *Critical Reviews in Microbiology*, 31:213-232, 2005.

33. H. C. Flemming. Biofouling in Water Systems--Cases, Causes and Countermeasures. *Applied Microbiology and Biotechnology*, 59:629-640, 2002.

34. M. Kostakioti, M. Hadjifrangiskou and S. J. Hultgren. Bacterial Biofilms: Development, Dispersal, and Therapeutic Strategies in the Dawn of the Postantibiotic Era. *Cold Spring Harbor Perspectives in Medicine*, 3(4):a010306, 2013.

35. X. Z. Li, J. S. Webb, S. Kjelleberg and B. Rosche. Enhanced Benzaldehyde Tolerance in *Zymomonas mobilis* Biofilms and the Potential of Biofilm Applications in Fine-Chemical Production. *Applied and Environmental Microbiology*, 72:1639-1644, 2006.

36. R. Singh, D. Paul and R. K. Jain. Biofilms: Implications in Bioremediation. *Trends Microbiology*, 14:389-397, 2006.

37. Z. W. Wang and S. Chen. Potential of Biofilm-Based Biofuel Production. *Applied Microbiology and Biotechnology*, 83:1-18, 2009.

38. M. R. Kunduru and A. Pometto III. Continuous Ethanol Production By *Zymomonas mobilis* and *Saccharomyces cerevisiae* in Biofilm Reactors. *Journal of Industrial Microbiology*, 16:249-256, 1996.

39. T. Coenye and H. J. Nelis. *In-Vitro* and *in-Vivo* Model Systems to Study Microbial Biofilm Formation. *Journal of Microbiological Methods*, 83:89-105, 2010.

40. H. Heukelekian and A. Heller. Relation between Food Concentration and Surface for Bacterial Growth. *Journal of Bacteriology*, 40:547-558, 1940.

41. C. E. Zobell. The Effect of Solid Surfaces Upon Bacterial Activity. *Journal of Bacteriology*, 46:39-56, 1943.

42. M. G. Paraje. Antimicrobial Resistance in Biofilms. *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances*, 2:736-744, 2011.

43. L. Hall-Stoodley, J. W. Costerton and P. Stoodley. Bacterial Biofilms: From the Natural Environment to Infectious Diseases. *Nature Reviews Microbiology*, 2:95-108, 2004.

44. S. Szczepanski and A. Lipski. Essential Oils Show Specific Inhibiting Effects on Bacterial Biofilm Formation. *Food Control*, 36:224-229, 2014.

45. G. Solmaz, F. Ozen, Y. Ekinci, P. S. Bird and M. Korachi. Inhibitory and Disruptive Effects of Shiitake Mushroom (Lentinula Edodes) Essential Oil Extract on Oral Biofilms. *Jundishapur Journal of Microbiology*, 6:1-6, 2013.

46. F. Gosselin, L. Madeira, T. Juhna and J. Block. Drinking Water and Biofilm Disinfection by Fenton-Like Reaction. *Water Research*, 47:5631-5638, 2013.

47. S. Dosler and E. Karaaslan. Inhibition and Destruction of *Pseudomonas aeruginosa* Biofilms by Antibiotics and Antimicrobial Peptides. *Peptides*, 62:32-37, 2014.

48. H. Saini, S. Chhibber and K. Harjai. Azithromycin and Ciprofloxacin: A Possible Synergistic Combination against *Pseudomonas aeruginosa* Biofilm-Associated Urinary Tract Infections. *International Journal of Antimicrobial Agents*, 45:359-367, 2014.

49. J. Tsibouklis, M. Stone, A. A. Thorpe, P. Graham, T. G. Nevell and R. J. Ewen. Inhibiting Bacterial Adhesion onto Surfaces: The Non-Stick Coating Approach. *International Journal of Adhesion and Adhesives*, 20:91-96, 2000.

50. M. Thouvenin, V. Langlois, R. Briandet, J. Y. Langlois, P. H. Guerin, J. J. Peron, D. Haras and K. Vallee-Rehel. Study of Erodable Paint Properties Involved in Antifouling Activity. *Biofouling*, 19:177-186, 2003.

51. D. W. Cook. Sensitivity of *Vibrio* Species in Phosphate-Buffered Saline and in Oysters to High-Pressure Processing. *Journal of Food Protection*, 66:2276-2282, 2003.

