ANTIBIOTIC RESISTANT *PSEUDOMONAS SP*. BIOMIG1 PROTECTS SUSCEPTIBLE BACTERIA FROM DISINFECTANTS

by

GÖKÇİN GÜL

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ANTIBIOTIC RESISTANT *PSEUDOMONAS SP*. BIOMIG1 PROTECTS SUSCEPTIBLE BACTERIA FROM DISINFECTANTS

APPROVED BY:

Assist. Prof. Ulaş TEZEL
(Thesis Supervisor) …….

Prof. Işıl BALCIOĞLU …….

Assoc. Prof. Serdar DOĞRUEL …….

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To make this life worth living…

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ANTIBIOTIC RESISTANT *PSEUDOMONAS SP.* BIOMIG1 PROTECTS SUSCEPTIBLE BACTERIA FROM DISINFECTANTS

Benzalkonium chlorides (BACs), are one of the biocides that are active ingredients of many disinfectants. A novel bacteria *Pseudomonas sp.* BIOMIG1 which is resistant to BACs and degrade BACs at high concentrations has recently been isolated. The objective of this research is to evaluate antibiotic resistance of BIOMIG1 and elucidate the impact of BIOMIG1 on the efficacy of BAC containing disinfectants. Tolerance of BIOMIG1s, *E. coli* and *Serratia marcescens* against 18 antibiotics was measured by using macro dilution and E-test methods. BAC resistant bacteria were more resistant to tested antibiotics than BAC susceptible bacteria which suggested that BAC resistance favor antibiotic resistance. Antibiotic biodegradation potential was tested in shake-flasks. While BIOMIG1 did not biodegrade antibiotics, *Serratia marcescens* degraded amoxicillin and penicillin g. Lastly, BAC susceptibility of *E.coli*, in the presence of BIOMIG1 in co-culture was investigated. Domestos® was used as BAC source. Experiments were performed in liquid medium. While *E. coli* alone was survived up to 4 mg/L initial BAC, it survived at up to 125 mg/L initial BAC in co-culture which suggested that BIOMIG1 protect *E. coli* from disinfectant.

ANTİTİBİYOTİK DİRENÇLİ *PSEUDOMONAS SP.* BIOMIG1 DUYARLI BAKTERİLERİ DEZENFEKTANLARDAN KORUR

Benzalkonyum klorürler (BAK), birçok ticari dezenfektanın aktif maddesidir. Yakın bir zamanda, BAK'lara karşı oldukça dirençli ve bu bileşikleri yüksek konsantrasyonlarda bile parçalayabilen *Pseudomonas sp.* BIOMIG1 bakterisi izole edilmiştir. Bu araştırmanın amacı BIOMIG1'in antibiyotik direncini belirlemek ve BAK içeren dezenfektanların etkisini nasıl değiştirdiğini anlamaktır. BIOMIG1, *E. coli* ve *Serratia marcescens'*in 18 antibiyotiğe karşı olan direnci makro seyrelme ve E-test metotlarıyla belirlenmiştir. BAK dirençli bakterilerin antibiyotiklere de direnç göstermesi BAK direnci ve antibiyotik direnci arasında bir ilişkili olabileceğini göstermiştir. Bakterilerin antibiyotik parçalama potansiyeli test edilmiştir. BIOMIG1 antibiyotiklerden hiçbirini parçalayamazken *Serratia marcescens* amoksisilin ve penisilin g'yi parçalayabilmiştir. Son olarak, BIOMIG1'in ortamda bulunmasının E.coli'nin BAK direnci üzerindeki etkisi belirlenmiştir. Deneylerde Domestos BAK kaynağı olarak kullanılmıştır. Deneyler sıvı besiyeri içerisinde gerçekleştirilmiştir. E coli yalnızken 4 mg/L ve üzeri başlangıç BAK konsantrasyonlarında büyüyemezken, BIOMIG1 varlığında *E. coli* test edilen en yüksek konsantrasyon olan 125 mg/L başlangıç BAK konsantrasyonunda bile büyüyebilmiştir. İnkübasyon sonucunda BIOMIG1 bulunduran kültürlerde BAK'in tespit edilememesi, BAK'in BIOMIG1 tarafından etkisiz hale getirildiğinin ve böylelikle *E. coli*'nin büyüyebileceği uygun bir ortamın BIOMIG1 tarafından sağlandığının bir göstergesidir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units Used
QAC	Quaternary Ammonium Compounds	
BAC	Benzalkonium Chloride	
HPLC	High Performance Liquid Chromatography	
Cl	Chloride	
HI	Hanna Instruments	
TFA	Trifluroacetic Acid	
AMX	Amoxicillin	
PEN G	Penicillin G	
CIP	Ciprofloxacin	
EFX	Enrofloxacin	
NOR	Norfloxacin	
OFL	Ofloxacin	
LEV	Levofloxacin	
TMP	Trimethoprim	
SMX	Sulfamethoxazole	
TET	Tetracycline	
DOX	Doxycycline	
CLR	Clarithromycin	
ERY	Erythromycin	
AZM	Azithromycin	
CLI	Clindamycin	
KAN	Kanamycin	
VAN	Vancomycin	
CHL	Chloramphenicol	
EU	European Union	
EEA	European Economic Area	
ECDC	European Center for Disease Prevention and Control	
DDD	Defined Daily Dose	

WHO	World Health Organization
EPA	Environmental Protection Agency
OECD	Organization for Economic Co-operation and Development
HPVs	High Production Volume Chemicals
WWTP	Wastewater Treatment Plant
RNA	Ribo Nucleic Acid
tRNA	Transfer Ribo Nucleic Acid
mRNA	Messenger Ribo Nucleic Acid
C_nTMA-X	Monoalkonium Halides
DC_{n-n}DMA-X	Dialkonium Halides
C_nBDMA-X	Benzalkonium Halides
C_{12}BDMA-Cl	Dodecyl Benzyl Dimethyl Ammonium Chloride
C_{14}BDMA-Cl	Tetradecyl Benzyl Dimethyl Ammonium Chloride
C_{16}BDMA-CI	Hexadecyl Benzyl Dimethyl Ammonium Chloride
CFU	Colony Forming Unit
DNA	Deoxyribo Nucleic Acid
rDNA	Ribosomal Deoxyribo Nucleic Acid
PCR	Polymerase Chain Reaction
TBE	Tris/Borate/EDTA
EDTA	Ethylenediaminetetraacetic Acid
HPLC	High Performance Liquid Chromatography
NCBI	National Center for Biotechnology
RFLP	Restriction Fragment Length Polymorphism
MUSCLE	Multiple Sequence Comparison by Log-Expectation
LB	Luria Bertani
ECC	Escherichia coli
PS	Pseudomonas
OR	Orientation
SCS	Single Carbon Source
DI	Deionized
bp	Base Pair
min	Minute
MIC	Minimum Inhibitory Concentration

NCCLS	National Committee for Clinical Laboratory Standards	
OD	Optical density	(nm)
CLSI	Clinical and Laboratory Standards Institute	
UV/Vis	Ultraviolet-Visible	
MH	Mueller-Hinton	
DMSO	Dimethyl Sulfoxide	

1. INTRODUCTION

Antimicrobials are chemicals that are used to kill microorganisms. Biocides and antibiotics are the two main classes of antimicrobials.

Biocides are composed of substances used as preservatives, insecticides, disinfectants and pesticides. They are used in order to disinfect, sanitize or sterilize surfaces or objects. In addition, they are added to personal care products, foods, marine antifouling paints, plastics, wood, and swimming pools and so on (Chapman, 2003a). Biocides can be categorized under four group according to their mode of actions; oxidants, electrophiles, lytic, protonophores (Chapman, 2003b). Halogens and peroxy compounds rapidly kill microorganisms via radical-mediated reactions to oxidize organic material (Dukan et al., 1999). The electrophilic agents such as; silver, copper, mercury react covalently with cellular nucleophiles to inactivate enzymes (Collier et al., 1990; Slawson et al., 1990). Chlorhexidine, alcohols and quaternary ammonium compounds (QACs) are cationic active biocides. They are destabilze membranes which resulting in cell lysis (Broxton et al., 1983; Chawner and Gilbert, 1989). Parabens, weak acids and pyrithione interfere the pH balance of cell membrane leading in acidification of the cell interior (Eklund, 1985; Ermoleayeva and Sanders, 1995).

Biocide usage escalates every day due to human society's new cleaning habits to sustain hygiene in our modern life. For instance, Dye et al. (2007) reported that the annual production of m-cresol is more than a thousand tons, and the annual triclosan production is up to a thousand tons in EU. Hauthal et al. (2004) reported the worldwide annual consumption of quaternary ammonium compounds (QACs) was about 500,000 tons. After biocides are used, they end up in wastewater. Since wastewater treatment plants are designed to remove easily degradable organic pollutants, most of biocides pass through wastewater treatment plants and are discharged into the environment. After biocides are released into nature, they are transported through distances and accumulate in different compartments (Martinez-Carballo et al., 2007a; Hughes et al., 2012). Due to dilution, the biocide concentrations are generally very low in the environment. As a result, bacteria in the environment are exposed to biocides at their sub-inhibitory concentrations which facilitate

the development and dissemination of biocide resistance in bacteria (Martinez, 2008). In the literature, there is a continuous increase in the number of bacteria which show resistance to biocides. Chapman (2003b) reported that tolerance of various bacteria to biocides such as QACs, chlorhexidine, phenolics, heavy metals and aldehydes has been increased. *E.coli* attained resistance to hydrogen peroxide and peracetic acid by induction of the *oxy*R or *sox*RS regulons (Dukan and Touati, 1996). Biofilm formation is another resistance mechanism to oxidizing biocides (Cochran et al., 2000; Gilbert et al., 1990). Microorganisms gain resistance to inorganic electrophilic biocides by reducing them to non or less toxic ions (Silver and Phung Le, 1996). It was found that chloromethylisothiazolone resistant *P. aeruginosa* isolate overproduced an outher membrane protein which is component of mexA-maxB-oprM efflux system (Chapman et al., 1998). It has been questioned that bacteria employing efflux mechanisms lead cross-resistance to antibiotics. The changes in the composition of the cytoplasmic membrane enable *Serratia marcescens* and *Providencii stuartiis* to become resistant to chlorhexidine (Lannigan and Bryan, 1985; Ismael et al., 1986).

Among biocides, QACs attracted attention in 2000s due to their unique physical/chemical properties such as; surface-active, detergency and antimicrobial properties (McDonnell and Pretzer, 2001). They are used as additives in consumer products as well as in disinfectants. Among QACs, benzalkonium chlorides (BACs) are the most commonly used biocides in disinfectants. Widespread use of BACs not only lead to proliferation of BAC resistance but also development of BAC degrading enzymes in microorganisms. *Pseudomonas fluorescens, Bacillus niabensis Thalassospira sp., Stenetrophomonas* spp., *Achromobacter* spp. and *Pseudomonas putida* and *Pseudomonas aeruginosa* were reported as BAC degrading bacteria (Nishihara et al., 2000; Bassey et al., 2011). *P. putida* and *P. aeruginosa* groups are dominant BAC degraders among all.

Although antibiotic resistance is not a new phenomenon, increasing amounts of biocides in wastewater and resulting contamination of natural water bodies have amplified the severity and complexity of the antibiotic resistance problem in the last decade (Levy et al., 2004; Coates et al., 2011; Kümmerer and Henninger, 2003). Forsberg et al. (2012) showed that antimicrobial resistance genes present in soil bacteria are similar to many

clinical pathogens suggesting that these genes are transferred to pathogens originally from some other non-pathogenic bacteria present in nature. As a result the environment is the platform where antibiotic/antimicrobial resistance evolve and spread. Nowadays connection between biocide resistances to antibiotic resistance is emerging (Aiello and Larson, 2003; Adelowo et al., 2008). In order to understand this relation, it is necessary to have knowledge about antibiotics. Antibiotics are used to treat or prevent bacterial infection. They are generally classified according to their mode of actions. For example, penicillins and cephalosporins target bacterial cell; rifamycin, lipiarmycins, quinolones and sulfonamides interfere with essential bacterial enzymes; macrolides, lincosamides and tetracyclines target protein synthesis (Finberg et al., 2004). Reduce permeability, increased efflux, changes in antibiotic targets by mutation, modification and protection of target, inactivation of antibiotics by hydrolysis and transfer of a chemical group are the main antibiotic resistance mechanisms (Blair et al., 2005).

Many biocide and antibiotic resistance mechanism are similar. Therefore, several biocide resistance mechanisms can also act against antibiotics, in other words biocide resistance favors antibiotic resistance (Scenihr, 2009; Hegstad et al., 2010; Tezel and Pavlostathis, 2012a). QAC resistant *Staphylococcus aureus* and triclosan resistant *Pseudomonas aeruginosa* also show resistance to several group of antibiotics (Akimitsu et al., 1999; Chuanchuen et al., 2001). *Pseudomonas stutzeri*, which was a chlorhexidine resistant bacteria, shows resistance to QACs, several antibiotics and also triclosan (Russell, 1998). Moreover, it was found that after long-term BAC exposure, microorganisms become more resistant to antibiotics (Tandukar et al., 2013). Above mentioned resistance occurs because most of biocide resistance mechanisms like efflux pumps and biodegradation are also effective against antibiotics, so acquisition of biocide resistance may also cause antibiotic resistance. He et al. (2004) reported that MATE family multidrug efflux pump PmpM targets both benzalkonium chloride and fluoroquinolone groups of antibiotics. In another report Fetar et al. (2011) found that RND family efflux pump MexEF-OprN accommodates a variety of biocides as well as trimethoprim and chloramphenicol antibiotics. *qac* A, *qac* B, *qac* C, *qac* D are plasmid carrying QAC resistance genes that encode proton dependent export proteins. It was reported that the *qac* A/B and tetracycline resistance genes show significant homology (Rouch et al., 1990).

Since constitutive degradative enzymes that are responsible for biocide degradation have the potential to degrade other chemicals like antibiotics, biodegradation can be considered as a major resistance mechanism against both biocides and antibiotics. Degradation of antibiotics and biocides decrease the efficacy of these antimicrobials. On the other hand biodegradation has the great potential to become a very important key tool in the control and suppression of antibiotic and biocide release into the environment, if qualifying microorganisms and/or their enzymes can be harvested and used effectively. This way we can reduce the evolution and dissemination of antimicrobial resistance in the environment and in medical settings. However, biocide and antibiotic degrading bacteria has yet attracted very limited interest in the literature. Although several studies have reported a number of microorganisms that are responsible of the biotransformation of benzalkonium chlorides (BACs) which are extensively used group of quaternary ammonium biocides, none of them investigated the antibiotic degradation potential of these microorganisms. A systematic understanding of key microorganisms responsible for both BAC and antibiotic biotransformation is crucial.

Recently, Ertekin et al. (2016) isolated a novel *Pseudomonas sp.*, strain BIOMIG1 from sewage, activated sludge, soil and sea sediment. This strain can degrade BACs, the active biocidal ingredient of many commercial disinfectants, and it is also resistant to many antibiotics and inhibitors such as minocycline, lincomycin, vancomycin, nalidixic acid, etc., yet not pathogenic. The genome of BIOMIG1 contains genes for biotransformation of many micropollutants such as benzalkonium chlorides, alkylbenzenesulfonates, hetero-atom containing drugs, mercury, arsenic and etc. Given the fact that the strain BIOMIG1 is common in the environmental biological systems, one of the objective of this thesis work is to elucidate the role of this bacterium on the fate of antibiotics in the environment. In our study the relation between BAC degradation, BAC resistance and antibiotic susceptibility is established by using the strain BIOMIG1 and other bacteria that show different tolerance to BACs. In the last part of the study, we examined how the presence of BAC degrading microorganism affects the survival of BAC susceptible bacteria in BAC containing media such as commercial cleaning liquids. Results obtained in this study are aimed to be used to develop post-treatment technologies or process modifications to achieve complete removal of antibiotics and biocides from the wastewater as well as to understand the role of biotransformation on the biocide resistance in a microbial community.

2. LITERATURE SURVEY

2.1. Quaternary Ammonium Compounds (QACs)

Biocides are antimicrobial molecules, like antibiotics, having bactericidal or bacteriostatic effects. They are used as active ingredients in disinfectant or antiseptic formulations as well as many consumer products such as: toothpastes, detergents, surface cleaners, emulsifiers, corrosion inhibitors and so on (Garcia et al., 1999; Steichen, 2001; Patrauchan and Oriel, 2003). They are also added to dishwashing liquids, hand soaps, window cleaners, floor cleaners, baby care products, disinfectant sprays, air fresheners (Hegstad et al., 2010). Moreover, they are used as surfactants, emulsifiers, fabric softeners, pesticides, corrosion inhibitors and components of personal care products (Hegstad et al., 2010; Tezel, 2009; Marple et al., 2014).

In 2000s, quaternary ammonium compounds (QACs) were attracted attention, and they started to be used extensively as an active ingredient in disinfectants since QACs maintain biocidal properties in a wide range of pH and QACs are not only effective against bacteria but also effective against fungi and viruses at very low concentrations. The applied concentration of QACs in disinfectants is typically between 400 and 500 mg/L and it is not exceeding 1, 000 mg/L (e.g., 0.1% w/v in Lysol®). Recommended application concentrations of Domestos® and Dixi® are 335 mg/L and 90 mg/L respectively. The chemical structure of quaternary ammonium compound composed of four functional groups which are either long alkyl chains, aryl or methyl groups bound to the nitrogen atom ($N^+R_1R_2R_3R_4$) (Figure 2.1). U.S. Environmental Protection Agency (U.S. EPA, 2006) and the Organization for Economic Co-operation and Development (OECD) reported QACs as high production volume chemicals (HPVs, i.e., chemicals manufactured or imported in amounts equal to or greater than one million pounds per year).

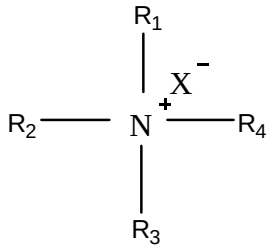
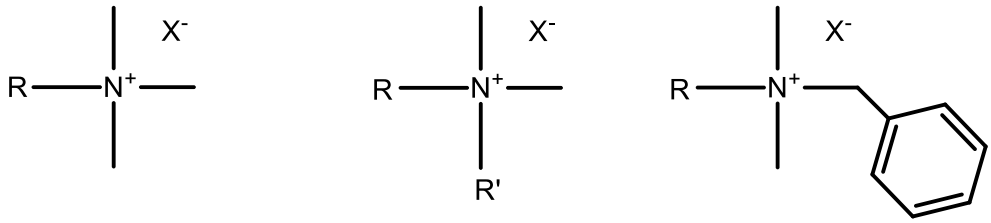


Figure 2.1. General molecular structure of a quaternary ammonium compound (R represents a functional group, X^- represents a halide such as Cl^-, Br^-) (Tezel and Pavlostathis, 2012a).

QACs are divided into three main groups depending on the type of functional groups: monoalkonium, dialkonium and benzalkonium halides (Figure 2.2). The most abundant type of QACs that are used as disinfectants are benzalkonium chlorides (BACs). Dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl), tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl) and hexadecyl benzyl dimethyl ammonium chloride (C_{16}BDMA-Cl) are the most extensively used BACs and they are abundantly found in wastewaters with concentrations changing between 20 and 300 µg/L, whereas monoalkonium and dialkonium QACs were detected at 9.9 and 40 µg/L, respectively (Martinez-Carballo et al., 2007b; Clara et al., 2007). In another research, C_{12}BDMA-Cl concentration in hospitals were reported to be between 0.05 and 6 mg/L (Kümmerer et al., 1997). Garcia et al. (1999) reported that concentrations of QACs may reach up to 50 mg/L in anaerobic digesters of sewage treatment plants. Also BACs exist in the effluents of wastewater treatment plants and surface waters. BACs' concentrations are reported to be between 1.2 and 36.6 µg/L in surface waters downstream to a wastewater treatment plant (WWTP) and between 21 and 260 µg/kg in sediments in riversides downstream to a WWTP (Ferrer et al., 2002).



Monoalkonium halides C_nTMA-X

Dialkonium halides $DC_{n-n}DMA$-X

Benzalkonium halides C_nBDMA-X

Figure 2.2. Representative QAC groups, their general structure and abbreviations used in this study (X^- is a halide counter-ion) (Tezel and Pavlostathis, 2012a).

Perturbation of the lipid bilayer of the bacterial cytoplasmic membrane and the other membrane of Gram-negative bacteria are the two main mode of action of QACs against bacterial cell which leads to cell lysis (Tezel and Pavlostathis, 2012a). Cell lysis happens when QAC concentration is at or above critical micelle concentrations (Heerklotz, 2008). Due to ionic interactions, cationic part of QAC (quaternary nitrogen) associate with the head groups of the acidic phospholipids within the membrane. Then, hydrophobic tail integrates the lipid core. As a result, cell membrane loses its osmoregulatory and physiological functions (Maillard, 2002; Gilbert and Moore, 2005). For example, benzalkonium chlorides (BACs) bind to the cell membrane of *Pseudomonas fluorescens* by ionic and hydrophobic interactions, bringing about changes of membrane properties and function, followed by cellular disruption, loss of membrane integrity, ultimately resulting in leakage of essential intracellular constituents (Ferreira et al., 2011; Morente et al., 2013). Inhibition of respiratory enzymes and the dissipation of proton motive force are the mode of actions of QACs at low concentrations which are affect the miccrobial metabolism, oxidative phosphorilation, adenosine triphosphate sythesis and active transport in bacteria (Knox et al., 1949; McDonnell and Russell, 1999; Maillard, 2002).