52. A. C. Quevedo, J. G. Smith, G. E. Rodrick and A. C. Wright. Ice Immersion as a Postharvest Treatment of Oysters for the Reduction of *Vibrio vulnificus*. *Journal of Food Protection*, 68:1192-1197, 2005.

53. H. O. Rashid, H. Ito and I. Ishigaki. Distribution of Pathogenic *Vibrios* and Other Bacteria in Imported Frozen Shrimps and Their Decontamination by Gamma-Irradiation. *World Journal of Microbiology and Biotechnology*, 8:494-499, 1992.

54. U. Klueh, V. Wagner, S. Kelly, A. Johnson and J. Bryers. Efficacy of Silver-Coated Fabric to Prevent Bacterial Colonization and Subsequent Device-Based Biofilm Formation. *Journal of Biomedical Materials Research, Part A*, 53:621-631, 2000.

55. H. Hashimoto. Evaluation of the Anti-Biofilm Effect of a New Anti-Bacterial Silver Citrate/Lecithin Coating in an *in-Vitro* Experimental System Using a Modified Robbins Device. *Kansenshogaku Zasshi*, 75:678-685, 2001.

56. K. A. Whitehead, J. Colligon and J. Verran. Retention of Microbial Cells in Substratum Surface Features of Micrometer and Sub-Micrometer Dimensions. *Colloids Surf B Biointerfaces*, 41:129-138, 2005.

57. A. Splendiani, A. G. Livingston and C. Nicolella. Control of Membrane-Attached Biofilms Using Surfactants. *Biotechnology and Bioengineering*, 94:15-23, 2006.

58. R. Patel. Biofilms and Antimicrobial Resistance. *Clinical Orthopaedics and Related Research*: 437:41-47, 2005.

59. B. D. Hoyle, C. K. Wong and J. W. Costerton. Disparate Efficacy of Tobramycin on Ca(2+)-, Mg(2+)-, and Hepes-Treated *Pseudomonas aeruginosa* Biofilms. *Canadian Journal of Microbiology*, 38:1214-1218, 1992.

60. I. G. Duguid, E. Evans, M. R. Brown and P. Gilbert. Effect of Biofilm Culture Upon the Susceptibility of *Staphylococcus epidermidis* to Tobramycin. *The Journal of Antimicrobial Chemotherapy*, 30:803-810, 1992.

61. O. Tresse, T. Jouenne and G. A. Junter. The Role of Oxygen Limitation in the Resistance of Agar-Entrapped, Sessile-Like *Escherichia coli* to Aminoglycoside and Beta-Lactam Antibiotics. *The Journal of Antimicrobial Chemotherapy*, 36:521-526, 1995.

62. R. M. Donlan, J. W. Costerton, R. M. Donlan and J. W. Costerton. Biofilms : Survival Mechanisms of Clinically Relevant Microorganisms. *Clinical Microbiology,* 15:167-193, 2002.

63. J. Bigger. Treatment of Staphylococcal Infections with Penicillin by Intermittent Sterilisation. *The Lancet,* 244:497-500, 1944.

64. I. Keren, N. Kaldalu, A. Spoering, Y. Wang and K. Lewis. Persister Cells and Tolerance to Antimicrobials. *FEMS Microbiology Letters,* 230:13-18, 2004.

65. K. Lewis. Persister Cells and the Riddle of Biofilm Survival. *Biochemistry (Moscow),* 70:267-274, 2005.

66. A. L. Spoering and K. Lewis. Biofilms and Planktonic Cells of *Pseudomonas aeruginosa* Have Similar Resistance to Killing by Antimicrobials. *Journal of Bacteriology,* 183:6746-6751, 2001.

67. H. S. Moyed and K. P. Bertrand. Hipa, a Newly Recognized Gene of *Escherichia coli* k-12 That Affects Frequency of Persistence after Inhibition of Murein Synthesis. *Journal of Bacteriology,* 155:768-775, 1983.

68. J. G. Leid, M. E. Shirtliff, J. W. Costerton and P. Stoodley. Human Leukocytes Adhere to, Penetrate, and Respond to *Staphylococcus aureus* Biofilms. *Infection and Immunity,* 70:6339-6345, 2002.