When all these reports about BACs pollution mentioned above are taken into consideration, it is evident that BACs are widely distributed and can be found in a broad range of environments such as industrial effluents, sewage sludge, activated sludge, treated wastewater and receiving waters. Therefore humans and microorganisms are in continuous contact with BACs. Although BAC concentration in a disinfectant application is not exceeding 1,000 mg/L, which is more than enough to kill many pathogens, environmental concentrations of BACs are much lower than their minimum inhibitory concentration (MICs) for many microorganism in the environment. Presence of sub-inhibitory concentrations of BACs in the environment has been suggested to result in emergence and dissemination of BAC resistance amongst bacteria.

Microorganisms show intrinsic and acquired resistance to QACs. When a microorganism show resistance to an antimicrobial agent due to its phenotypic, physiological or biochemical properties, it is called as intrinsic resistance. Advance membrane permeability barriers and chromosomally transcribed efflux pumps are the phenotypic properties that confer intrinsic resistance to QACs. For example Gram-negative bacteria is less susceptible to QACs since it has an outer membrane which surrounds the cell membrane (Tezel and Pavlostathis, 2012a). Other physiological traits that confer QAC resistance are complex lipid containing cell walls, less acidic outer membrane lipopolysaccharides (LPS), small porins resulting from strong LPS–LPS links, fewer porins, and a slime layers (McDonnell and Russell, 1999; Hegstad et al., 2010). Nonspesific efflux pumps are another intrinsic resistance mechanism against QACs. Resisrance nodulation division family efflux pumps; AcrAB-TolC, SdeXY and MexAB-OprM are pumps QACs out of the cell (Piddock, 2006; Hegstad et al., 2010). Mc Cay et al. (2010) reported the activity of MexAB-OprM and MexCD-OprJ efflux pumps lead *P. aeruginosa* NCIMB 10421 to gain QAC resistance.

Acquired resistance mechanisms to QACs include biodegradation, reduction of number of porins, mobile genetic elements, enhanced biofilm formation, and overexpression of efflux pumps as a result of QACs exposure (Tezel and Pavlostathis, 2015; Buffet-Bataillon et al., 2011). Microorganisms acquired tolerance to QACs by reducing the permeability of the outer cell layer by changing the composition of the cell membrane fatty

acids, phospholipids, and outer membrane lipopolysaccharides (Denyer and Maillard, 2002; Dubois-Brissonnet et al., 2001; Loughlin et al., 2002; Boeris et al., 2007).

Efflux-mediated QAC resistance has gained significant interest because it has a genetic origin, confers co-resistance to antibiotics and is transferable among microbial species through horizontal gene transfer. QAC resistance via efflux pumps follows two mechanisms. First, QAC resistance is induced by overexpression of efflux pumps upon exposure to QAC or as a result of QAC-induced stress. These efflux pumps are generally chromosomally encoded and act against a wide array of antimicrobial agents (Guo et al., 2014; Buffet-Bataillon et al., 2012; Holdsworth and Law, 2013; Mc Cay et al., 2010; Morita et al., 2014). Overexpression of these efflux pumps results in a two to eight fold increased tolerance of the adapted microorganism to QACs and other substrates of these pumps.

Acquisition of genes for specialized QAC efflux pumps, which belong to the SMR family is the other QAC resistance via efflux pumps. Among them, EmrE, smr and SugE are multidrug efflux pumps (He et al., 2011), whereas QacE, QacΔE, QacF, QacG, QacH, QacI, QacJ and QacZ are QAC-specific efflux determinants (Braga et al., 2011).

Since efflux protein genes are found in mobile genetic elements; transposons, plasmids and integrons, they can be horizontally transferred between microorganisms of the same or different genera (Gaze et al., 2005; Schluter et al., 2007). Integrons, which are promoterless mobile recombinational elements, play a significant role in the acquisition and mobilization of QAC resistance genes (Crambray et al., 2010). Integrons are found in many environmental bacterial species, particularly in those exposed to QACs and/or antibiotic residues (Gaze et al., 2011). QAC contamination is also responsible for the stabilization of integrons and their gene cassettes (Gillings et al., 2009). A direct link between SOS response and the expression of integron integrases was demonstrated. SOS regulation enhances cassette swapping and capture under stressful conditions, such as during QAC exposure (Cambray et al., 2011).

Plasmids also play a significant role in harboring and disseminating genes related to resistance to QACs and other biocides. Plasmids of the incompatibility (Inc) group IncP-1, also called IncP, as extrachromosomal genetic elements can be transferred and replicated virtually in all Gram-negative bacteria. IncP plasmids commonly harbor QAC resistance genes along with many other resistance genes such as sul1 (sulfonamide resistance gene) (Popowska and Krawczyk-Balska, 2013; Dutta et al., 2013; Elhanafi et al., 2010; Marti and Balcazar, 2012). In addition, plasmid-associated QAC resistance genes were transferred between non-pathogenic and pathogenic bacteria exposed to QACs, a process that also leads to the co-selection of resistance to other contaminants (Katharios-Lanwermeyer et al., 2012).

Biodegradation is another resistance mechanism. There are several studies about biodegradation of QACs. Nishihara et al. (2000) reported a *Pseudomonas fluorescens* strain that degrades BAC. Patrauchan and Oriel (2003) reported an *Aeromonas* strain degrading BAC by utilizing it as a sole carbon and nitrogen source. *Bacillus niabensis* and *Thalassospira sp.* were reported as C_{16}BDMA-Cl degrading bacteria isolated from marine sediments (Bassey et al., 2011). *Stenetrophomonas* spp. (γ-proteobacteria), *Achromobacter* spp. (α-proteobacteria) and *Pseudomonas* spp., particularly the *P. putida* and *P. aeruginosa* groups are predominant species in QAC/BAC degradation based on the phylogenetic tree prepared by using 16 rDNA sequences of QAC degrading isolates (Figure 2.4.).

Recently in our laboratory, *Pseudomonas sp.* BIOMIG1, which can mineralize BACs, was isolated from sewage (Ertekin et al., 2016). *Pseudomonas sp.* BIOMIG1 is the only bacteria amongst BAC degraders that have the ability to grow on non-alkylated amines which are products of dealkylation (Tezel and Pavlostathis, 2015) (Figure 2.3). According to Ertekin et al. (2016), the enzyme that is responsible for the conversion of C_nBDMA-Cl to BDMA, in other words the dealkylation process, is a Rieske-type oxygenase.

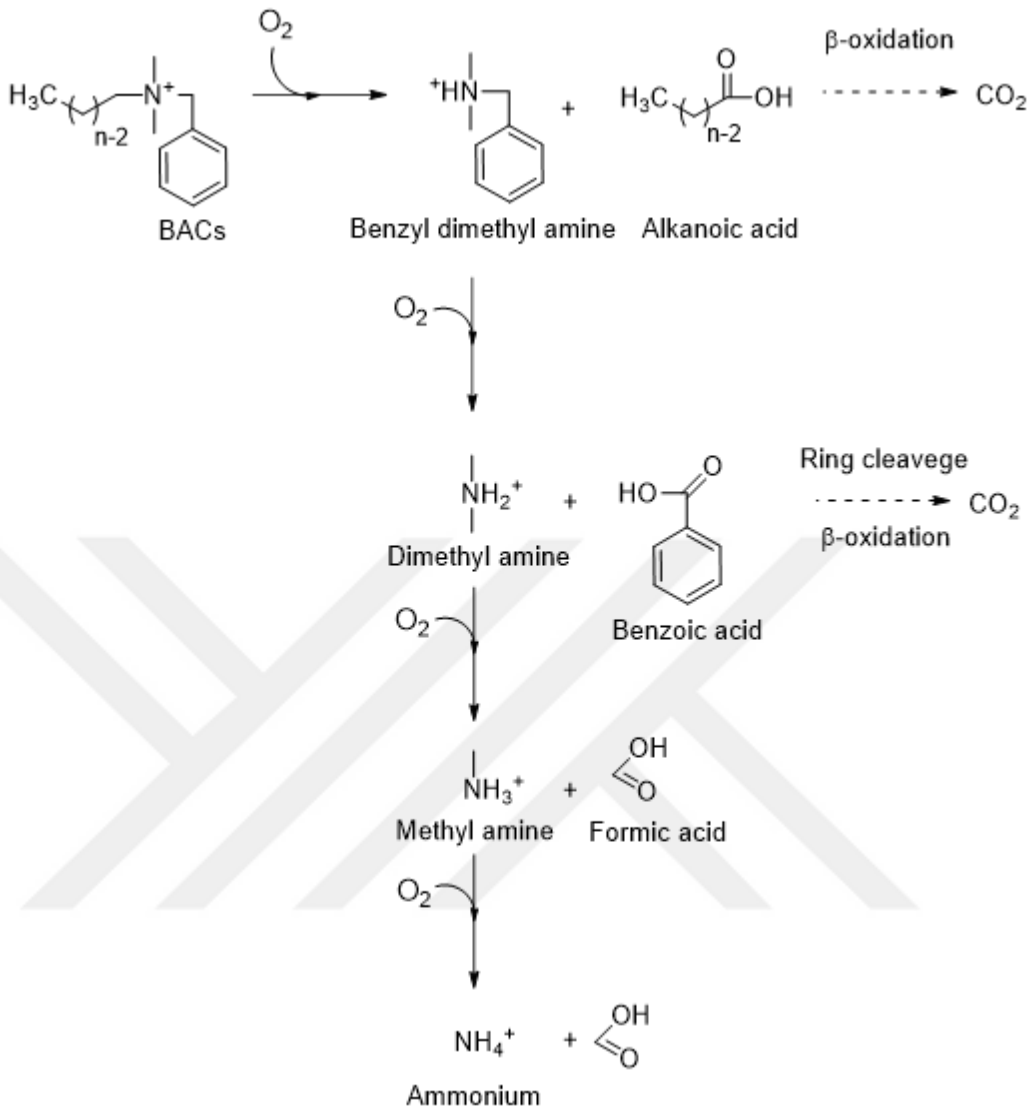


Figure 2.3. Proposed BAC biotransformation pathway by enrichment *Pseudomonas sp.* community (n: carbon number in the alkyl chain) (Yılmaz, 2014).

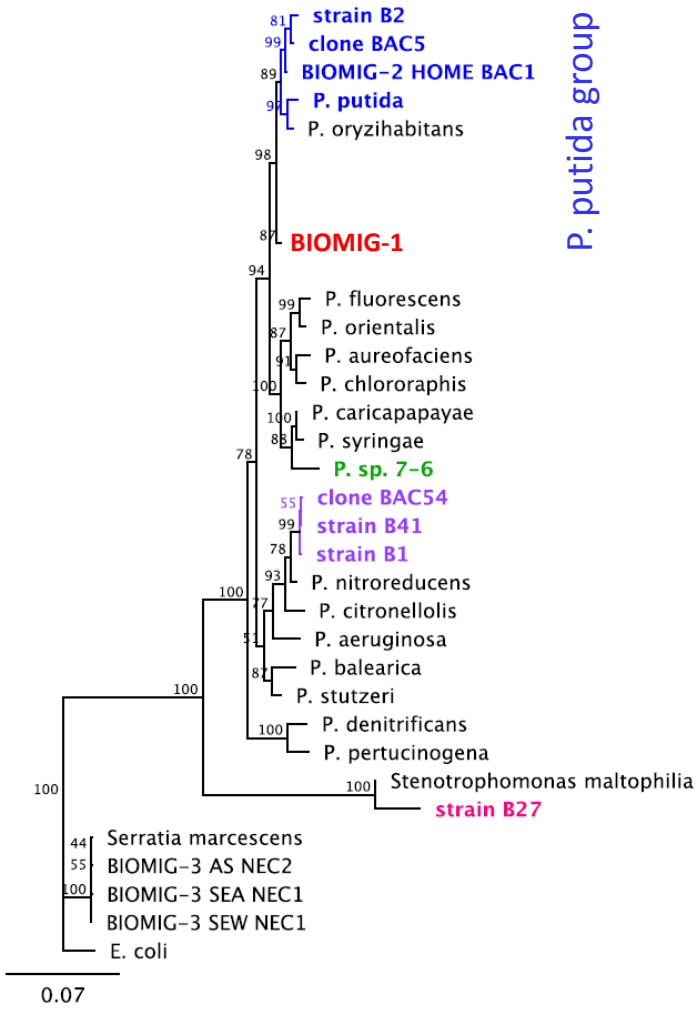


Figure 2.4. Phylogenetic tree of relationships of 16S rDNA sequence of BAC/QAC degraders, determined by maximum likelihood followed by neighbor joining tree building method and Hasegawa-Kishino-Yano genetic distance model. The scale bar represents 0.07 substitution per nucleotide position. *E. coli* (Z83204) was used as outgroup (Yılmaz, 2014).

Although BAC degraders play an important role in elimination of BACs from the environment, it pose a risk since proliferation and transfer of this resistance mechanism to indoor bacteria may cause decrease in BACs' antimicrobial efficacy. Enzymes that are responsible of BAC degradation may also play a role in antibiotic degradation.

2.2. Antibiotics in General

For a long time, innumerable number of people died due to infectious diseases caused by pathogenic bacteria. Tuberculosis, diphtheria, syphilis, plague, scarlet fever were most common cases for human deaths in the pre-antibiotic era (Pruden, 2014). In the late 19 century, as soon as the germ theory of disease showed the relationship between some diseases and pathogenic microorganisms, scientists began to search for compounds that would kill disease causing bacteria (Zaffiri et al., 2012). Antibiotics are molecules that are used to kill or hinder the growth of bacteria. They can either be natural products or synthetic chemicals (Walsh, 2003). Introduction of antibiotics, substantially decreased the mortality from diseases caused by bacterial infections. Discovery of penicillin and sulfonamide antibiotics were followed by new classes of antibiotics, broad range antimicrobials and the antimicrobials modified from previously discovered antibiotics (Powers, 2004). Today, hundreds of antibiotics present in 22 main antibiotic classes (Coates, 2002). In the last decade, global antibiotic consumption increased 30% in between 2000 and 2010as reported by Center for Disease Dynamics, Economics & Policy (CDDEP). In 2014, European Center for Disease Prevention and Control (ECDC) reported that the mean consumption of antibacterial in EU/EEA was 21.5 DDD (defined daily dose) per one thousand inhabitants per day. In the same study penicillin, macrolides and tetracyclines were reported as the most commonly used antibiotic groups. According to the 2014 WHO/Europe-ESAC project group report, antibiotic consumption in Turkey (42.3 DDD per 1,000 inhabitants and per day) is the highest among non-EU countries and penicillins, macrolides and the quinolones are the mostly used antibiotic groups in Turkey.

In our study, 18 antibiotics belonging to 9 different antibiotic groups were used. These antibiotics were selected according to the list of compounds that were presented within the study of the global occurrence of pharmaceuticals in river waters prepared by Hughes et al. (2012): amoxicillin, azithromycin, penicillin g, chloramphenicol, ciprofloxacin, clarithromycin, clindamycin, doxycycline, enrofloxacin, erythromycin, kanamycin, levofloxacin, norfloxacin, ofloxacin, sulfamethoxazole, tetracycline, trimethoprim and vancomycin. Their median concentrations in freshwater ecosystems in Spain, USA, UK, Germany, Canada were 59.9, 188.4, 142, 560, 163 673.5, 16.5, 20.6, 25.9, 5754, 50.8, 97,

208.1, 11 412.4, 628.6, 83, 41.5, 53.2, 26.3 ng/L respectively. In Table 2.1., these antibiotics were grouped according to their mode of actions and chemical structures of antibiotics, as indicated. Glycopeptide and penicillin group of antibiotics inhibit cell wall synthesis of the bacteria by inhibiting peptidoglycan layer synthesis. Peptidoglycan is the primary component of the cell wall of bacteria. While penicillins prevent cell wall biosynthesis via inhibiting transpeptidase enzyme, vancomycin inhibits transglycosylase enzyme (Walsh, 2003). On the other hand tetracyclines, macrolides, licosamides, aminoglycosides and chloramphenicol inhibit protein synthesis of the bacteria. The primary target of tetracyclines is the 30S ribosome, preventing binding of aminoacyl tRNA to the acceptor site on mRNA. Macrolides and lincosamides bind to the 50S ribosomal subunit blocking the binding of tRNA to the acceptor site which blocks translocation of the peptide chain. The primary target of aminoglycosides is the 30S ribosome causing premature chain termination and RNA codon misreading. Chloramphenicol binds to the 50S ribosome preventing attachment to the acceptor site on mRNA (Blair et al., 2015). Sulfamethoxazole and trimethoprim inhibit folic acid synthesis. While sulfamethoxazole inhibits folate synthesis, trimethoprim inhibits folate reduction (Walzer et al., 1988). Quinolones/Floroquinolones inhibit DNA synthesis via binding to the subunit of DNA gyrase (topoisomerase) (Baron, 1996).

Table 2.1. Structures and the mode of actions of 18 antibiotics used in the experiments.

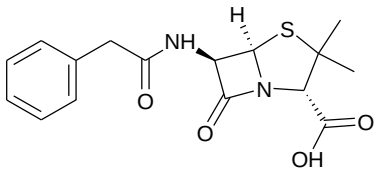
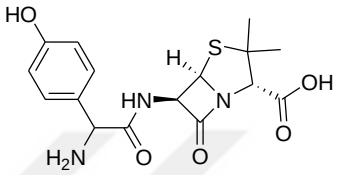
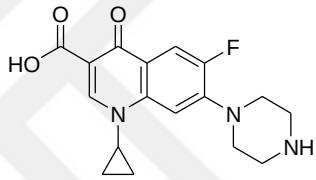
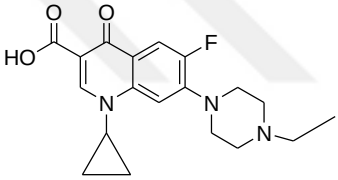
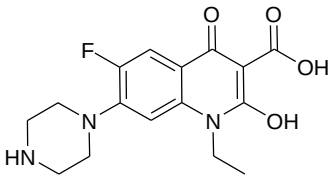
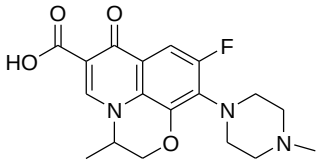
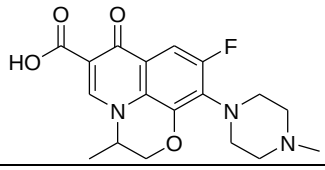
ANTIBIOTIC GROUP	ANTIBIOTIC NAME	STRUCTURE OF ANTIBIOTIC
Penicillins	Penicillin G (PEN G)	
	Amoxicillin (AMX)	
Mode of Action	Inhibitors of cell wall synthesis	
Quinolones/Flouroquinolones	Ciprofloxacin (CIP)	
	Enrofloxacin (EFX)	
	Norfloxacin (NOR)	
	Ofloxacin (OFL)	
	Levofloxacin (LEV)	
Mode of Action	Inhibitors of nucleic acid synthesis	

Table 2.1. Continued.

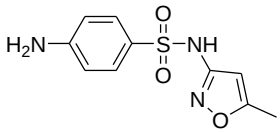
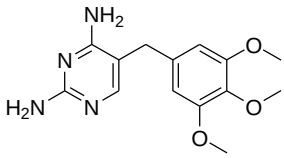
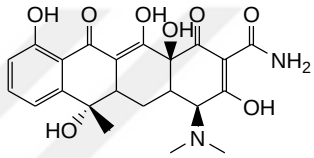
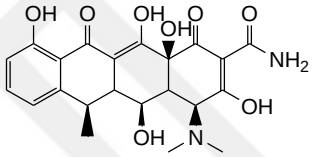
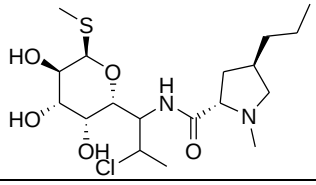
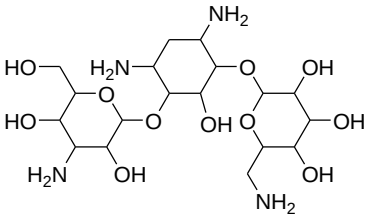
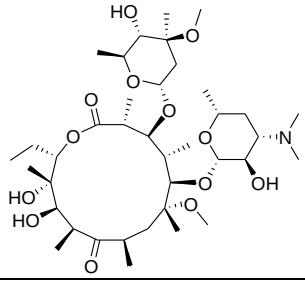
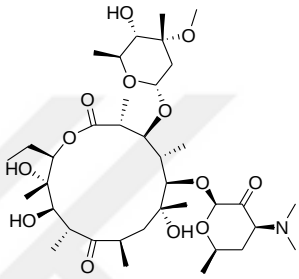
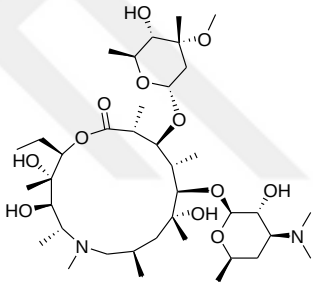
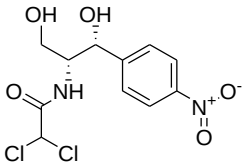
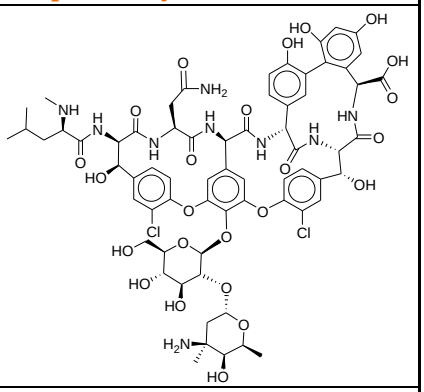
ANTIBIOTIC GROUP	ANTIBIOTIC NAME	STRUCTURE OF ANTIBIOTIC
Sulfonamides	Sulfamethoxazole (SMX)	
	Trimethoprim (TMP)	
Mode of Action	Inhibitors of folic acid synthesis	
Tetracyclines	Tetracycline (TET)	
	Doxycycline (DOX)	
Mode of Action	Inhibitors of protein synthesis	
Lincosamides	Clindamycin (CLI)	
Mode of Action	Inhibitors of protein synthesis	
Aminoglycosides	Kanamycin (KAN)	
Mode of Action	Inhibitors of protein synthesis	

Table 2.1. Continued.