69. G. Midelet and B. Carpentier. Impact of Cleaning and Disinfection Agents on Biofilm Structure and on Microbial Transfer to a Solid Model Food. *Journal of Applied Microbiology,* 97:262-270, 2004.

70. T. Bjarnsholt, O. Ciofu, S. Molin, M. Givskov and N. Hoiby. Applying Insights from Biofilm Biology to Drug Development - Can a New Approach Be Developed? *Nature Reviews Drug Discovery,* 12:791-808, 2013.

71. G. Rampioni, L. Leoni and P. Williams. The Art of Antibacterial Warfare: Deception through Interference with Quorum Sensing-Mediated Communication. *Bioorganic Chemistry*, 55:60-68, 2014.

72. T. H. Jakobsen, M. van Gennip, R. K. Phipps, M. S. Shanmugham, L. D. Christensen, M. Alhede, M. E. Skindersoe, T. B. Rasmussen, K. Friedrich, F. Uthe, P. O. Jensen, C. Moser, K. F. Nielsen, L. Eberl, T. O. Larsen, D. Tanner, N. Hoiby, T. Bjarnsholt and M. Givskov. Ajoene, a Sulfur-Rich Molecule from Garlic, Inhibits Genes Controlled by Quorum Sensing. *Antimicrobial Agents and Chemotherapy*, 56:2314-2325, 2012.

73. H. Jenssen, P. Hamill and R. E. Hancock. Peptide Antimicrobial Agents. *Clinical Microbiology Reviews*, 19:491-511, 2006.

74. C. de la Fuente-Nunez, F. Reffuveille, E. F. Haney, S. K. Straus and R. E. Hancock. Broad-Spectrum Anti-Biofilm Peptide That Targets a Cellular Stress Response. *PLoS Pathogens*, 10(5):e1004152, 2014.

75. A. D. Valderrey, M. J. Pozuelo, P. A. Jimenez, M. D. Macia, A. Oliver and R. Rotger. Chronic Colonization by *Pseudomonas aeruginosa* of Patients with Obstructive Lung Diseases: Cystic Fibrosis, Bronchiectasis, and Chronic Obstructive Pulmonary Disease. *Diagnostic Microbiology and Infectious Disease*, 68:20-27, 2010.

76. D. S. Blanc, C. Petignat, B. Janin, J. Bille and P. Francioli. Frequency and Molecular Diversity of *Pseudomonas aeruginosa* Upon Admission and During Hospitalization: A Prospective Epidemiologic Study. *Clinical Microbiology and Infection*, 4:242-247, 1998.

77. M. G. Page and J. Heim. Prospects for the Next Anti-*Pseudomonas* Drug. *Current Opinion in Pharmacology*, 9:558-565, 2009.

78. T. Bjarnsholt, P. O. Jensen, M. J. Fiandaca, J. Pedersen, C. R. Hansen, C. B. Andersen, T. Pressler, M. Givskov and N. Hoiby. *Pseudomonas aeruginosa* Biofilms in the Respiratory Tract of Cystic Fibrosis Patients. *Pediatr Pulmonol*, 44:547-558, 2009.

79. C. de la Fuente-Nunez, F. Reffuveille, L. Fernandez and R. E. Hancock. Bacterial Biofilm Development as a Multicellular Adaptation: Antibiotic Resistance and New Therapeutic Strategies. *Current Opinion in Microbiology*, 16:580-589, 2013.

80. L. Ma, M. Conover, H. Lu, M. R. Parsek, K. Bayles and D. J. Wozniak. Assembly and Development of the *Pseudomonas aeruginosa* Biofilm Matrix. *PLoS Pathogens*, 5(3):e1000354, 2009.

81. K. D. Jackson, M. Starkey, S. Kremer, M. R. Parsek and D. J. Wozniak. Identification of Psl, a Locus Encoding a Potential Exopolysaccharide That Is Essential for *Pseudomonas aeruginosa* Pao1 Biofilm Formation. *Journal of Bacteriology*, 186:4466-4475, 2004.

82. C. B. Whitchurch, T. Tolker-Nielsen, P. C. Ragas and J. S. Mattick. Extracellular DNA Required for Bacterial Biofilm Formation. *Science*, 295:1487, 2002.

83. M. Allesen-Holm, K. B. Barken, L. Yang, M. Klausen, J. S. Webb, S. Kjelleberg, S. Molin, M. Givskov and T. Tolker-Nielsen. A Characterization of DNA Release in *Pseudomonas aeruginosa* Cultures and Biofilms. *Molecular Microbiology*, 59:1114-1128, 2006.

84. E. C. Pesci, J. P. Pearson, P. C. Seed and B. H. Iglewski. Regulation of Las and Rhl Quorum Sensing in *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 179:3127-3132, 1997.

85. R. S. Harbron and C. A. Kent. Aspects of Cell Adhesion. In: L. F. Melo, Bott T. R., and Bernardo C. A. (eds) *Fouling Science and Technology*, pages 125-140. Springer Netherlands, Dordrecht, 1988.

86. C. Beloin, A. Roux and J. M. Ghigo. *Escherichia coli* Biofilms. *Current Topics in Microbiology and Immunology*, 322:249-289, 2008.

87. R. Van Houdt and C. W. Michiels. Role of Bacterial Cell Surface Structures in *Escherichia coli* Biofilm Formation. *Research in Microbiology*, 156:626-633, 2005.

88. J. B. Kaper, J. P. Nataro and H. L. Mobley. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, 2:123-140, 2004.

89. J. Wingender and H. C. Flemming. Biofilms in Drinking Water and Their Role as Reservoir for Pathogens. *International Journal of Hygiene and Environmental Health*, 214:417-423, 2011.

90. T. K. Wood. Insights on *Escherichia coli* Biofilm Formation and Inhibition from Whole-Transcriptome Profiling. *Environmental Microbiology*, 11:1-15, 2009.

91. D. Ren, L. A. Bedzyk, S. M. Thomas, R. W. Ye and T. K. Wood. Gene Expression in *Escherichia coli* Biofilms. *Applied Microbiology and Biotechnology*, 64:515-524, 2004.

92. V. Hancock and P. Klemm. Global Gene Expression Profiling of Asymptomatic Bacteriuria *Escherichia coli* During Biofilm Growth in Human Urine. *Infection and Immunity*, 75:966-976, 2007.

93. M. A. Schembri, K. Kjaergaard and P. Klemm. Global Gene Expression in *Escherichia coli* Biofilms. *Molecular Microbiology*, 48:253-267, 2003.

94. B. C. Das, P. Thapa, R. Karki, C. Schinke, S. Das, S. Kambhampati, S. K. Banerjee, P. Van Veldhuizen, A. Verma, L. M. Weiss and T. Evans. Boron Chemicals in Diagnosis and Therapeutics. *Future Medicinal Chemistry*, 5:653-676, 2013.

95. P. P. Power and W. G. Woods. The Chemistry of Boron and Its Speciation in Plants. *Plant and Soil*, 193:1-13, 1997.

96. D. Schubert. Boron Oxides, Boric Acid, and Borates. *Kirk-Othmer Encyclopedia of Chemical Technology*, 2011.

97. K. Warington. The Effect of Boric Acid and Borax on the Broad Bean and Certain Other Plants. *Annals of Botany*, 37:629-672, 1923.

98. R. Barr, M. Böttger and F. Crane. The Effect of Boron on Plasma Membrane Electron Transport and Associated Proton Secretion by Cultured Carrot Cells. *Biochemistry and Molecular Biology International*, 31:31-39, 1993.

99. D. G. Blevins and K. M. Lukaszewski. Boron in Plant Structure and Function. *Annual Review of Plant Physiology and Plant Molecular Biology*, 49:481-500, 1998.

100. M. A. O'Neill, S. Eberhard, P. Albersheim and A. G. Darvill. Requirement of Borate Cross-Linking of Cell Wall Rhamnogalacturonan Ii for Arabidopsis Growth. *Science*, 294:846-849, 2001.

101. P. H. Brown, N. Bellaloui, M. A. Wimmer, E. S. Bassil, J. Ruiz, H. Hu, H. Pfeffer, F. Dannel and V. Römheld. Boron in Plant Biology. *Plant Biology*, 4:205-223, 2002.

102. T. A. Devirian and S. L. Volpe. The Physiological Effects of Dietary Boron. *Critical Reviews in Food Science and Nutrition*, 43:219-231, 2003.