ANTIBIOTIC GROUP	ANTIBIOTIC NAME	STRUCTURE OF ANTIBIOTIC
Macrolides	Clarithromycin (CLR)	
	Erythromycin (ERY)	
	Azithromycin (AZM)	
Mode of Action	Inhibitors of protein synthesis	
Chloramphenicol	Chloramphenicol (CHL)	
Mode of Action	Inhibitors of protein synthesis	
Glycopeptides	Vancomycin (VAN)	
Mode of Action	Inhibitors of cell wall synthesis	

Occurrence of antibiotics in the wastewater increase substantially in parallel to antibiotic consumption. Since conventional wastewater treatment plants are designed for the treatment of easily degradable organics, most of the micropollutants pollutants including antibiotics pass through wastewater treatment without any transformation (Pruden, 2014). In conventional wastewater treatment processes, the removal efficiency of antibiotics is fairly varied from zero to hundred percent and operating conditions such as solid retention time, hydraulic retention time and temperature drastically affects the removal efficiency of antibiotics (Le-Minh et al., 2010). Activated sludge process is the prominent wastewater treatment process that shows high antibiotic removal efficiency mainly achieved by adsorption, hydrolysis and/or biotransformation (Gobel et al., 2004; Batt et al., 2006; Li et al., 2010), where adsorption is the most dominant mechanism (Xu et al., 2010). Although removal of antibiotics in wastewater treatment systems has been demonstrated, Hughes et al. (2013) showed that surface waters receiving discharges from wastewater treatment plants contained a wide array of antibiotics. Results of Marti et al. (2013) suggest that considerable amount of antibiotics, regardless of where they were used and if they passed through treatment or not, find their ways to aquatic environments and meet the natural flora of microorganisms.

2.3. Antibiotic Resistance

Introduction of first antibiotics substantially decreased the mortality from diseases caused by bacterial infections. Discovery of penicillin and sulfonamide antibiotics were followed by new classes of antibiotics, broad range antimicrobials and antimicrobials that were modified from previously discovered antibiotics (Powers, 2004). Extensive and often inappropriate uses of antibiotics lead to their release into the environment and it is suggested to be a contributory factor in the development and dissemination of antibiotic resistance (Scenihr 2009). As a result, antimicrobial resistance has become a major health threat that human society is facing today, and many authorities try to find a solution for this growing problem (Hegstad et al., 2010; Tezel and Pavlostathis, 2012a).

Unfortunately, high rates of antimicrobial use combined with their release into the environment due to insufficient treatment has brought up a bigger problem; “antimicrobial resistance”. Antimicrobial resistance is the ability of microorganisms to develop tolerance to an antimicrobial drug which they were susceptible before. Whenever an antibiotic started to be prescribed, sooner or later some species of bacteria gain resistance to that antibiotic. For instance, streptomycin was discovered in 1943 and the first resistance to it was observed in 1946. In addition, fidaxomicin was discovered in 1948 and the first resistance strain was recorded in 1977 (Walsh, 2003; Coates et al., 2011; Lewis, 2013). Presence of different kinds of antibiotics in the market and their unintended use by patients eventually lead to the evolution of multidrug resistant bacteria. For instance, ESCAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) -a group of pathogenic bacteria that evokes many nosocomial infections- are the prominent bacteria that evolves resistance mechanisms to multiple drugs (Rice, 2010). Bacteria can be intrinsically resistant to certain antibiotics or develop antibiotic resistance by undergoing some mutations in their genes or by acquiring those genes via horizontal gene transfer. Common antibiotic resistance mechanisms that microorganisms develop include reduced cellular permeability, increased efflux, changes in antibiotic targets by mutation or modification, modification of antibiotics (Blair et al., 2015). By reducing permeability of the outer membrane, entrance of antibiotic into the cell is prevented. Over expression of efflux pumps facilitates the transportation of the antibiotics out and away from the cell, thus improves bacteria’s resistance to antibiotics. The mutation in the gene encoding the target site of the antibiotic or alteration at the antibiotic binding site also confer antibiotic resistance. Apart from these, the most common antibiotic resistance mechanism is the enzyme catalyzed modification of antibiotics. Many of the organisms have enzymes that can degrade antibiotic totally or modify the chemical structure of the antibiotics.

Two main methods are used to detect antibiotic resistant microorganisms in the environment: culture-based and molecular-based (metagenomics) methods (Amabile-Cuevas, 2016). In the culture based method, target bacteria are first isolated and then the antimicrobial concentration that inhibit the growth of isolated microorganism is determined by applying various antimicrobial concentrations under specific growth conditions (McLain et al., 2016). Broth dilution, disk diffusion, agar dilution and gradient diffusion are the

frequently used culture-based methods provided by the National Committee for Clinical Laboratory Standards (NCCLS) to determine antibiotic resistance (Franklin et al., 1999). Culture based methods have some limitations such as: not all of the bacteria can be grown in available culture media. In molecular-based methods, first all microbial DNA in the environmental sample is extracted and then resistance genes are detected in that sample. Although one can detect resistance genes belonging to the non-culturable bacteria, designing oligonucleotide primers suitable to capture all DNA sequences in the sample is a challenging work (Amabile-Cuevas, 2016; Lin et al., 2015). In this study culture based method was used to determine antibiotic resistance of selected microorganisms.

2.4. The Link between QACs and Antibiotics in Terms of Resistance

Many authorities suggest that extensive use of biocides may lead to biocide resistance and would have an impact on dissemination of antibiotic resistance amongst microorganisms (Fraise 2002; Russell et al., 1999; Levy 2000; Schweizer 2001). Main biocide resistance mechanisms are related to impermeability, efflux and catabolic enzymes that are also effective in antibiotic resistance mechanisms. If one resistance mechanism, such as efflux pumps, makes microorganisms resistant to other substances, such a process is accepted as cross-resistance. Akimitsu et al. (1999) reported that *Staphylococcus aureus* shows β-lactam resistance related with QAC resistance which point out the occurrence of biocide and antibiotic co-resistance. *Pseudomonas aeruginosa* which exhibits triclosan resistance, is also resistant to many antibiotics (Chuanchuen et al., 2001). *Staphylococcus aureus*, *Pseudomonas aeruginosa* and several other microorganisms belong to *Enterobacteriaceae* family that have plasmid mediated resistance to QACs. The responsible genes are qacA, B, C, D and E and they are suggested to be related to the efflux mechanism. They are also affective against many antibiotics.

QAC resistance is acquired at sub-inhibitory QAC concentrations via modification of the outer membrane, cell membrane, density and structure of porins, regulatory hyper expression of efflux pumps, biodegradation and acquisition of QAC-specific efflux pumps through mobile recombinational elements such as plasmid and integrons (Tezel and Pavlostathis, 2012a). Efflux mediated QAC resistance is especially important since it has a

genetic origin. Efflux pumps may be effective against other antimicrobials (cross-resistance), they may confer co-resistance to antibiotics, and the resistance can be transferred to other microbial species thorough horizontal gene transfer (Buffet-Bataillon et al., 2011). During the transfer of a resistance gene from one bacterium to another, genes that are responsible of resistance against other antibacterial agents, which are located near the main resistance gene, may also be transferred along. Multidrug efflux pumps work via transfer of biocides from inside to outside of the cell and they are usually active against many other antimicrobial agents (Guo et al., 2014; Buffet-Bataillon et al., 2012; Holdsworth and Law, 2013; McCay et al., 2010; Morita et al., 2014). Microorganisms which gain QAC resistance by overexpression of drug transporter gene might be effective against several antibiotics (Grkovic et al., 2002). He et al. (2004) found that PmpM, which is a MATE family multidrug efflux pump, effective against BACs, is also effective against fluoroquinolone group of antibiotics, ethidium bromide, acriflavine, and tetraphenylphosphonium chloride. Fetar et al. (2011) reported that MexEF-OprN (RND family efflux pump) accommodates both BACs and a variety of antimicrobials including trimethoprim and chloramphenicol antibiotics.

Extensive use of QACs as biocides end up with a wide array of QAC resistant bacteria which lead to sanitary issues. QAC resistant bacteria pose a serious threat to human health since QAC induced resistance mechanisms also confer cross and co-resistance to many antibiotics.

2.5. Biodegradation of Antibiotics and the Link between QACs and Antibiotics in Terms of Biodegradation

Biodegradation is another antibiotic resistance mechanism and there are numerous literature examples related to antibiotic biodegradation by bacteria. *Pseudomonas cepacia* and *Pseudomonas fluorescens* strains were able to use benzylpenicilin as a carbon source (Beckman et al., 1979; Johnsen, 1977). In addition, Barnhill et al. (2010) demonstrated that 140 out of 572 isolates of *Salmonella* from animal samples degraded at least one antibiotic which are used in veterinary medicines such as amikacin, ampicillin, cefepime, cefiofur, ciprofloxacin, florfenicol, kanamycin, streptomycin, sulfisoxazole, trimethoprim, and vancomycin. Baghapour et al. (2014) reported that amoxicillin was biodegraded by aerobic

microbial consortia with submerged biological aerated filter. Several penicillin resistant bacteria biodegrade penicillin by β-lactamase and penicillinase enzymes (Maulin et al., 1986; Dougherty et al., 1980). Liu et al. (2015) reported that *Microcystis aerosinosa* biodegrades amoxicillin. Dantas et al. (2008) identified a large number of bacteria that are capable of degrading 18 different antibiotics in total including amikacin, gentamicin, kanamycin, sisomycin, carbenicillin, penicillin g, dicloxacillin, ciprofloxacin, levofloxacin, nalidixic acid, sulfisoxasole, mafenide, sulfamethizole, trimethoprim, vancomycin, D-cycloserine, thiamphenicol and chloramphenicol. The resulting phylogenetic distribution of bacteria that subsist on those antibiotics is shown below (Figure 2.5.). They also reported that more than half of the isolates that subsist on antibiotic belongs to *Burkholderiales* and *Pseudomonadales* order. According to their study isolates belongs to *Pseudomonadales* order can degrade Chloramphenicol, trimethoprim, vancomycin, mafenide, carbenicillin, dicloxacillin antibiotics. The phylogenetic relationship of *Pseudomonas sp.* BIOMIG1 and for each bacteria that can subsist on vancomycin, trimethoprim and chloramphenicol were checked, it was found that *Pseudomonas sp.* BIOMIG1 clustered with antibiotic degrading isolates (Figure 2.6-2.8.). Considering *Pseudomonas sp.* BIOMIG1 is a common BAC degrader and it has close phylogenetic relationship with other antibiotic degraders, it can be speculated that *Pseudomonas sp.* BIOMIG1 may degrade antibiotics.

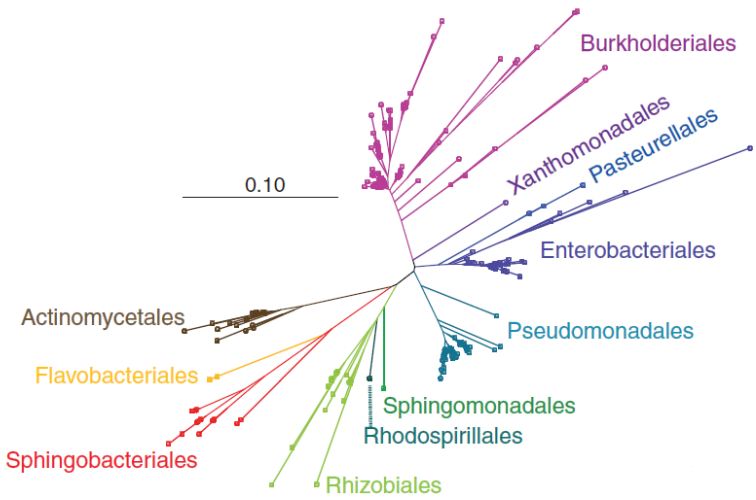


Figure 2.5. Phylogenetic distribution of bacterial isolates subsisting on antibiotics (Dantas et al., 2008).

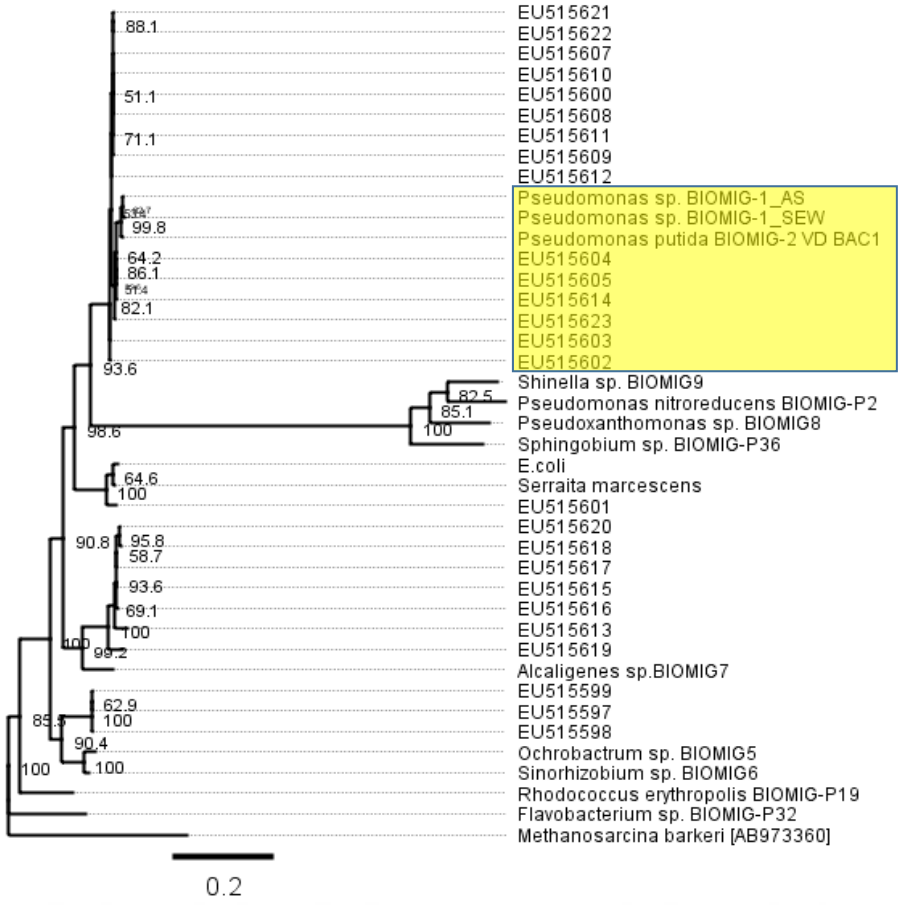


Figure 2.6. Phylogenetic tree of relationships of bacteria isolated in our lab, determined by maximum likelihood followed by neighbor joining tree building method and Hasegawa-Kishino-Yano genetic distance model, relative to bacteria reported as vancomycin degraders according to Dantas et al. (2008). Bootstrap values represents 100 replicates. The scale bar represents 0.2 substitution per nucleotide position. *M. barkeri* (AB973360) was used as the out group. (Branches were shown in yellow, when the bacteria isolated in our lab clustered with bacteria which can degrade vancomycin).

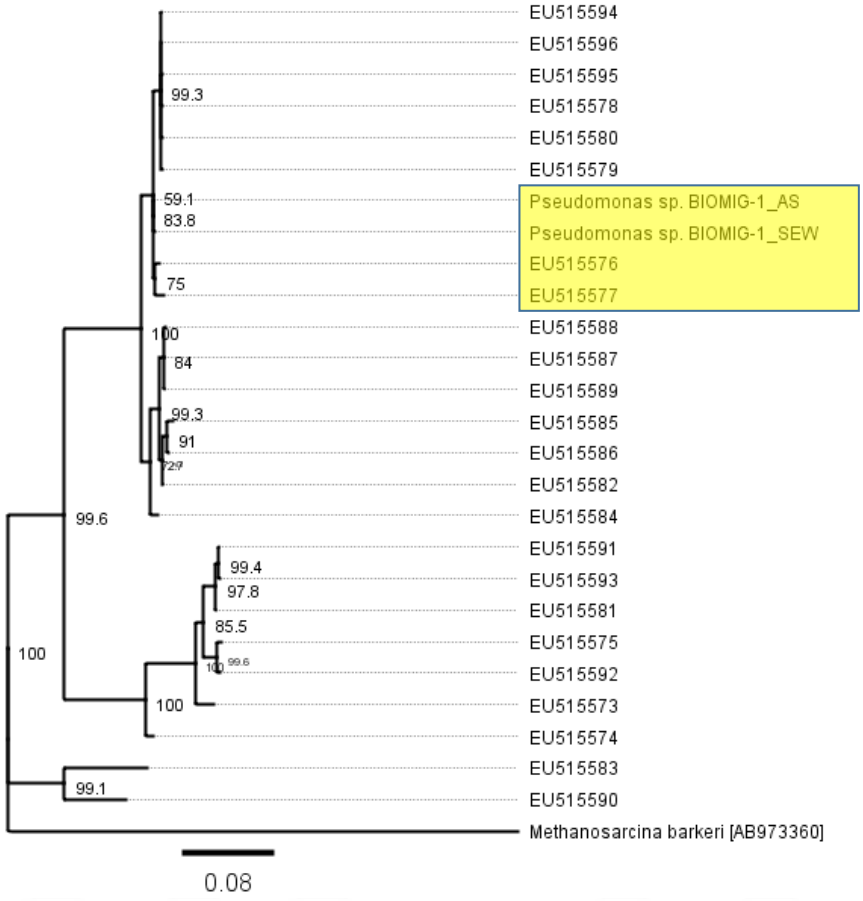


Figure 2.7. Phylogenetic tree of relationships of bacteria isolated in our lab, determined by maximum likelihood followed by neighbor joining tree building method and Hasegawa-Kishino-Yano genetic distance model, relative to bacteria reported as trimethoprim degraders according to Dantas et al. (2008). Bootstrap values represents 100 replicates. The scale bar represents 0.08 substitution per nucleotide position. *M. barkeri* (AB973360) was used as the out group. (Branches were shown in yellow, when the bacteria isolated in our lab clustered with bacteria which can degrade trimethoprim).

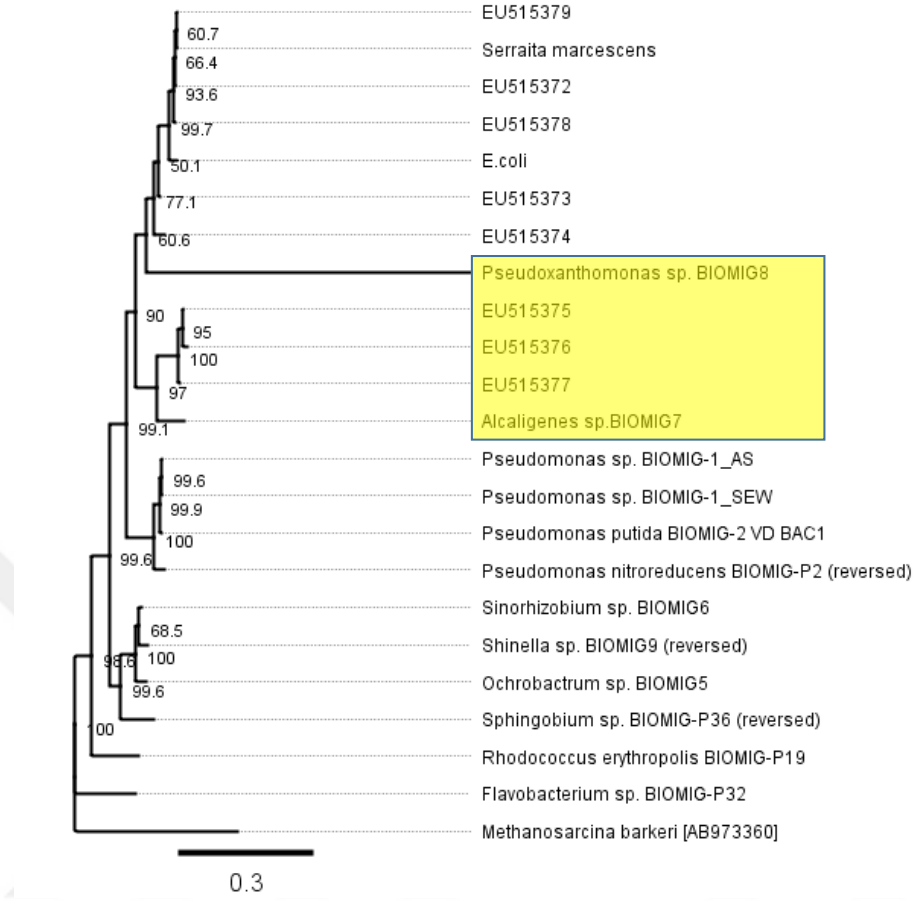


Figure 2.8. Phylogenetic tree of relationships of bacteria isolated in our lab, determined by maximum likelihood followed by neighbor joining tree building method and Hasegawa-Kishino-Yano genetic distance model, relative to bacteria reported as chloramphenicol degraders according to Dantas et al. (2008). Bootstrap values represents 100 replicates. The scale bar represents 0.3 substitution per nucleotide position. *M. barkeri* (AB973360) was used as the out group. (Branches were shown in yellow, when the bacteria isolated in our lab clustered with bacteria which can degrade chloramphenicol).