103. F. S. Kot. Boron Sources, Speciation and Its Potential Impact on Health. *Reviews in Environmental Science and Bio/Technology*, 8:3-28, 2009.

104. V. K. Bharti, M. Gupta and D. Lall. Ameliorative Effects of Boron on Serum Profile in Buffalo (Bubalus Bubalis) Fed High Fluoride Ration. *Tropical Animal Health and Production*, 40:111-116, 2008.

105. S. Meacham, S. Karakas, A. Wallace and F. Altun. Boron in Human Health: Evidence for Dietary Recommendations and Public Policies. *The Open Mineral Processing Journal*, 3:36-53, 2010.

106. C. D. Hunt. Dietary Boron: Progress in Establishing Essential Roles in Human Physiology. *Journal of Trace Elements in Medicine and Biology*, 26:157-160, 2012.

107. W. T. Barranco and C. D. Eckhert. Boric Acid Inhibits Human Prostate Cancer Cell Proliferation. *Cancer Letters*, 216:21-29, 2004.

108. S. Mahabir, M. R. Spitz, S. L. Barrera, Y. Q. Dong, C. Eastham and M. R. Forman. Dietary Boron and Hormone Replacement Therapy as Risk Factors for Lung Cancer in Women. *American Journal of Epidemiology*, 167:1070-1080, 2008.

109. M. Korkmaz, E. Uzgoren, S. Bakirdere, F. Aydin and O. Y. Ataman. Effects of Dietary Boron on Cervical Cytopathology and on Micronucleus Frequency in Exfoliated Buccal Cells. *Environmental Toxicology*, 22:17-25, 2007.

110. R. Scorei, R. Ciubar, C. M. Ciofrangeanu, V. Mitran, A. Cimpean and D. Iordachescu. Comparative Effects of Boric Acid and Calcium Fructoborate on Breast Cancer Cells. *Biological Trace Element Research*, 122:197-205, 2008.

111. R. L. Travers, G. C. Rennie and R. E. Newnham. Boron and Arthritis: The Results of a Double-Blind Pilot Study. *Journal of Nutritional Medicine*, 1:127-132, 1990.

112. P. D. Meers and C. K. Chow. Bacteriostatic and Bactericidal Actions of Boric Acid against Bacteria and Fungi Commonly Found in Urine. *Journal of Clinical Pathology*, 43:484-487, 1990.

113. R. Hütter, W. Keller-Schien, F. Knüsel, V. Prelog, G. C. Rodgers, P. Suter, G. Vogel, W. Voser and H. Zähner. Stoffwechselprodukte Von Mikroorganismen. 57. Mitteilung. Boromycin. *Helvetica Chimica Acta*, 50:1533-1539, 1967.

114. T. Hemscheidt, M. P. Puglisi, L. K. Larsen, G. M. Patterson, R. E. Moore, J. L. Rios and J. Clardy. Structure and Biosynthesis of Borophycin, a New Boeseken Complex of

Boric Acid from a Marine Strain of the Blue-Green Alga *Nostoc linckia*. *The Journal of Organic Chemistry*, 59:3467-3471, 1994.

115. T. Okazaki, T. Kitahara and Y. Okami. Studies on Marine Microorganisms. Iv. A New Antibiotic Ss-228 Y Produced by Chainia Isolated from Shallow Sea Mud. *The Journal of Antibiotics*, 28:176-184, 1975.

116. N. P. Nies and R. W. Hulbert. Solubility Isotherms in the System Sodium Oxide-Boric Oxide-Water. Revised Solubility-Temperature Curves of Boric Acid, Borax, Sodium Pentaborate, and Sodium Metaborate. *Journal of Chemical and Engineering Data*, 12:303-313, 1967.

117. D. M. Schubert and R. J. Brotherton. Boron: Inorganic Chemistry. In: *Encyclopedia of Inorganic Chemistry*. John Wiley and Sons, Ltd, 2006.

118. F. Haesebrouck, M. Baele, H. De Keyser, K. Hermans and F. Pasmans. Antimicrobial Activity of an Acetic and Boric Acid Solution against *Staphylococcus pseudintermedius*. *Vlaams Diergeneeskundig Tijdschrift*, 78:89-90, 2009.

119. F. De Seta, M. Schmidt, B. Vu, M. Essmann and B. Larsen. Antifungal Mechanisms Supporting Boric Acid Therapy of *Candida vaginitis*. *Journal of Antimicrobial Chemotherapy*, 63:325-336, 2009.

120. J. D. Sobel and W. Chaim. Treatment of *Torulopsis glabrata vaginitis*: Retrospective Review of Boric Acid Therapy. *Clinical Infectious Diseases*, 24:649-652, 1997.

121. S. Boiko. Treatment of Diaper Dermatitis. *Dermatologic Clinics*, 17:235-240, 1999.

122. M. Schmidt, J. Z. Schaumberg, C. M. Steen and M. P. Boyer. Boric Acid Disturbs Cell Wall Synthesis in *Saccharomyces cerevisiae*. *International Journal of Microbiology*, 2010, 2010.

123. M. T. Yilmaz. Minimum Inhibitory and Minimum Bactericidal Concentrations of Boron Compounds against Several Bacterial Strains. *Turkish Journal of Medical Sciences*, 42:1423-1429, 2012.

124. I. Porter and J. Brodie. Boric Acid Preservation of Urine Samples. *British Medical Journal*, 2:353-355, 1969.

125. D. L. Vyszenski-Moher and L. G. Arlian. Effects of Wet Cleaning with Disodium Octaborate Tetrahydrate on Dust Mites (Acari: Pyroglyphidae) in Carpet. *Journal of Medical Entomology*, 40:508-511, 2003.

126. T. P. Schultz, H. Militz, M. H. Freeman, B. Goodell and D. D. Nicholas. *Development of Commercial Wood Preservatives: Efficacy, Environmental, and Health Issues*. ACS Publications, 2008.

127. J. D. Lloyd, K. L. Kirkland and T. D. Malone. Surface Cleaning Method and Composition. Google Patents, 2007.

128. N. P. Nies and D. S. Taylor. Anhydrous Crystalline Borate and Process for Producing Same. Google Patents, 1959.

129. A. Sava and S. Kritzler. Process and Composition for Cleaning Medical Instruments. Google Patents, 2007.

130. S. L. Edmonds, C. Zapka, D. Kasper, R. Gerber, R. McCormack, D. Macinga, S. Johnson, S. Sambol, C. Fricker and J. Arbogast. Effectiveness of Hand Hygiene for Removal of *Clostridium difficile* Spores from Hands. *Infection Control and Hospital Epidemiology*, 34:302-305, 2013.

131. D. G. Blevins and K. M. Lukaszewski. Proposed Physiologic Functions of Boron in Plants Pertinent to Animal and Human Metabolism. *Environmental Health Perspectives*, 102:31, 1994.

132. S. A. Hubbard and F. M. Sullivan. Toxicological Effects of Inorganic Boron Compounds in Animals: A Review of the Literature. *The Journal of Trace Elements in Experimental Medicine*, 9:165-173, 1996.

133. H. Foreman. Toxicology: Inorganic. *Annual Review of Pharmacology*, 2:341-362, 1962.

134. F. H. Nielsen. Boron in Human and Animal Nutrition. *Plant and Soil*, 193:199-208, 1997.

135. S. Bakirdere, S. Orenay and M. Korkmaz. Effect of Boron on Human Health. *The Open Mineral Processing Journal*, 3, 2010.

136. C. Locatelli, C. Minoia, M. Tonini and L. Manzo. Human Toxicology of Boron with Special Reference to Boric Acid Poisoning. *Giornale italiano di Medicina del Lavoro ed Ergonomia*, 9:141-146, 1986.

137. D. M. Goeres, T. Palys, B. B. Sandel and J. Geiger. Evaluation of Disinfectant Efficacy against Biofilm and Suspended Bacteria in a Laboratory Swimming Pool Model. *Water Research*, 38:3103-3109, 2004.

138. P. Naves, G. Del Prado, L. Huelves, M. Gracia, V. Ruiz, J. Blanco, V. Rodríguez-Cerrato, M. Ponte and F. Soriano. Measurement of Biofilm Formation by Clinical Isolates of *Escherichia coli* Is Method-Dependent. *Journal of Applied Microbiology*, 105:585-590, 2008.