3. OBJECTIVES

It has been reported that efflux pumps may confer co and cross resistance to antibiotics (Buffet-Bataillon et al., 2011). Several studies reported that multidrug efflux pumps are usually active against other microbial agents (Guo et al., 2014; Buffet-Bataillon et al., 2012; Holdsworth and Law, 2013; McCay et al., 2010; Morita et al., 2014). Therefore efflux mediated QAC resistance might be effective against antibiotics (Grkovic et al., 2002). On the other, there is no detailed research about the relationship between QACs resistance and antibiotic susceptibility. BAC-degrading bacterial strain *Pseudomonas sp.* BIOMIG1 was recently isolated from activated sludge in our lab. In this study, this bacteria is going to be used to test whether BAC resistance confer antibiotic resistance or not. Also antibiotic biotransformation potential of BIOMIG1 is going to be elucidated since BIOMIG1 is phylogenetically close to several microorganisms which are able to degrade several antibiotics.

Although several studies have reported a number of microorganisms that are responsible of the biotransformation of benzalkonium chlorides (BACs) none of them investigated the antibiotic degradation potential of these microorganisms. A systematic understanding of key microorganisms responsible for both BAC and antibiotic biotransformation is crucial. Therefore antibiotic biotransformation potential of BIOMIG1 is going to be elucidated. Then it will be investigated that how BAC degrading bacteria presence affect the BAC susceptibility of other microorganisms present in the same community with BAC degraders. The hypotheses below are going to be tested in this study.

Hypothesis 1: BAC degrading microorganisms may be present in activated sludge

Approach: An activated sludge sample was taken from a municipal wastewater treatment plant. An enrichment community feeding with BACs was generated. BAC degrading species were isolated from the BAC enrichment activated sludge microbial community and identified based on 16S rDNA sequence.

Objective: Isolation of BAC degraders in an activated sludge microbial community.

Hypothesis 2: QAC-induced resistance mechanisms may confer cross and co-resistance to many antibiotics.

Approach: Microorganisms having different tolerance to benzalkonium chlorides (BACs) were selected. Their minimum inhibitory concentration (MIC) of 18 antibiotics belonging to different antibiotic groups were determined by using both macro dilution and E-test method. These finding were analyzed to find out whether QAC resistance play a role in antibiotic resistance or not.

Objective: Investigation of the relationship between QACs resistance and antibiotic susceptibility.

Hypothesis 3: Strain BIOMIG1 may subsist on antibiotics.

Approach: Modified E-test was performed to determine antibiotics that are biodegradable by BIOMIG1 isolated from sewage, and activated sludge (ecotypes). Biotransformation kinetics and pathways of degradable antibiotics were elucidated using shake-flask experiments.

Objective: To determine the spectrum of antibiotics degradable by strain BIOMIG1.

Hypothesis 4: Presence of strain BIOMIG1 may cause susceptible bacteria to become BAC resistant.

Approach: BAC susceptible bacteria *E.coli* (BAC MIC: 16 mg/L) and *Pseudomonas sp.* BIOMIG1 -BAC resistant and BAC degrading bacteria- used in co-culture. This co-culture

and bacteria were separately fed with BAC containing surface cleaner Domestos® and the susceptibility of bacteria was monitored.

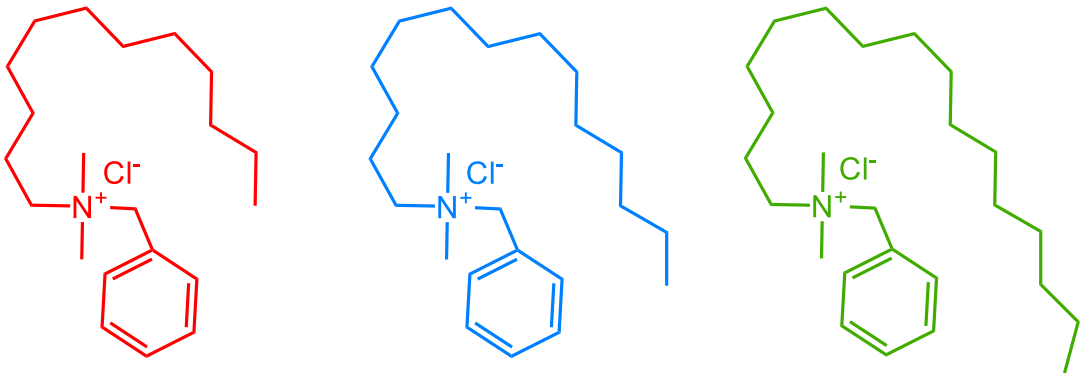
Objective: To elucidate the role of biotransformation on BAC resistance in co-culture.

4. MATERIALS AND METHODS

4.1. Chemicals

Penicillin g (penicillin g sodium salt, $C_{16}H_{17}N_2NaO_4CS$, 356.37 g/mole), amoxicillin (amoxicillin trihydrate, $C_{16}H_{19}N_3O_5S \cdot 3H_2O$, 419.45 g/mole), ciprofloxacin (ciprofloxacin hydrochloride monohydrate, $C_{17}H_{18}FN_3O_3 \cdot HCl \cdot H_2O$, 385.82 g/mole), enrofloxacin (enrofloxacin, $C_{19}H_{22}FN_3O_3$, 359.39 g/mole), norfloxacin (norfloxacin, $C_{16}H_{18}FN_3O_3$, 319.33 g/mole), ofloxacin (ofloxacin, $C_{18}H_{20}FN_3O_4$, 361.37 g/mole), levofloxacin (levofloxacin, $C_{18}H_{20}FN_3O_4$, 361.37 g/mole), trimethoprim (trimethoprim, $C_{14}H_{18}N_4O_3$, 290.32 g/mole), sulfamethoxazole (sulfamethoxazole, $C_{10}H_{11}N_3O_3S$, 253.28 g/mole), tetracycline (tetracycline hydrochloride, $C_{22}H_{24}N_2O_8 \cdot HCl$, 480.90 g/mole), doxycycline (doxycycline hydrochloride, $C_{22}H_{24}N_2O_8 \cdot HCl$, 480.90 g/mole), clarithromycin (clarithromycin, $C_{38}H_{69}NO_{13}$, 747.95 g/mole), erythromycin (erythromycin, $C_{37}H_{67}NO_{13}$, 733.93 g/mole), azithromycin (azithromycin dihydrate, $C_{38}H_{72}N_2O_{12} \cdot 2H_2O$, 396.1 g/mole), clindamycin (clindamycin hydrochloride, $C_{18}H_{33}ClN_2O_5S \cdot HCl \cdot H_2O$, 479.46 g/mole), kanamycin (kanamycin sulfate, $C_{18}H_{36}N_4O_{11} \cdot H_2O_4S$, 582.58 g/mole), chloramphenicol (chloramphenicol, $C_{11}H_{12}Cl_2N_2O_5$, 323.13 g/mole) and vancomycin (vancomycin hydrochloride hydrate, $C_{66}H_{75}C_{l2}N_9O_{24} \cdot HCl \cdot xH_2O$, 1485.71 g/mole anhydrous basis) were obtained in high purity from Fluka and Sigma Aldrich Chemicals Company. E-test of these 18 antibiotics were purchased from bioMerieux Inc.

Dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl, $C_{21}H_{38}NCl$, 340 g/mole), tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl, $C_{23}H_{42}NCl$, 368 g/mole) and hexadecyl benzyl dimethyl ammonium chloride (C_{16}BDMA-Cl, $C_{25}H_{46}NCl$, 396.1 g/mole),were obtained in high purity from TCI Chemicals (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (Figure 4.1.).



(A) **(B)** **(C)**

Figure 4.1. Molecular structures of (A) C_{12}BDMA-Cl, (B) C_{14}BDMA-Cl and (C) C_{16}BDMA-Cl.

Mineral salts and organic solvents used in experiments were purchased from Merck and Sigma Aldrich Chemicals Company.

4.2. Microorganisms

4.2.1. Microorganisms Used During the Experiments

BAC resistant and degrading ecotypes of *Pseudomonas sp.* BIOMIG1 were used in antibiotic resistance, degradation and co-culture resistance experiments. These *Pseudomonas sp.* BIOMIG1 ecotypes are the same species but they were isolated from different environments: sewage, activated sludge and soil. Since they are ecotypes, they may exhibit phenotypic differences.

BIOMIG1 has an MIC of 1024 for BACs and mineralizes BACs. A non-BAC degrading mutant of BIOMIG1, BIOMIG1^{N} which has BAC MIC of 600 mg/L was used to identify the role of BAC degradation on antibiotic resistance.

The *Pseudomonas putida* BIOMIG2 VD was isolated from Vileda® samples that are used in cleaning residential floors with BAC containing surface cleaners. It is genetically very close to BIOMIG1. The MIC value of BACs for the *Pseudomonas putida* BIOMIG2 VD is 125 mg/L.

Serratia marcescens BIOMIG4 was isolated from sewage. The MIC value of BACs for BIOMIG4 is more than 1024 mg/L.

E.coli BIOMIG3 was isolated from sewage and the corresponding MIC value of BACs against BIOMIG3 is 16 mg/L.

4.2.2. Preparation of Cultures

To prepare the culture for assays, the frozen isolate was thawed at room temperature for 2 hours. Serial dilutions were applied in microcentrifuge tubes with 0.85% saline solutions. 100 µL sample from each dilution was spread on LB agar or CHROM®Agar Orientation plate. The plate was placed in an incubator at 30 °C overnight. Then the plate was stored at 4 °C. A single colony was taken from the plate using a sterile wooden applicator and it was transferred to an appropriate liquid media for experiments.

4.3. Preparation of Media, Broth and Agar Plates

4.3.1. Mineral Salt Medium

The composition of mineral salt medium (MSM) is given in Table 4.1. Briefly, 7.4 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl and 1 mL trace metal solution (Table 4.2.) were added into deionized (DI) water and the content was autoclaved at 121°C for 15 min. After the solution cooled down to 60 ^oC, 10 mL of sterile 0.1 M $MgSO_4.7H_2O$ and 0.01 M $CaCl_2.2H_2O$ were added into the solution to make the total volume 1L.

Table 4.1. Composition of medium used in this study.

Ingredient	**Concentration**
K_2HPO_4	7.4 g/L
KH_2PO_4	3.0 g/L
NaCl	0.5 g/L
NH_4Cl	1.00 g/L
Trace metal stock solution	1 mL/L
$MgSO_4$.7H2O	0.25 g/L
$CaCl_2$	0.01 g/L

Table 4.2. Composition of trace metal stock solution used in this study.

Ingredient	**Concentration**
$ZnCl_2$	0.50 g/L
$MnCl_2.4H_2O$	0.30 g/L
H_3BO_3	3.0 g/L
$CoCl_2.6H_2O$	2.0 g/L
$CuCl_2.2H_2O$	0.1 g/L
$NiSO_4.6H_2O$	0.2 g/L
$Na_2MoO_4.2H_2O$	0.3 g/L

4.3.2. Luria Bertani (LB) Broth and LB-BAC Broth

LB broth was prepared by adding 10 g tryptone, 5 g yeast extract, 5 g NaCl into 1L DI water and autoclaving the solution at 121°C for 15 min (Table 4.3.)

Table 4.3. Composition of LB broth used in this study.

Ingredient	Concentration
Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	5 g/L

LB-BAC broth containing 50 mg/L BAC was prepared by adding 5 mL of 10 g/L BAC stock solution into 1 L LB broth after the sterilization of LB broth.

4.3.3. Plates

a. LB Agar Plates: LB agar plates were prepared with the same way as LB broth but with an additional step in which 1.5% (w/v) agar was added to the solution before the mixture was autoclaved. After the solution was cooled approximately to 55°C, it was poured into petri dishes aseptically.

b. LB-BAC Plates: LB-BAC agar plates were prepared by following the procedure of LB agar preparation, but with an additional step in which BAC mixture was added into LB agar following sterilization, at a final concentration of 50 mg/L BAC.

c. CHROM®Agar Pseudomonas™ Plates: Chromagar Pseudomonas (PS agar; CHROMagar Microbiology, France) was prepared by the addition of 8.30 g of PS agar into 250 mL DI water followed by stirring and boiling the mixture on a hot plate.

d. CHROM®Agar Orientation Plates: Chromagar Orientation (OR agar; CHROMagar Microbiology, France) was prepared by the addition of 8.25 g of OR agar into 250 mL deionized (DI) water followed by stirring and boiling the mixture on a hot plate.

e. CHROMAgar™ ECC Agar Plates: Chromagar ECC (ECC agar; CHROMagar Microbiology, France) was prepared by the addition of 8.2 g of ECC agar into 250 mL deionized (DI) water followed by stirring and boiling the mixture on a hot plate.

f. MSM Agar Plates: MSM agar plates were prepared by adding 15 g agar into 1L of MSM medium and autoclaving the content at 121°C for 15 min.

g. MSM Agarose Plates: MSM agarose plates were prepared by adding 10 g agarose into 1L of MSM medium and autoclaving the content at 121°C for 15 min.

h. Single Carbon Source (SCS) Plates: Single Carbon Source (SCS) media containing 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 15 mg EDTA, 4.5 mg $ZnSO_4 \cdot 7H_2O$, 4.5 mg $CaCl_2 \cdot 2H_2O$, 3 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 4H_2O$, 1 mg H_3BO_3, 0.4 mg $Na_2MoO_4 \cdot 2H_2O$, 0.3 mg $CuSO_4 \cdot 5H_2O$, 0.3 mg $CoCl_2 \cdot 6H_20$ and 0.1 mg KI and 15 g agar per liter water. The pH was adjusted to 5.5 using HCl, and media was sterilized by autoclaving.

4.3.4. BACs Stock Solution

A 10 g/L of BACs stock solution was prepared by mixing 0.4 g C_{12}BDMA-Cl, 0.5 g C_{14}BDMA-Cl and 0.1 g C_{16}BDMA-Cl in 100 mL sterile medium under laminar hood.

4.3.5. 0.85% Saline Solution

To prepare a 100 mL of saline solution, 0.85 g sodium chloride was added into 100 mL of DI water in a volumetric flask. The solution was autoclaved at 121 °C for 15 minutes for sterilization.

4.4. Analytical Methods

4.4.1. Antibiotic Analysis

Presence and concentrations of antibiotics (amoxicillin, vancomycin, trimethoprim, sulfamethoxazole, chloramphenicol, clindamycin and penicillin g) in samples were analyzed using an Agilent 1260 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with an Eclipse Plus C18 (100 x 4.6 mm, 3.5μ) (Agilent Technologies, Palo Alto, CA, USA). HPLC was performed at a flow rate of 1.0 mL/min with an acetonitrile gradient going from 5% to 65% in the presence of 0.1% trifluoroacetic acid (TFA). Column was maintained at 35 °C. Detection was achieved with UV-VIS diode array detector at a wavelength of 210 nm.

A representative HPLC chromatogram, UV-VIS spectra and calibration curves of 7 antibiotics are given in Figure 4.2, 4.3. and 4.4 respectively.

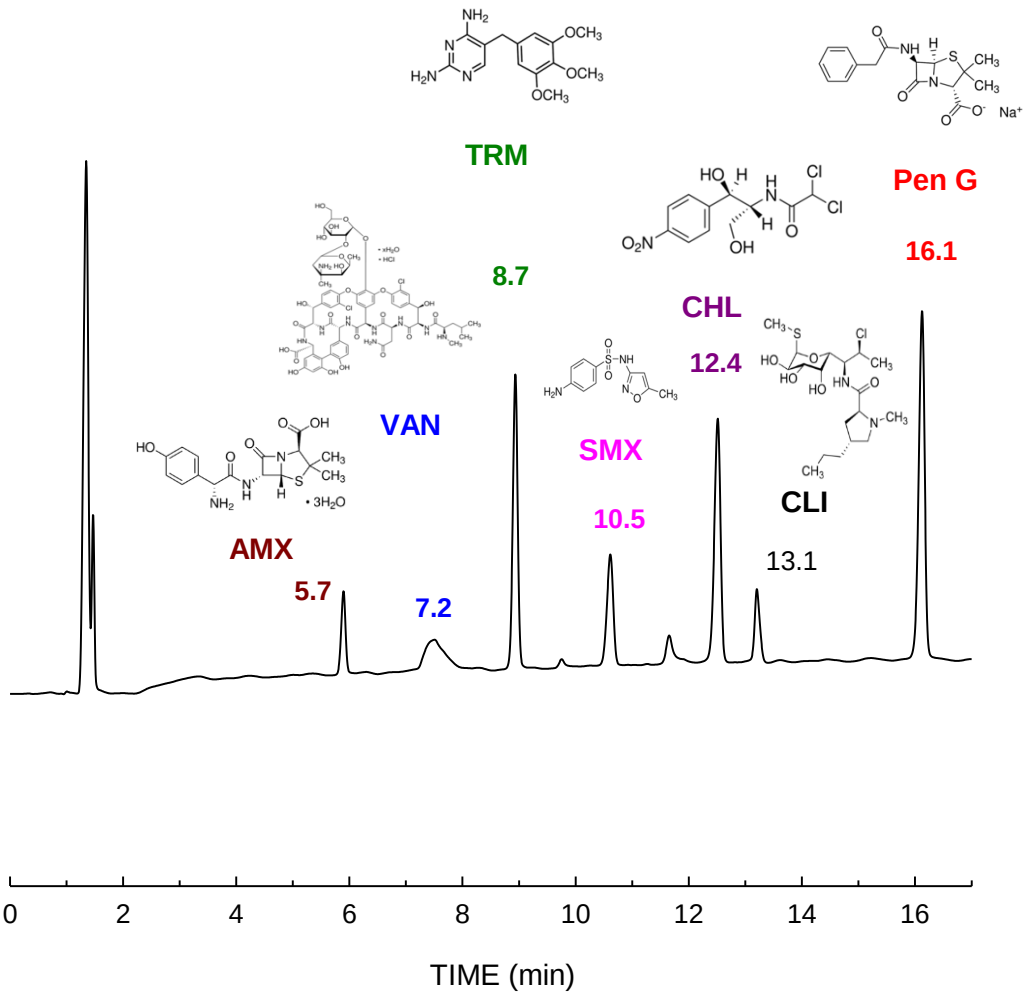


Figure 4.2. HPLC chromatogram of a sample containing trimethoprim, clindamycin, sulfamethoxazole, penicillin g, chloramphenicol, amoxicillin, and vancomycin at 100 mg/L concentration each.

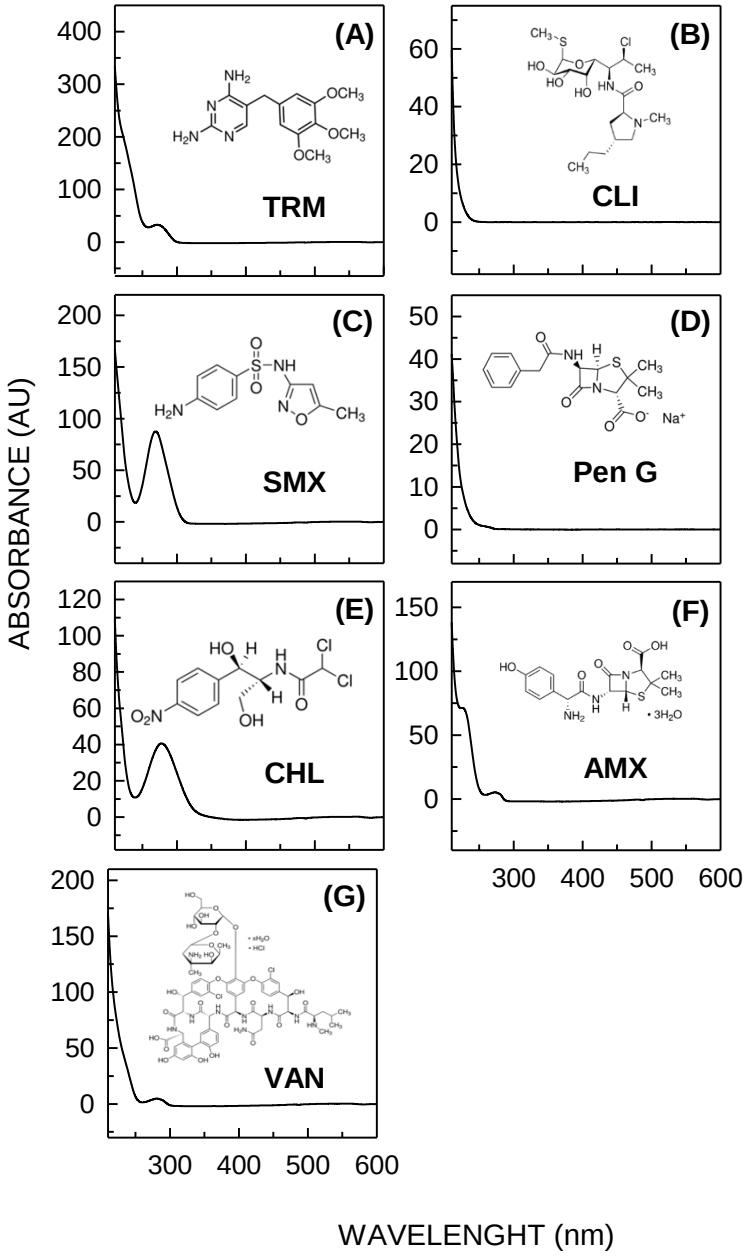


Figure 4.3. UV-VIS spectrum of (A) trimethoprim, (B) clindamycin, (C) sulfamethoxazole, (D) penicillin g, (E) chloramphenicol, (F) amoxicillin, and (G) vancomycin antibiotics.

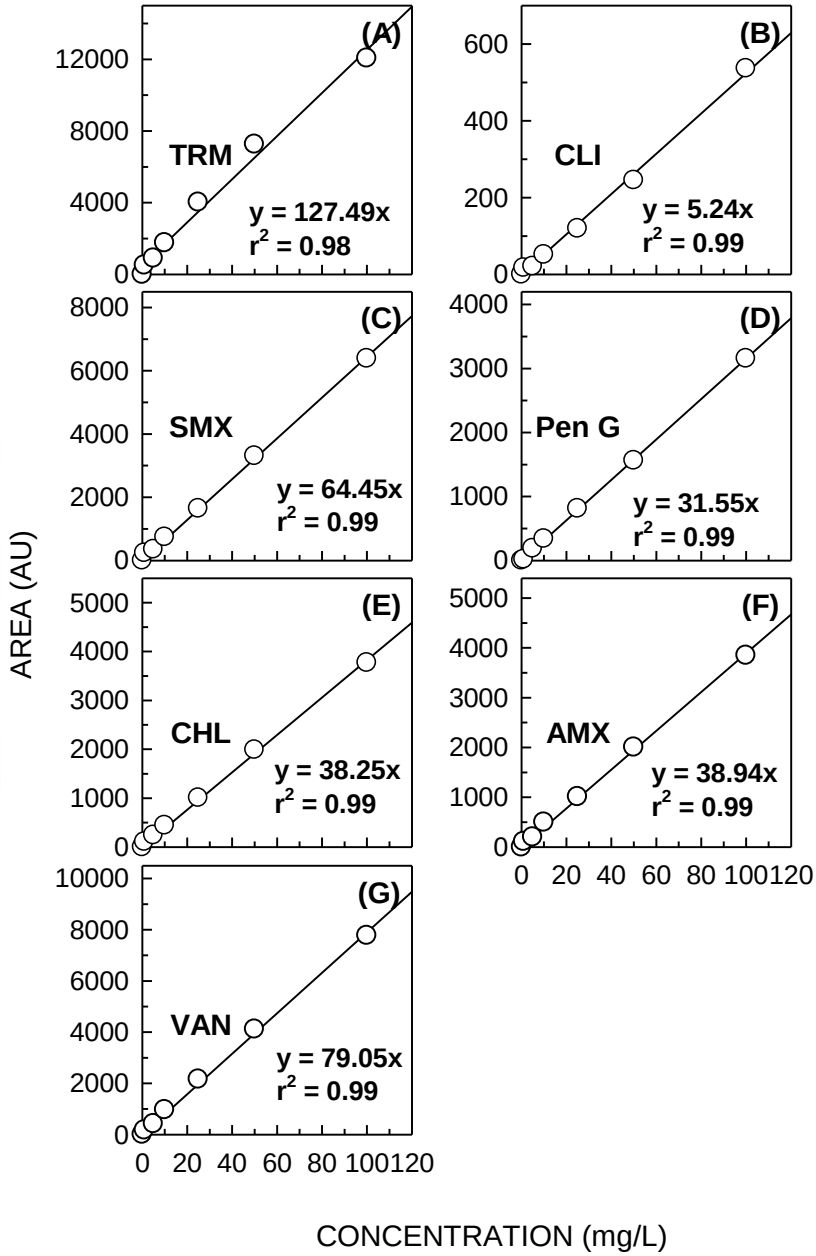


Figure 4.4. Calibration curves of (A) trimethoprim, (B) clindamycin, (C) sulfamethoxazole, (D) penicillin g, (E) chloramphenicol, (F) amoxicillin, and (G) vancomycin antibiotics.

4.4.2. QAC Analysis

The presence and concentrations of benzalkonium chlorides in samples were analyzed using an Agilent 1260 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a Phenomenex Luna SCX column (250 x 4.6 mm, 5µ) (Phenomenex, Inc.,Torrance, CA) followed by a Polaris C_{18}A column (50 × 4.6 mm, 3.2 µ) (Agilent Technologies, Palo Alto, CA, USA). A Phenomenex SCX SecurityGuard cartridge (4 × 3.0 mm) is used as a precolumn. A 60:40 (v/v) mixture of acetonitrile and 50 mM phosphate buffer (pH 2.5) is used as the mobile phase at a flow rate of 1.0 mL/min and the columns were maintained at 35 °C. Detection is achieved with UV-Vis diode array detector at a wavelength of 210 nm.

A representative HPLC chromatogram of a sample containing C_{12}BDMA-Cl, C_{14}BDMA-Cl and C_{16}BDMA-Cl is shown in Figure 4.5. The calibration curves of C_{12}BDMA-Cl, C_{14}BDMA-Cl and C_{16}BDMA-Cl used in this study are given in Figure 4.6.

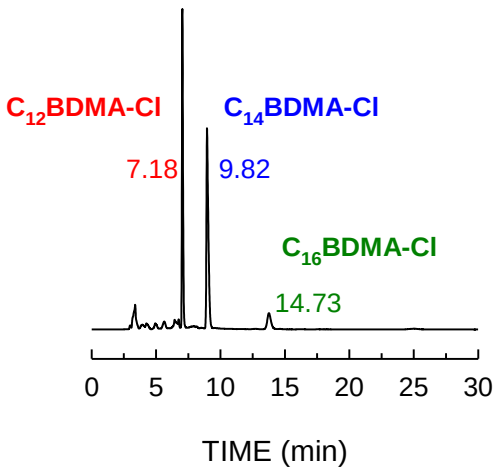


Figure 4.5. HPLC chromatogram of a sample containing C_{12}BDMA-Cl, C_{14}BDMA-Cl and C_{16}BDMA-Cl.

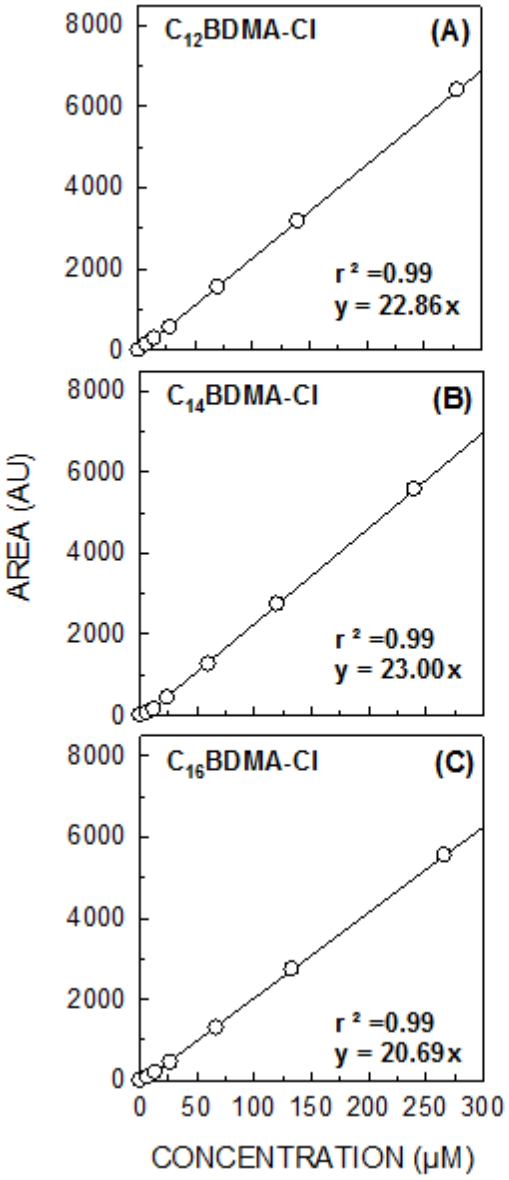


Figure 4.6. Calibration curve of C_{12}BDMA-Cl, C_{14}BDMA-Cl and C_{16}BDMA-Cl used in this study.

5. DEVELOPMENT OF A BAC DEGRADING CULTURE AND ISOLATION OF A BAC DEGRADER

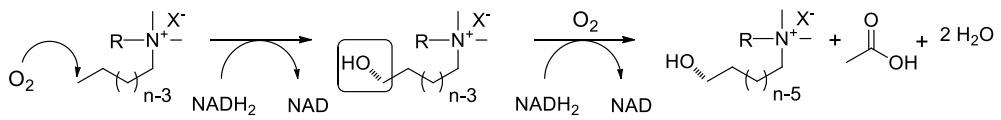
5.1. Introduction

Benzalkonium chlorides (BACs) are antimicrobial biocides belonging to quaternary ammonium compounds (QACs). These chemicals are the active disinfectant agents present in commercial cleaning products that we use both in our daily lives and in industrial applications (Tezel and Pavlostathis, 2012a). BACs are the most common quaternary ammonium compounds in municipal wastewater and their concentrations are changing between 20 and 300 µg/L (Martinez-Carballo et al., 2007a; Clara et al., 2007). On the other hand BAC concentrations were found to be up to 10 mg/L in wastewaters originating from hospitals and poultry processing facilities (Kümmerer et al., 1997; Martinez-Carballo et al., 2007b). Whether treated or not, wastewaters are discharged into receiving environment and BACs release into natural systems where they accumulate in different compartments of that environment (Oh et al., 2013). It is believed that the exposure of bacteria to such antimicrobials that are present at sub inhibitory concentrations in the environment results in the development and dissemination of antimicrobial resistance. Finding efficient methods that remove BACs from wastewaters can play a key role in the suppression of such cases. In wastewater treatment plants, biotransformation can be the most important mechanism affecting the environmental fate of BACs.

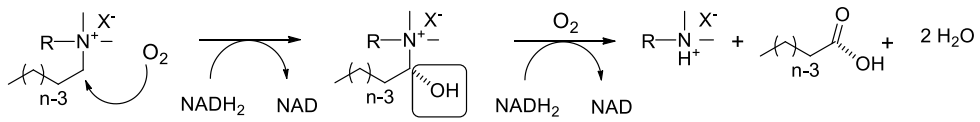
Species in genera *Xanthomonas, Aeromonas, Pseudomonas, Bacillus* and *Thalassospira* were reported as QAC degraders (Tezel and Pavlostathis, 2012a; Dean-Raymond, 1977; Patrauchan and Oriel, 2003; Tandukar et al., 2013). QAC degrading microbial community was isolated from river sediment and it was found that more than 50% of species in enriched culture belongs to *Pseudomonas* genera (Oh et al., 2013; Tezel et al., 2012b; Tandukar et al., 2013). *P. putida* and *P. aeruginosa* groups were found to be dominant species based on the phylogenetic tree prepared using the 16S rDNA sequences of QAC degrading isolates and predominant species in QAC degrading microbial communities

(Yılmaz, 2014). Also *Stenetrophomonas* spp., and *Achromobacter* spp. were identified as QAC degrading bacteria (Oh et al., 2013). More recently, *Pseudomonas putida* ATCC 12633 have been identified that are capable of QAC degradation and Tetradecyl trimethyl ammonium bromide monooxygenase (TTABMO) was identified as the enzyme responsible for BAC degradation (Liffourrena and Lucchesi, 2014). On the other hand, a FAD-using amine oxidase (AOx-BAC) was identified as the enzyme responsible for dealkylating BACs by *Pseudomonas nitroreducens* (Oh et al., 2014).

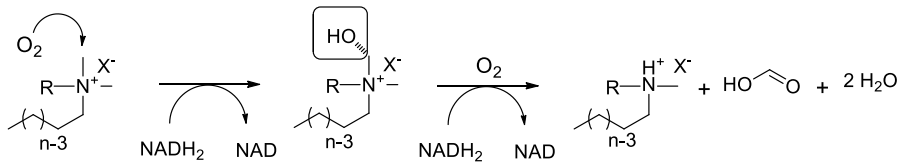
According to the location of the initial reaction QAC biotransformation pathways differ (Figure 5.1.): a) hydroxylation of the terminal C of the alkyl chain (ω-hydroxylation), followed by multiple β-oxidation cycles, progressing toward the hydrophilic moiety; b) hydroxylation of the C adjacent to the central N (α-hydroxylation), followed by central fission, resulting in the separation of the hydrophobic from the hydrophilic moiety; and c) hydroxylation of the methyl-C attached to the central N, followed by fission of the methyl group. Pathway-b is the best way to cope with the toxic effects of QACs since the products of pathway-b, a tertiary amine, is less toxic than the products of pathway-a and –c. (Tezel et al., 2012b).



a. ω-hydroxylation of terminal C of alkyl group



b. α-hydroxylation of C adjacent to N of alkyl group



c. α-hydroxylation of C of methyl group

Figure 5.1. Aerobic QAC biotransformation pathways (Tezel et al., 2012a).

Tezel et al. (2012b) reported the biotransformation of n-tetra decyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl), under aerobic conditions by an enriched microbial community growing on benzalkonium chlorides (BACs). In contrast to previous findings, C_{14}BDMA-Cl was converted to benzyldimethylamine (BDMA) and then BDMA was fully mineralized. Recently Ertekin et al. (2016) investigated the prevalent BAC degrader in the environment. To do that four microbial community were developed from sewage, activated sludge, soil and sea sediment samples. *Pseudomonas* and *Achromobacter* genera were found the dominant species in all microbial communities. Moreover, it was found that the relative abundance of *Pseudomonas sp.* BIOMIG1 were positively correlated with BAC biotransformation rates of the enriched microbial community. Also, the dioxygenase enzyme present in Contig_BAC1 of BIOMIG1 genomes was reported as a candidate enzyme which is responsible from BAC biotransformation.

Under the light of the above information, specific objectives of the research reported in this chapter were to: (a) develop a BAC degrading microbial community; (b) identify major species in that community.

5.2. Materials and Methods

5.2.1. Development of BAC Degrading Activated Sludge Microbial Community

A 50 mL of the activated sludge sample, which was provided from biological treatment unit of Paşaköy Municipal Wastewater Treatment Plant, Istanbul, was transferred aseptically into a sterile 1 L glass bottle with a screw-cap containing 150 mL of MSM. 1 mL from 10,000 mg/L BAC stock solution was amended aseptically to the bottle as a carbon and energy source to maintain 50 mg/L (ca 140 μM) initial total BAC concentration in the bottle. Another bottle was prepared as control reactor which contained 200 mL of MSM and 1 mL of 10,000 mg/L BAC stock solution. This bottle did not contain any sample and used as control bottle.

Bottles were placed on an orbital shaker at 130 rpm at room temperature (22 °C). On a daily basis, BAC concentration in the bottle was monitored using HPLC method described in 4.2.2. As soon as all BAC was consumed in the bottle, 100 mL sample from this bottle was transferred aseptically into 100 mL sterile MSM media, and total BAC concentration in the bottle was set to 50 mg/L, by spiking 1 mL from a 10,000 mg/L BAC solution. We continued to operate the BAC-degrading community in fed-batch mode by replacing 100 mL mixed liquor with fresh MSM and amending 50 mg/L BAC two times a week. This serial dilution procedure was repeated for 21 times to obtain a specialized BAC-degrading activated sludge microbial community. The pH of the culture was also measured periodically before adding the BAC.

5.2.2. Isolation BAC-degraders in BAC Enriched Microbial Community

Once the aforementioned microbial community reached a steady-state, a sample was taken and diluted 10^4 times with 0.85% saline solution. A 100 µL of diluted sample from the BAC-degrading microbial community was spread on a CHROM®Agar Pseudomonas™ plate. After one day of incubation at 28 °C, about fifty blue/green growing colonies on the agar plate were picked and patched on LB-BAC agar plates. After one day of incubation at 28 °C, half of each patches were transferred from agar plate into sterile glass test tubes with 2 mL sterile MSM which contains 50 mg/L BAC in order to test BAC biotransformation potential of each isolate. Test tubes were placed on an orbital shaker at 130 rpm at room temperature (22 °C). BAC concentration in each test tube was measured at 3rd and 7th days.

The other half of the grown patch were transferred into sterile microcentrifuge tubes with 0.5 mL LB which contains 50 mg/L BAC. These tubes were placed on an orbital shaker and agitated at 130 rpm at room temperature (22 °C). After one day of incubation, 180 µL of glycerol (80%) was added to each microcentrifuge tube and they were stored at -20 °C for use in subsequent experiments.

5.2.3. Phylogenetic Classification of BAC Degraders

Previously isolated and stored fifty colonies were removed from the cabinet and allowed to thaw. Then samples were taken by a sterile loop from each isolate and streaked on to LB-BAC plates. After one day of incubation at 28 °C, one single colony was taken from each plate and patched on to a new LB-BAC plates using a sterile wooden applicator. After one day of incubation at 28 °C, half of the each patch was transferred into sterile microcentrifuge tubes containing 0.5 mL of LB-BAC broth and 90 µL of glycerol (80%) then they were stored at -20 °C. The other halves of grown patches were transferred into sterile microcentrifuges tubes containing 100 µL DI (deionized) water. After samples were boiled around 15 minute at 100 °C, they were centrifuged at 10,000 rpm for 10 minutes. Supernatants were transferred to new sterile microcentrifuge tubes and the genomic DNA in the samples was quantified using Implen® P360 nanophotometer. Its quality was also checked visually on 0.7% agarose gel after electrophoresis. 16S rRNA gene in each genomic DNA sample was amplified by PCR using TaKaRa Premix Taq^{TM} Kit (TaKaRa Bio, Shiga, Japan) with the following primers: 27F forward primer (27F: 5'-AGAGTTTGATCMTGGCTCAG-3') (0.4 µM), and 1492R reverse primer (1492R: 5'-TACGGYTACCTTGTTACGACTT-3') (0.4 µM). During this procedure, PCR Master Mix was prepared using 600 µL DI water, 50 µL 27F forward primer, 50 µL 1492R reverse primer and 500 µL Premix Taq (Ex Taq). Then, 49 µL PCR Master Mix and 1 µL DNA sample was put into a PCR tube. PCR conditions included 35 cycles at 94 °C (30 sec), 55 °C (30 sec) and 72 °C (1min), with a final extension at 72 °C for 7 min by using SureCycler 8800 Thermal Cycler. Amplified 16S rDNA was electrophoresed on 1% agarose gel (SeaKem LE Agarose, Lonza Inc., Basel, Switzerland) prepared with 1X TBE buffer solution, stained with 6X DNA loading dye (New England Bıolabs® Inc., MA, USA) and Pronasafe (CONDA, Madrid, Spain). . A 100 bp DNA Ladder (New England Bıolabs® Inc., MA, U.S.A.) used as marker. After, gel was run with 100V for 20 min on Mupid® One, gel was monitored using BioRad EZ-DOC.

The similarity of the isolated strains determined by Restriction Fragment Length Polymorphism analysis (RFLP) as follows: PCR products were digested with an endonuclease MspI in a reaction mixture containing 5 µL of PCR product, 21 µL H_2O, 3 µL 10X Cut Smart buffer (New England Bıolabs® Inc., MA, USA) and 1 µL of MspI (restriction enzyme) (New England Bıolabs® Inc., MA, USA). Reaction was set in a PCR tube at 37 °C for 15 min. RFLP products stained with 6X DNA loading dye (New England Bıolabs® Inc., MA, USA) was loaded onto a 2% agarose gel (SeaKem LE Agarose, Lonza Inc., Basel, Switzerland) prepared with 1X TBE buffer solution. A 100 bp DNA Ladder (New England Bıolabs® Inc., MA, U.S.A.) used as marker. After, gel was run at 100V for 20 min on Mupid® One, gel was visualized using BioRad EZ-DOC.

After RFLP analysis, samples with similar fragment banding patterns were grouped and purified. Purified 16S rRNA PCR samples were sequenced by MacroGen Inc. Europe (Amsterdam, Netherlands). Forward and reverse sequences were trimmed and assembled using Geneious Software (Biomatters Ltd., Auckland, New Zealand) to yield about 1350 bp length and above 95% quality 16S rDNA sequence. The sequence was then queried against the NCBI database (NCBI, 2012) using MEGABLAST algorithm and closest neighbor sequence to queried sequence was determined.

5.3. Results and Discussion

5.3.1. Activity of BAC Degrading Community

A microbial community has been obtained from the sample taken from the biological treatment unit of Paşaköy Municipal Wastewater Treatment Plant, İstanbul, with dilution-to-extinction method and using BAC as a carbon and energy source. After eight days of first BAC amendment, BAC concentration dropped to zero in the reactor (Figure 5.2.). Then the reactor was fed twice a week with BACs. The resulting microbial community that reached steady-state after twenty-one dilution was consistently degrading BACs without accumulating any by-product (Figure 5.2.). Besides BACs, the pH of the reactor was

checked during the course and measured to be at around 7.0. Using BAC as the sole carbon source caused the extinction of microorganisms that cannot degrade BAC in the sample. Given the fact that BAC concentration did not change in the control reactor set up without inoculum from activated sludge, the disappearance of BACs in the microbial community bottle was attributed to biodegradation.

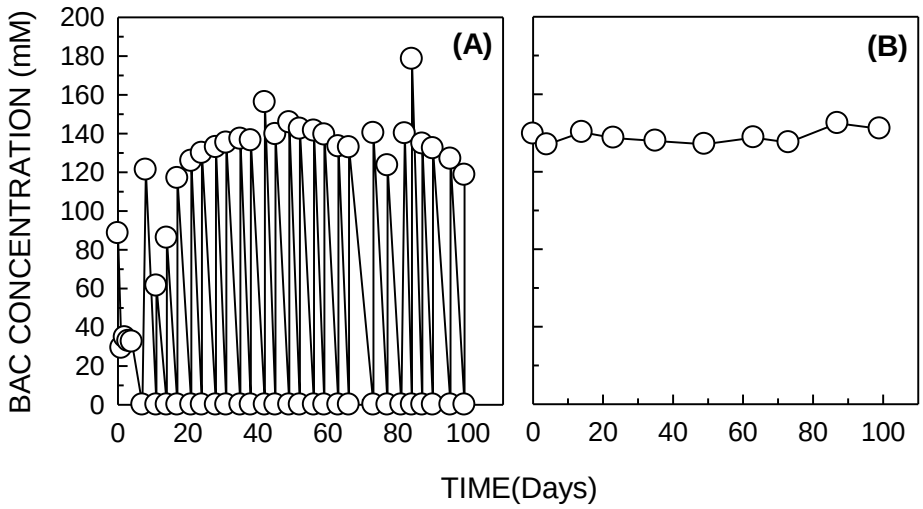


Figure 5.2. BAC utilization profile in (A) microbial community generated from activated sludge and (B) control during start-up and fed-batch operation period.

Given the fact that *Pseudomonas* genus of bacteria can degrade QACs and other xenobiotics (Tezel and Pavlostathis, 2015), *Pseudomonas* spp. in the community were isolated by plating a sample on a CHROM®Agar Pseudomonas™ plate (Figure 5.3.). This method assumes that colonies growing with blue/green color on this agar are those bacteria belonging to *Pseudomonas* spp.

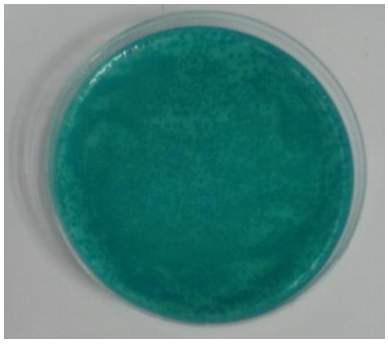


Figure 5.3. A grown sample obtained from microbial community on CHROM®Agar Pseudomonas™.

About 50 blue colonies grown on the selective agar plate were picked and patched on LB-BAC plates. After 24 hrs of incubation at 28 °C, a single colony of each isolate was transferred into a sterile glass test tube containing 2 mL MSM at 50 mg/L initial BAC concentration. BAC concentration in each tube was measured at 3rd and 7th days of incubation. 44 out of 50 isolates degraded BACs 100% in 7 days (Figure 5.4.). BAC concentration in tubes inoculated with the isolates did not change significantly which implies that those isolates were not capable of BAC biotransformation. By this method bacteria that can degrade BAC was isolated from activated sludge and stored.

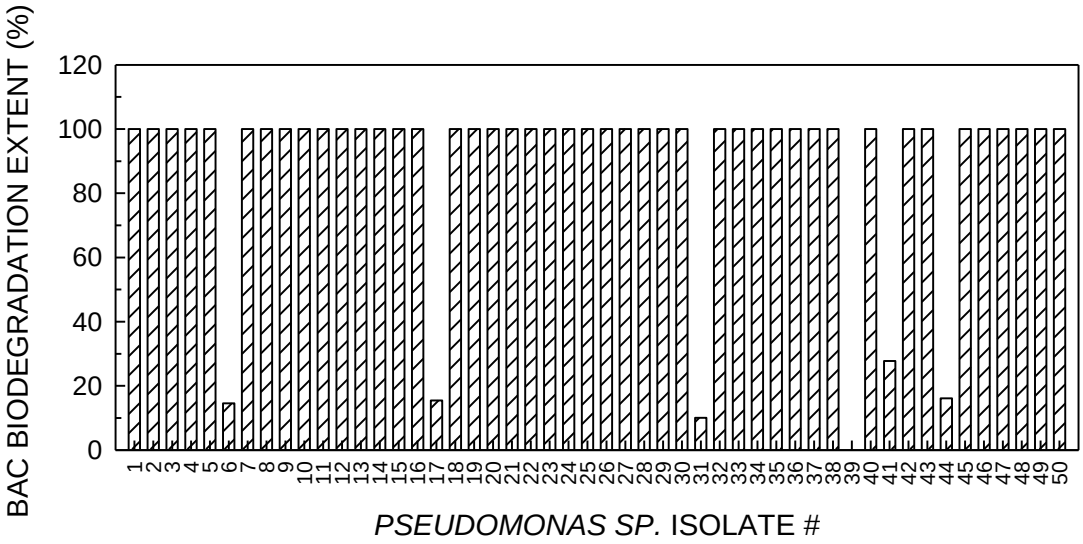


Figure 5.4. BAC degradation extent of the isolates obtained from BAC-degrading microbial community.

5.3.2. Identification of BAC Degraders Based on 16S rRNA Gene Sequence

The DNA of the above mentioned BAC degrading isolates was extracted and the concentration was measured by using Implen® P360 Nanophotometer. Because the average DNA concentration was around 118.43 ng/µL and the average A260/280 value was 1.89, it was continued directly with the PCR without the necessity of performing DNA purification. The 16S ribosomal DNA was amplified by PCR method and PCR products were run on 1% agarose gel (Figure 5.5.). Each 16S rDNA amplicon was digested with MspI endonuclease and restriction fragment length polymorphism (RFLP) patterns of each digest were visualized on 2% agarose gel (Figure 5.6.).

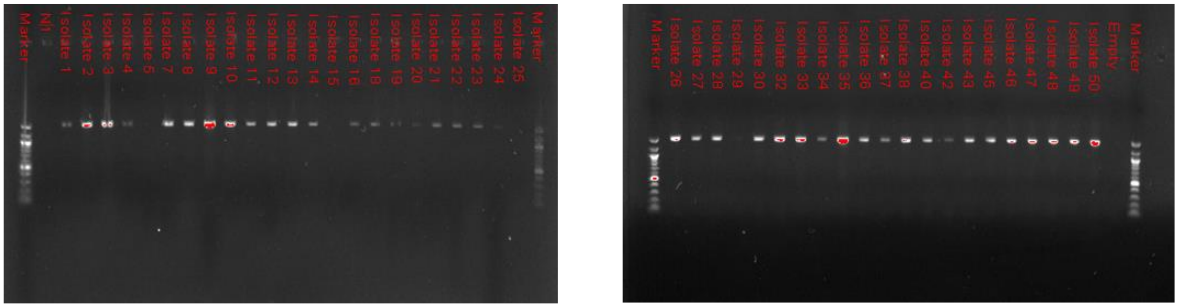


Figure 5.5. The image 16S rDNA amplicons of isolated strains on 1% agarose gel.

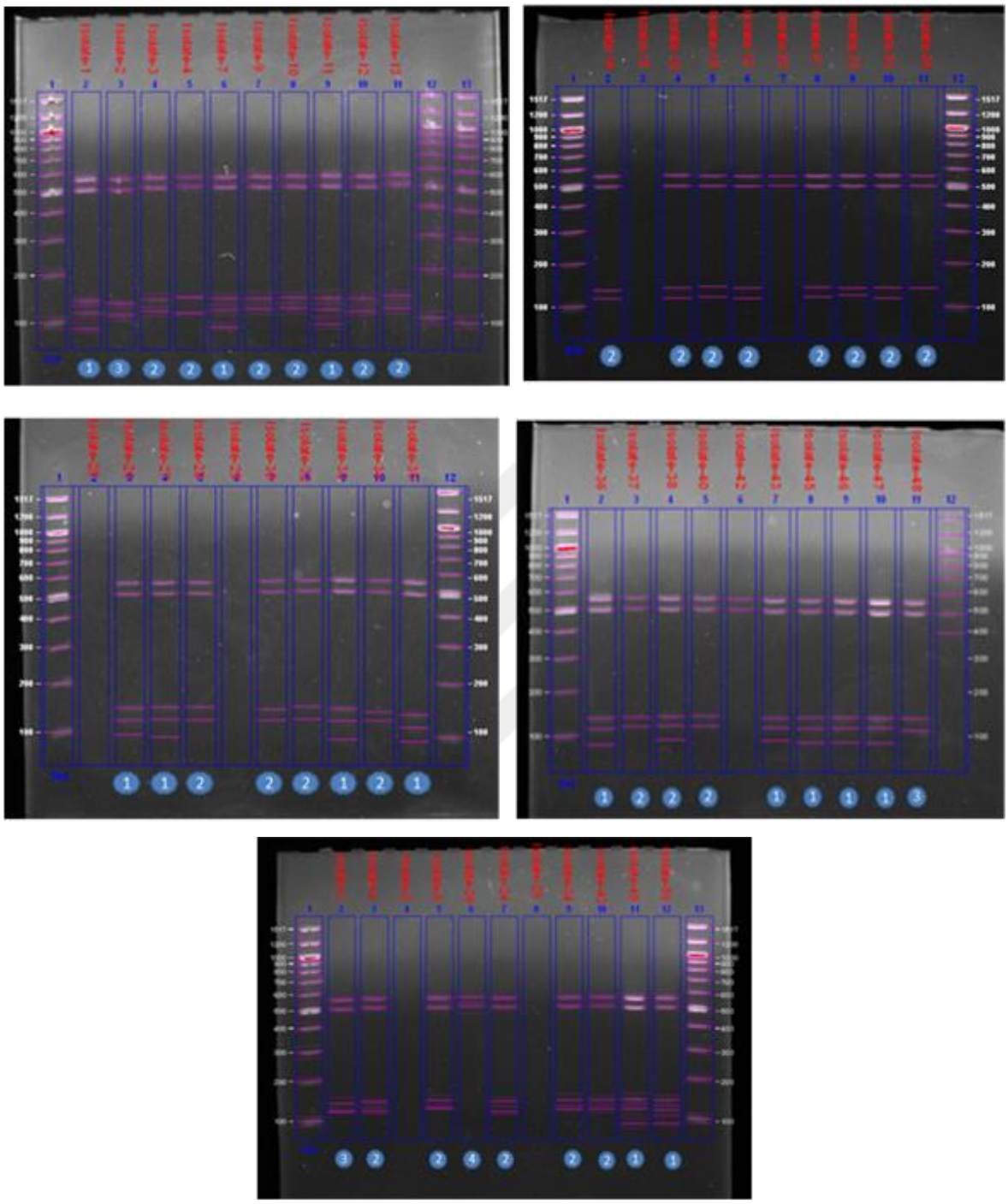


Figure 5.6. The 16S rDNA sequence of the restriction enzyme cut length distribution profiles of isolates.

When RFLP patterns of 16S rRNA gene of each strain were compared, they were similar to each other. Nevertheless, 30 of 50 16S rRNA amplicons were sequenced in MacroGen Inc. Europe.

16S rDNA sequence of each strain was aligned using MUSCLE algorithm and similarity between the sequences was calculated. Comparison showed that all sequenced 16S rDNA were similar to each other > 99.9%. (Table 5.1.) This result shows that BAC degrading *Pseudomonas sp.* isolated from activated sludge belongs to the same strain of *Pseudomonas sp.* which is identical to *Pseudomonas sp. BIOMIG1* reported by Ertekin et al. (2016).

Table 5.1. Comparison of the strains of 16S rDNA sequences obtained from this and previous study. (The numbers in the boxes indicates the % nucleotide similarity)

	AS-1	AS-2	AS-3	AS-4	AS-5	AS-6	AS-7	AS-8	AS-9	AS-10	AS-11	AS-12	AS-13	AS-14	AS-15
AS-1		100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-2	100		100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-3	100	100		100	100	100	100	100	100	100	100	100	100	99.9	100
AS-4	100	100	100		100	100	100	100	100	100	100	100	100	99.9	100
AS-5	100	100	100	100		100	100	100	100	100	100	100	100	99.9	100
AS-6	100	100	100	100	100		100	100	100	100	100	100	100	99.9	100
AS-7	100	100	100	100	100	100		100	100	100	100	100	100	99.9	100
AS-8	100	100	100	100	100	100	100		100	100	100	100	100	99.9	100
AS-9	100	100	100	100	100	100	100	100		100	100	100	100	99.9	100
AS-10	100	100	100	100	100	100	100	100	100		100	100	100	99.9	100
AS-11	100	100	100	100	100	100	100	100	100	100		100	100	99.9	100
AS-12	100	100	100	100	100	100	100	100	100	100	100		100	99.9	100
AS-13	100	100	100	100	100	100	100	100	100	100	100	100		99.9	100
AS-14	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9		99.9
AS-15	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	
AS-16	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-17	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-18	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.9
AS-19	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-20	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-21	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-22	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-23	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-24	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-25	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-26	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-27	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-28	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-29	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
AS-30	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100

	AS-16	AS-17	AS-18	AS-19	AS-20	AS-21	AS-22	AS-23	AS-24	AS-25	AS-26	AS-27	AS-28	AS-29	AS-30
AS-1	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-2	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-3	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-4	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-5	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-6	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-7	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-8	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-9	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-10	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-11	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-12	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-13	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-14	99.9	99.9	99.8	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9
AS-15	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-16		100	99.9	100	100	100	100	100	100	100	100	100	100	100	100
AS-17	100		100	100	100	100	100	100	100	100	100	100	100	100	100
AS-18	99.9	100		99.9	100	100	100	100	100	100	100	100	100	100	99.9
AS-19	100	100	99.9		100	100	100	100	100	100	100	100	100	100	100
AS-20	100	100	100	100		100	100	100	100	100	100	100	100	100	100
AS-21	100	100	100	100	100		100	100	100	100	100	100	100	100	100
AS-22	100	100	100	100	100	100		100	100	100	100	100	100	100	100
AS-23	100	100	100	100	100	100	100		100	100	100	100	100	100	100
AS-24	100	100	100	100	100	100	100	100		100	100	100	100	100	100
AS-25	100	100	100	100	100	100	100	100	100		100	100	100	100	100
AS-26	100	100	100	100	100	100	100	100	100	100		100	100	100	100
AS-27	100	100	100	100	100	100	100	100	100	100	100		100	100	100
AS-28	100	100	100	100	100	100	100	100	100	100	100	100		100	100
AS-29	100	100	100	100	100	100	100	100	100	100	100	100	100		100
AS-30	100	100	99.9	100	100	100	100	100	100	100	100	100	100	100	

When BIOMIG1's 16S rDNA sequence was queried at NCBI GenBank database, the closest (99% nucleotide similarity) bacterium to BIOMIG1 was identified as *Pseudomonas sp*. CMR21a (FJ652622). CMR21a is a *Pseudomonas* that was originally isolated from the root of the red cocoyam plant and it is capable of synthesizing phenazine and biological surface-active agents. In the literature, there is no report indicating that CMR12a can degrade BACs. (Perneel et al., 2007)

5.4. Summary

A BAC-degrading microbial community was developed from an activated sludge. This microbial community can utilize BACs (50 mg/L) within at most 7 days under aerobic conditions. Samples were taken from this microbial community and spread to a CHROM®Agar Pseudomonas™ plate and a LB-BAC plate. BAC biotransformation assay was done for each discrete colony that appeared on the agar plate and 44 out of 50 colonies could degrade BACs. As a result of 16S rDNA based molecular biology method it was found

that all of these isolates belong to the same strain which was then named *Pseudomonas sp.* BIOMIG1 AS. *Pseudomonas sp.* BIOMIG1 AS has the same sequence to *Pseudomonas sp.* BIOMIG1 SEW, a species which was isolated from sewage (Ertekin et al., 2016). These two *Pseudomonas* strains were found to be ecotypes.

6. ANTIBIOTIC RESISTANCE OF *PSEUDOMONAS SP.* BIOMIG1: A COMPARATIVE APPROACH

6.1. Introduction

Antimicrobial resistance is resistance of a microorganism to antimicrobials which was previously could treat it (WHO, 2011). Antimicrobial resistance is a major health threat that human society is facing today.

The methods that are used to determine antibiotic susceptibility or resistance of microorganisms differ (NCCLS 2012; BSAC 1991). Mostly used methods were dilution method, disk diffusion method and another diffusion technique called E-test method (NCCLS 2012; Davison et al., 2000). These tests are done by using different concentrations of antibiotics in either broth or agar culture media or on paper discs (Jorgensen and Ferraro, 1998). In paper disc diffusion test, disc containing agent is putting the Mueller Hinton plate after inoculation of plate with bacteria. Resistance is determined by measuring the inhibition zone diameter. If the zone diameter is greater than 21 mm, the microorganism reported as susceptible to tested antibiotic. If the zone diameter is less than 16 mm, the microorganism reported as resistant to tested antibiotic. If the zone diameter is in between these two values, microorganism reported as intermediate. Since it is a qualitative technique, after performing disk diffusion test one may need to do other test to determine resistance quantitatively. Minimum inhibitory concentration (MIC) is determined in order to measure the susceptibility of microorganism against antibiotics quantitatively. MIC is the lowest concentration of the antibiotic that inhibit the growth of bacteria. Micro and macro broth dilution, agar dilution and E-test methods can be used to find MIC of an antibiotic against one microorganism. In agar dilution method, the plates that are containing different concentration of antibiotics are inoculated. But this test generally is not preferred since it is labor intensive and time consuming method (Jiang. 2011). In broth microdilution test, a tray containing 96 wells inoculated with bacteria. A standard tray contains a range of 8 two-fold dilution of 12 antimicrobial agent. Although this method is not time consuming, one have to

stick with drug selections available in standard commercial trays (Jorgensen and Ferraro, 2009). E-test is another method to determine MIC of an antibiotic. Plastic E-test strips are containing a dried antibiotic concentration gradient. These strips are placed into a Mueller Hinton agar plate after plate is inoculated. The corresponding antibiotic concentration, at the point where cell growth diminished has been reported as MIC of an antibiotic. Generally results obtained from E-test method correlated with MICs determined by broth or agar dilution methods (Huang et al., 1992; Jorgensen et al., 1994; Citron et al., 1991; Baker et al., 1991; Rennie et al., 2008). Macro dilution is another method which is used to determine MIC of antibiotics. Macro dilution test is done in standard test tubes and the tubes contain a range of two-fold dilution of antibiotics are inoculated with isolate. The lowest concentration at which the isolate growth is completely inhibited is reported as MIC.

When the growth of isolate is not inhibited by the usually achievable concentrations of one antibiotic, the isolate called as resistant to that antibiotic (CLSI 2012). *Pseudomonas* spp. is considered one of the bacteria that show resistance to different antibiotic groups. Gajadhar et al. (2003) isolated *Pseudomonas* sp. from disinfectants/antiseptics used in hospitals. This *Pseudomonas* strain found to be resistant to ciprofloxacin, norfloxacin, tobramycin and gentamicin antibiotics. Agerso et al. (2005) reported that tetracycline resistant genes are cotransferred from *Pseudomonas* ssp. to *E.coli*. It was found that *Pseudomonas aeruginosa* have MexJK-OprM, MexCD-OprJ MexAB-OprM, MexEF-OprN, MexHI-OprD, MexXY-OprM and MexVW-OprM efflux pumps systems which provide resistance to ciprofloxacin, tetracycline, erythromycin and trimethoprim, β-lactams, aminoglycosides, clindamycin, floroquinolones. (Alekshun and Levy, 2007; Aeschlimann and Pharm, 2003; Morita et al., 2012; Alvarez-Ortega et al., 2011). It was reported that *P. aeruginosa* show resistance to β-lactams since it has ampC β-lactamase enzyme (Strateva and Yordonov, 2009). The structural changes in target enzymes and active efflux in *P. aeruginosa* were reported as two major mechanisms lead to fluoroquinolone resistance in *P. aeruginosa* (Hooper, 2001).

Moreover, it is believed that the main cause of antimicrobial resistance development is the exposure of microorganisms to biocides such as quaternary ammonium compounds (QACs) that are present at low concentrations in the environment (McBain et al., 2002). It

was suggested that QAC-induced resistance mechanisms also confer cross- and co-resistance to many antibiotics. (Buffet-Bataillon et al., 2012, Maillard, 2007; Gaze et al., 2005; Schluter et al., 2007). Loughlin et al. (2002) reported that adaptation *of Pseudomonas aeruginosa* to BACs cause increasing resistance against chloramphenicol.

The aim of the research presented in this chapter is to clarify the relationship between QACs resistance and antibiotic susceptibility by using different microorganisms having different tolerance to benzalkonium chlorides (BACs), which are the most extensively used QACs. To find the relationship, minimum inhibitory concentration (MIC) of 18 antibiotics belonging to different antibiotic groups for above mentioned bacteria was calculated and compared by using both macro dilution and E-test methods.

In order to find that relationship four microorganisms were used; a novel *Pseudomonas sp*., strain BIOMIG1 (BIOMIG1 SEW), *Serratia marcescens* strain BIOMIG4 and *E.coli* strain BIOMIG3. *Pseudomonas sp*., BIOMIG1 SEW and *Serratia marcescens* strain BIOMIG4 strains are tolerant to C_{12}BDMA-Cl, C_{14}BDMA-Cl and C_{16}BDMA-Cl (BACs) up to 1024 mg/L, whereas *E.coli* strain BIOMIG3 is susceptible to BACs (BACs MIC: 16 mg/L). In addition, BIOMIG1 SEW can degrade BACs under aerobic condition. *Pseudomonas sp*., strain BIOMIG1 AS isolated from activated sludge in the previous chapter of this study was found to be the ecotype of *Pseudomonas sp*., strain BIOMIG1 isolated from sewage. Also like BIOMIG1 SEW, *Pseudomonas sp*., strain BIOMIG1 AS shows resistance to BACs up to 1024 mg/L and it has the capability to degrade BACs. The phylogenetic relationship of these microorganisms are shown at Figure 6.1.

Tested antibiotics were selected according to the data set showing the global occurrence of pharmaceuticals in river waters prepared by Hughes et al. (2012) as: amoxicillin, azithromycin, penicillin g, chloramphenicol, ciprofloxacin, clarithromycin, clindamycin, doxycycline, enrofloxacin, erythromycin, kanamycin, levofloxacin, norfloxacin, ofloxacin, sulfamethoxazole, tetracycline, trimethoprim and vancomycin.

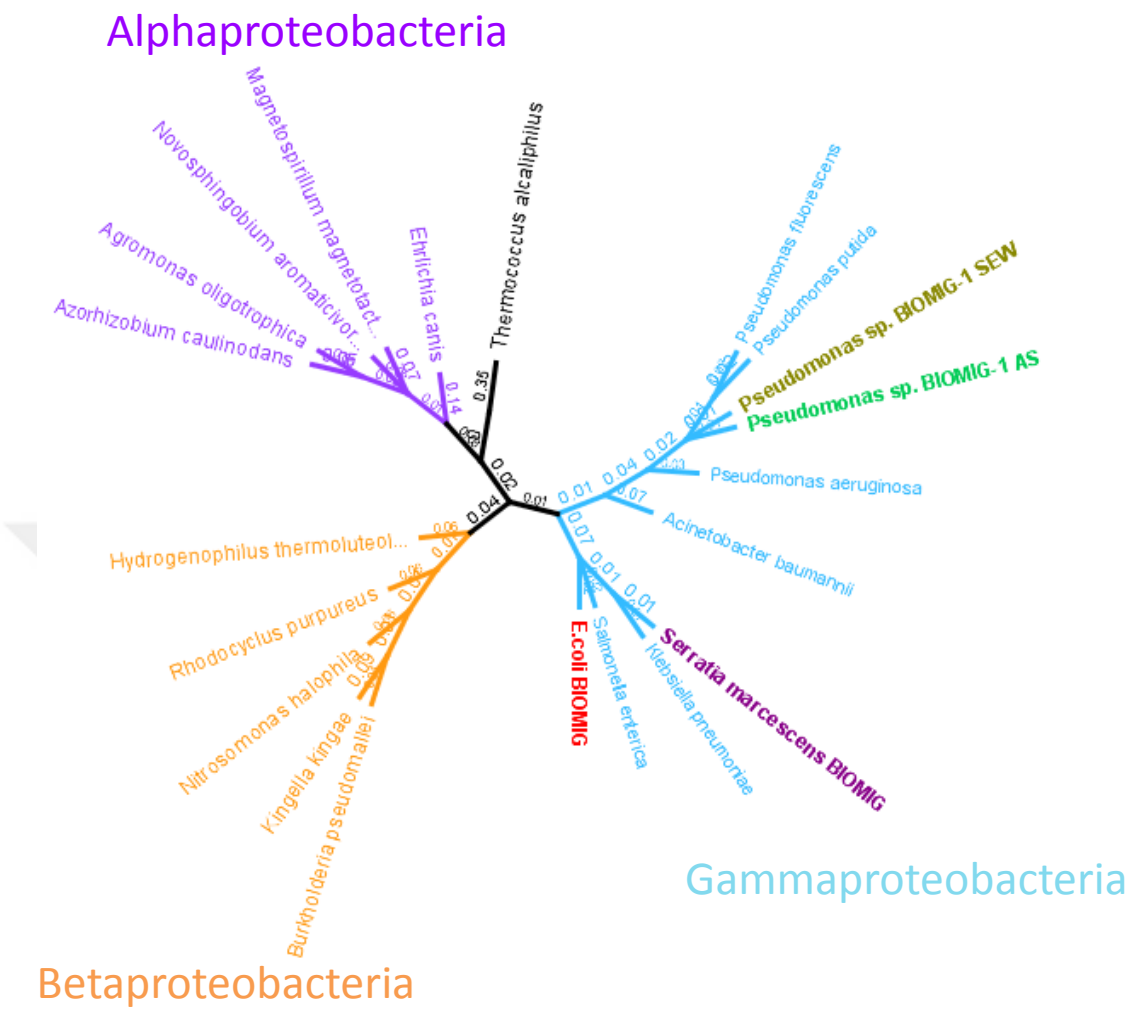


Figure 6.1. The phylogenetic relationship of 3 different types of bacteria (*Pseudomonas sp.* BIOMIG1, *E.coli* BIOMIG3 and *Serratia marcescens* BIOMIG4) that susceptibility measured against 18 antibiotic.

6.2. Materials and Methods

6.2.1. Susceptibility Testing by Macro Dilution Method

The susceptibilities of each microorganism against 18 antibiotics were determined using macro dilution assay as described by Clinical and Laboratory Standards Institute (CLSI 2012). A single colony of each microorganism was transferred into sterile falcon tube containing 5 mL Mueller-Hinton broth. Colonies were taken from CHROM®Agar Orientation plates prepared as described in 4.2.2. After microorganism were grown overnight in Mueller-Hinton broth, they were diluted in Mueller-Hinton broth to a turbidity comparable to that of a 0.5 McFarland turbidity standard (c.a. 0.5 x 10^8 CFU/mL). This suspension was further diluted 1:100 with Mueller-Hinton broth. A 1 mL of the diluted culture sample was transferred to culture tubes containing 1 mL broth and a range of antibiotics concentrations from 1 to 1024 mg/L (Antibiotics is diluted by factor 2) (Figure 6.2.). The tubes were incubated at room temperature (22 °C) for 24 hours and the growth measured with a UV/Vis spectrometer at 600 nm wavelength. Tubes containing nutrient broth having the same antibiotics concentrations but without any culture were used as blanks.

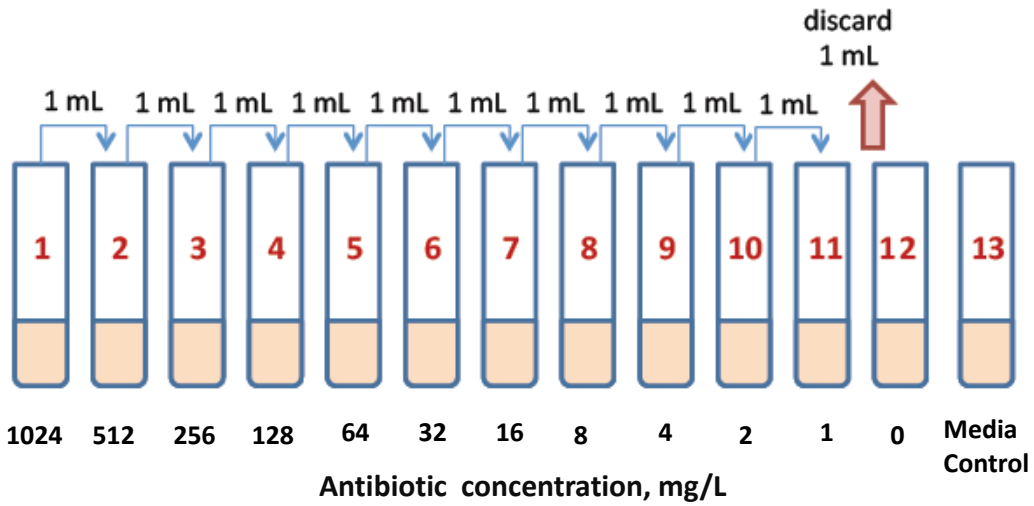


Figure 6.2. Preparation of susceptibility testing tubes using serial dilution.

6.2.2. Presentation of Data and Calculation of MICs

The susceptibility of each microorganism against 18 antibiotics was tested using macro dilution assay as described by Clinical and Laboratory Standards Institute (CLSI 2012). The minimum inhibitory concentrations (MICs) for each antibiotic was calculated by first calculating the relative optical density. The relative optical density was calculated for each set by dividing growth measurement results into the growth measurement result of the tube containing culture without antibiotic. By doing this, the difference between the growth rates of bacteria was eliminated. Then Berkeley Madonna software and Runge-Kuttl method was used according to the below equation 1. In the equation OD denotes relative optical density, C denotes antibiotic concentration. Since 1024 mg/L was the highest antibiotic concentration present in solutions, a homogeneous growth on test tube has been reported as greater than 1024 mg/L (> 1024 mg/L).

(Eq. 1);

d/dt (OD) = -a*(R-C) 1

a = IF C < Inh THEN 0 ELSE b
R = IF C >= MIC THEN C ELSE MIC
b = 0.000001
Inh = 100
MIC = 1500
INIT OD = 1

6.2.3. Susceptibility Testing by E-Test Method

The susceptibility of each microorganism against 18 antibiotics was determined using E-Test assay as described by Clinical and Laboratory Standards Institute (CLSI 2012). A single colony of each microorganism was transferred into sterile falcon tube containing 5 mL Mueller-Hinton broth. Colonies were taken from CHROM®Agar Orientation plates prepared as described in 4.2.2. After microorganism were grown overnight in Mueller-

Hinton broth, they were diluted in Mueller-Hinton broth to a turbidity comparable to that of a 0.5 McFarland turbidity standard (c.a. 0.5 x 10^8 CFU/mL). This suspension was further diluted 1:100 with Mueller-Hinton broth. Optimally, within 15 minutes after adjusting turbidity of inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swap was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This removed excess inoculum from the swab. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure repeated by streaking two more times, rotating the plate approximately 60 ° each time to ensure an even distribution of inoculum. Once the agar plate was completely dry, the antibiotic strip (E-Test strip) was applied aseptically with the help of sterile tweezer and plates were incubated overnight at 22 °C. After 24 hrs of incubation, plates were photographed by using BioRad EZ-DOC imager with Image Lab program. The corresponding antibiotic concentration, at the point where no cell growth was observed on E-Test strips, has been reported as the minimum inhibitory concentration of antibiotic (MIC).

6.3. Results and Discussion

6.3.1. Macro Dilution Assay

Macro dilution assay results were shown in Figure 6.3. In Figure 6.3, numbers denotes microorganisms and letter denotes antibiotics. While circles that are shown in the graph indicate the results of macro dilution, curved lines belong to curve fitting results which were obtained by Berkeley Madonna software. In these graphs, x-axis is showing antibiotic concentration and y-axis is showing relative optical density. In most of the graphs, curved lines become horizontal after a particular point related to increasing antibiotic concentration. That point was taken as minimum inhibitory concentration (MIC) for the particular microorganism which indicates that at higher concentrations than MIC, microorganism do not show any growth. The MIC was also calculated by Eq. 1. The susceptibility of four microorganisms which are *Pseudomonas sp.* BIOMIG1 AS, *Pseudomonas sp.* BIOMIG1 SEW, *E.coli*, *Serratia marcescens* against 18 antibiotic were reported in terms of MIC (mg/L) in Table 6.1.

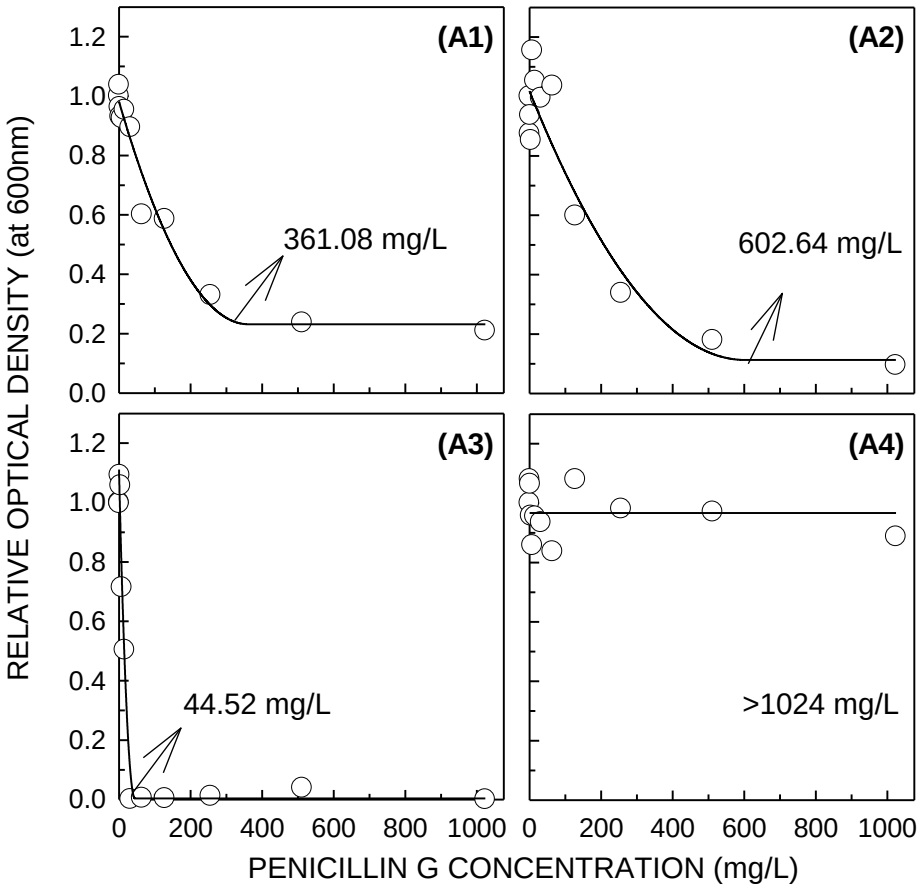
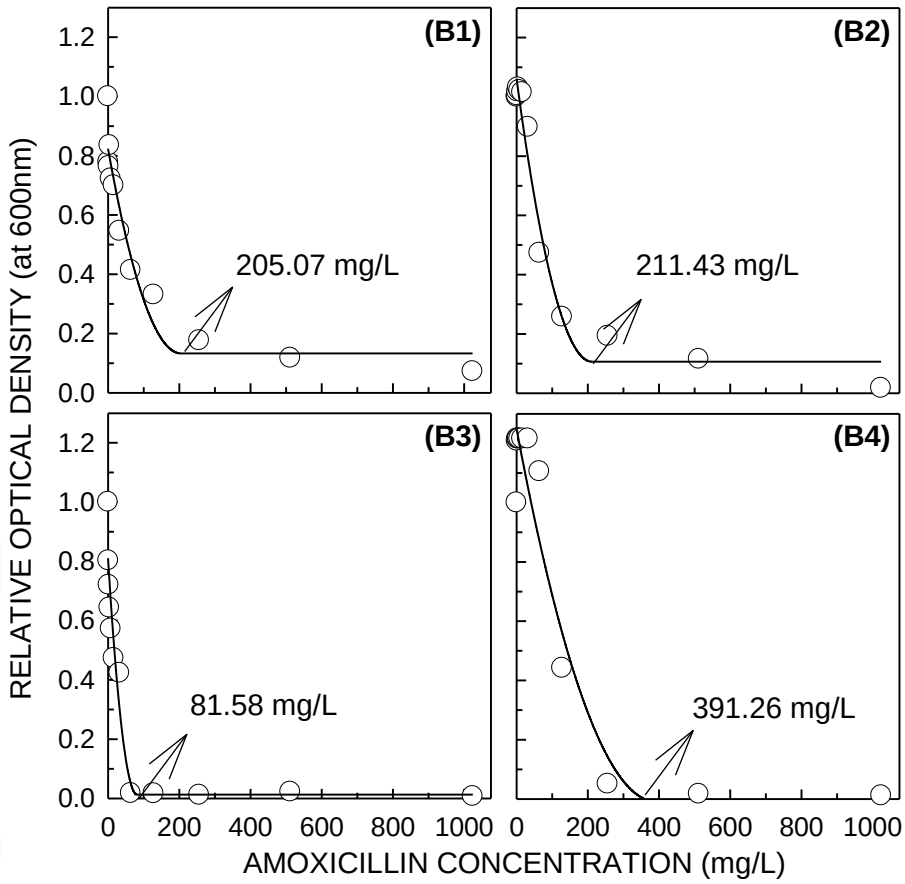


Figure 6.3. MIC of (A) penicillin g, (B) amoxicillin, (C) clindaycin, (D) vancomycin, (E) enrofloxacin, (F) norfloxacin, (G) ofloxacin, (H) levofloxacin , (I) ciprofloxacin, (J) chloramphenicol, (K) erythromycin, (L) azithromycin, (M) clarithromycin, (N) kanamycin, (O) tetracycline, (P) doxycycline, (Q) sulfamethoxazole and (R) trimethoprim against (1) *Pseudomonas sp.* BIOMIG1 SEW, (2) *Pseudomonas sp.* BIOMIG1 AS, (3) *E.coli* and (4) *Serratia marcescens.*



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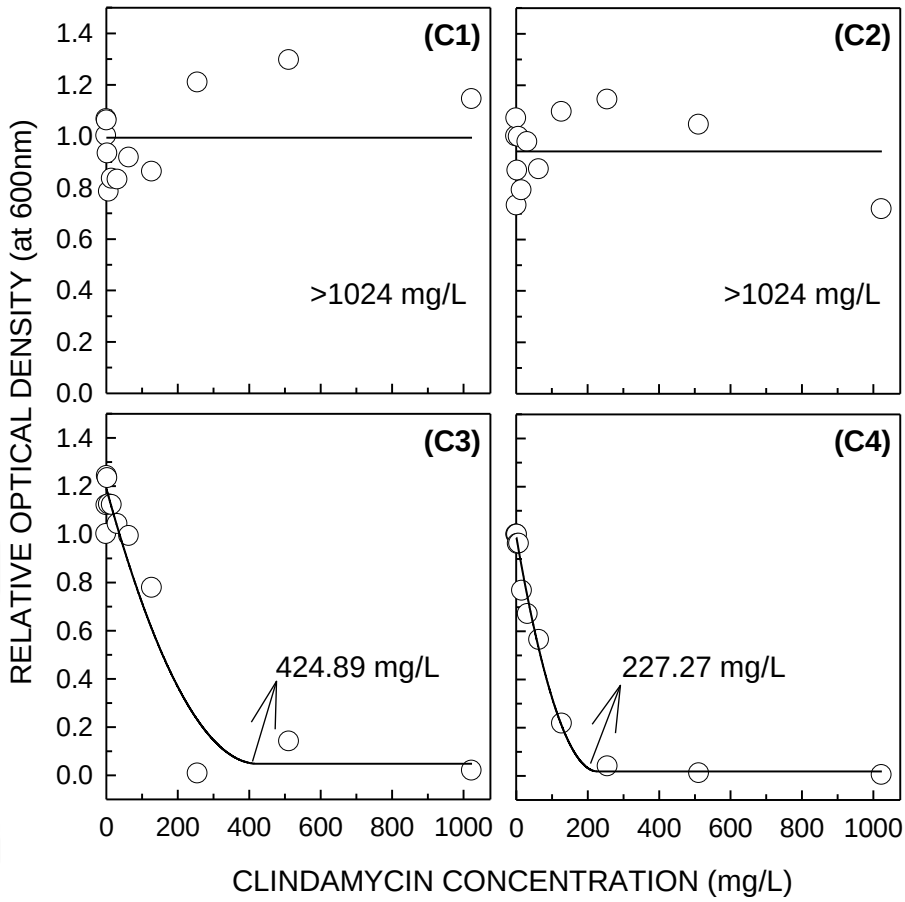

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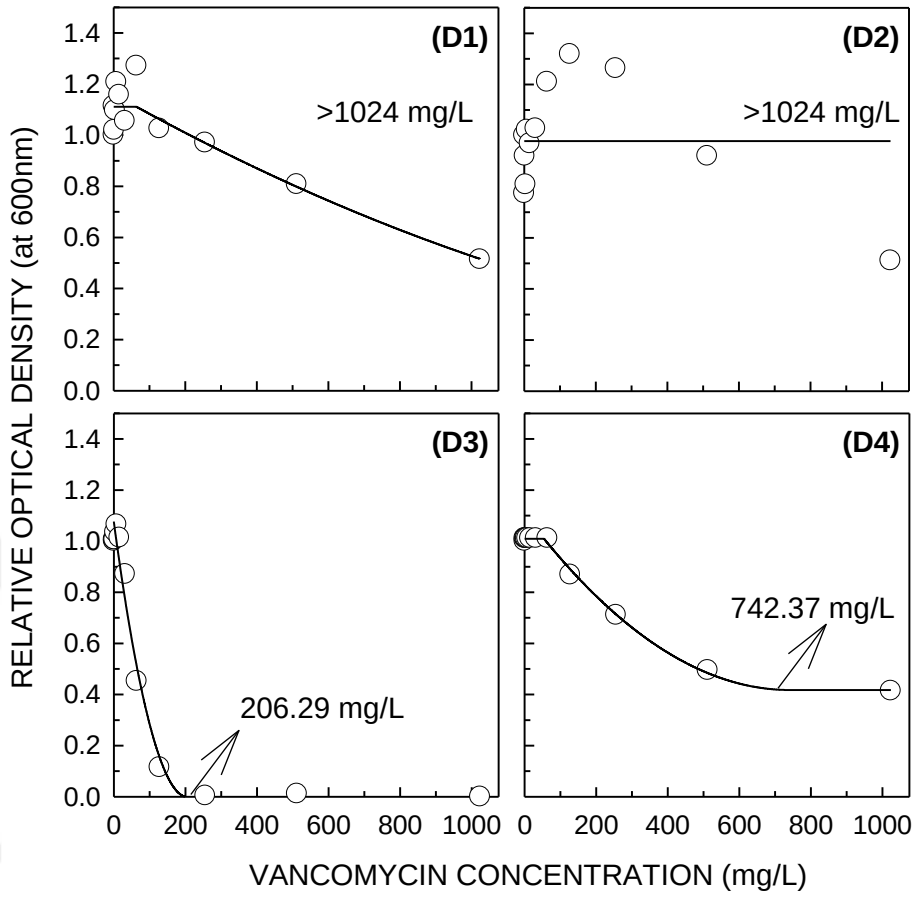

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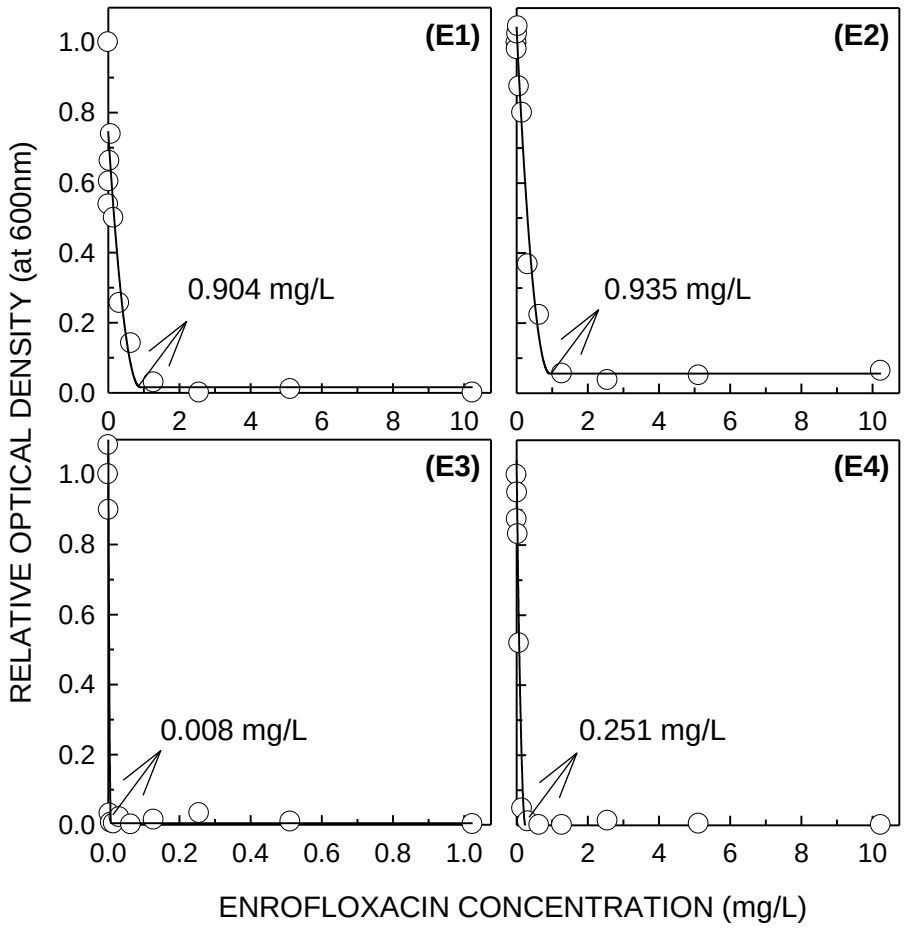

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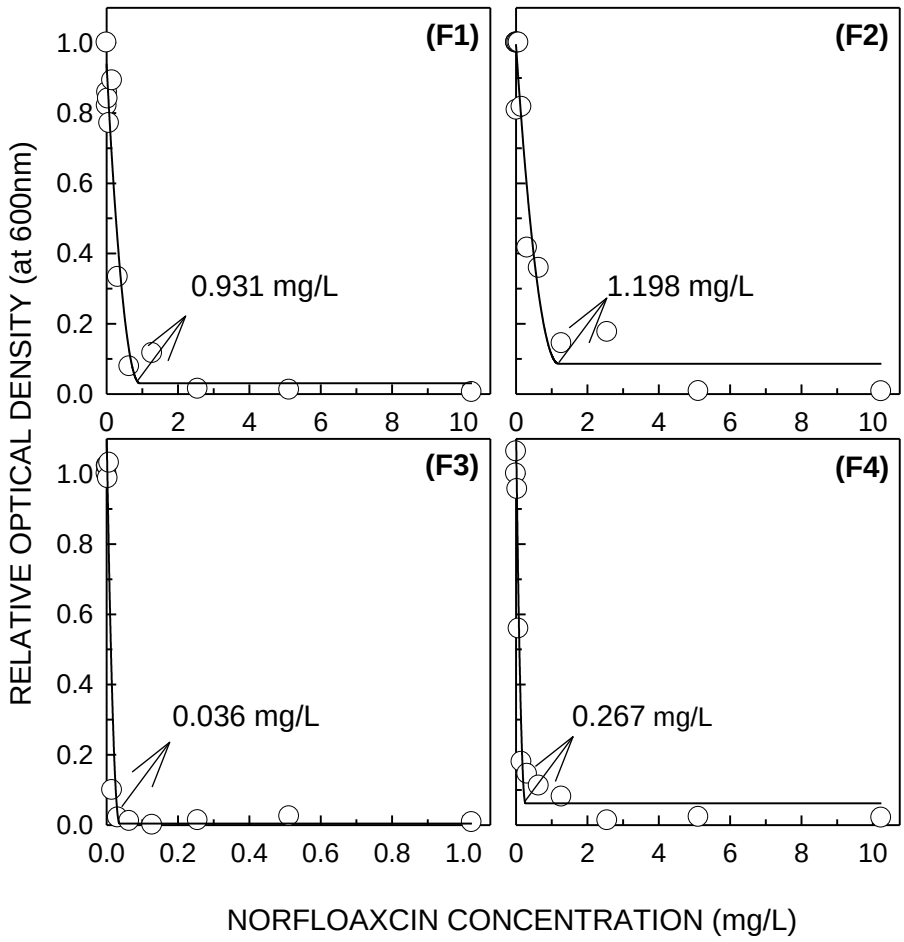

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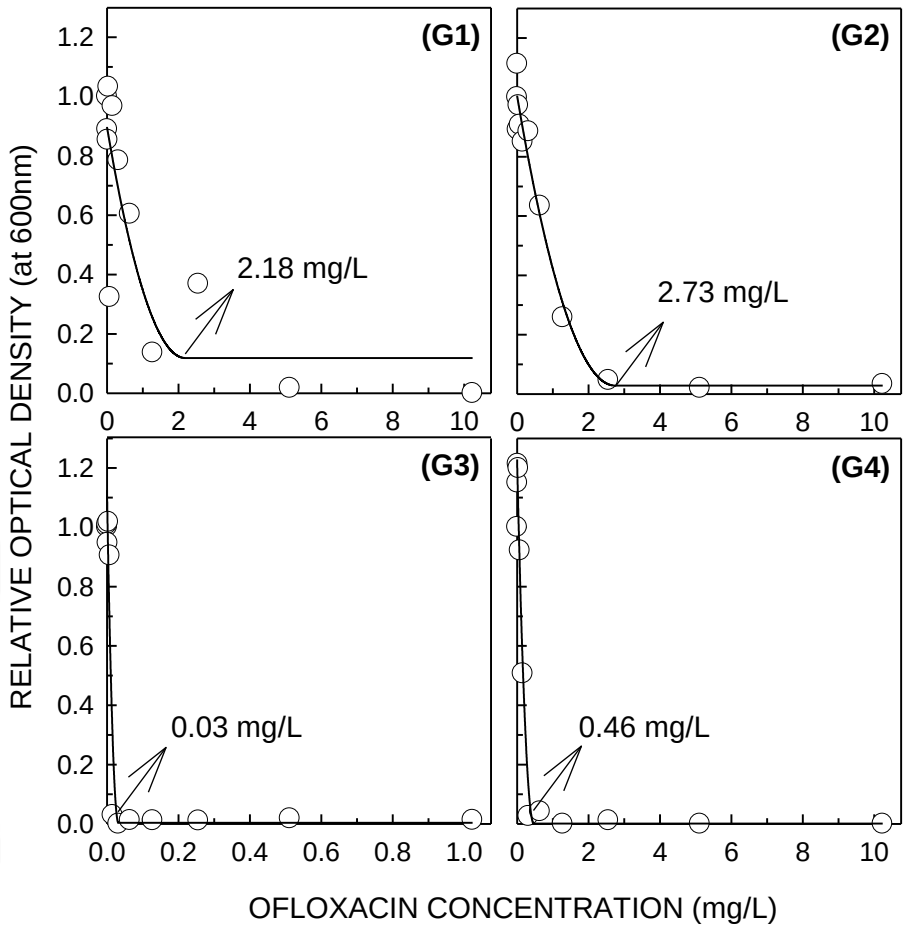

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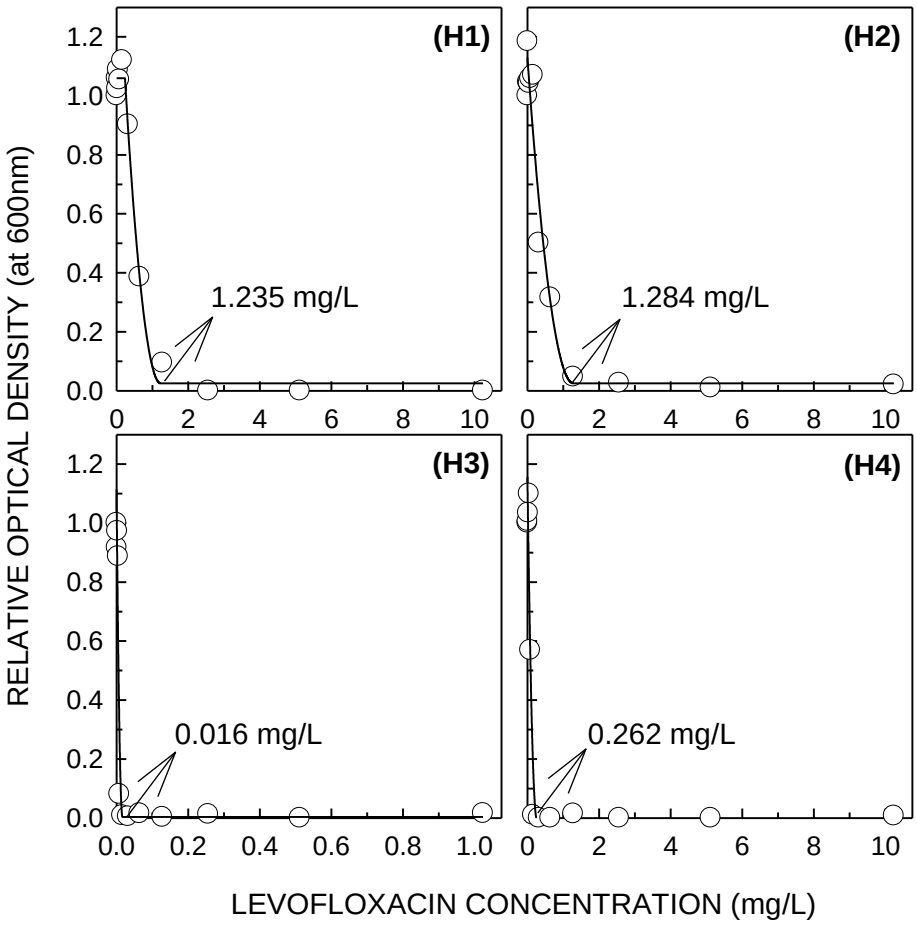

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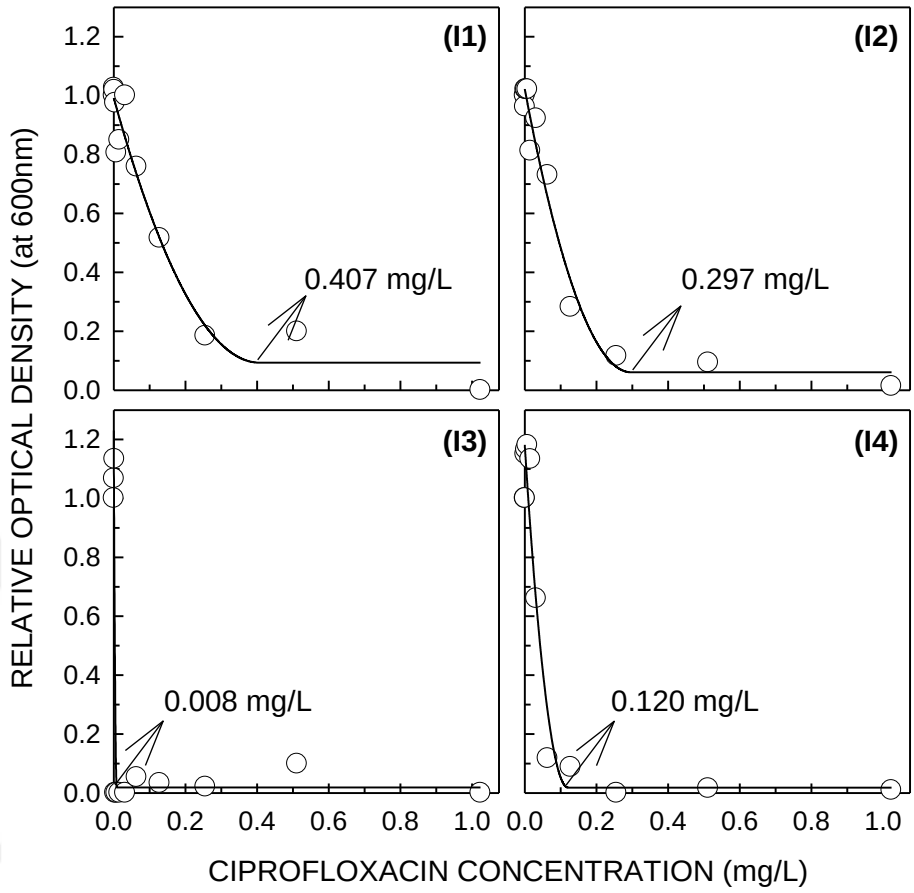

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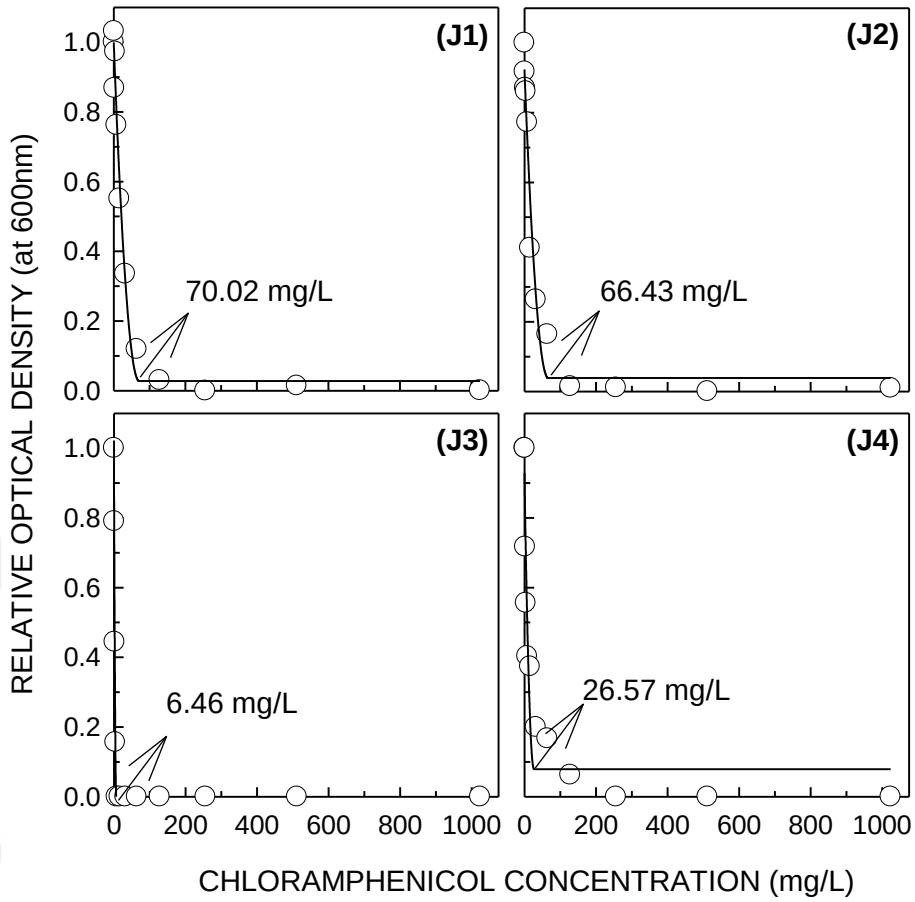

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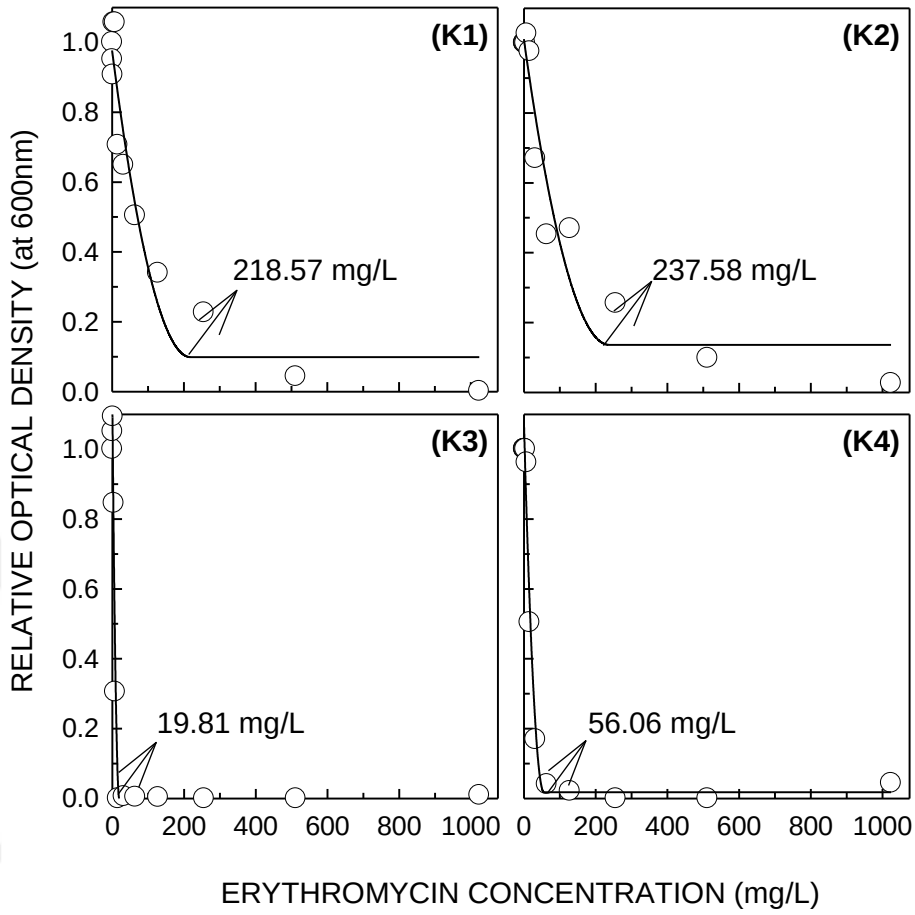

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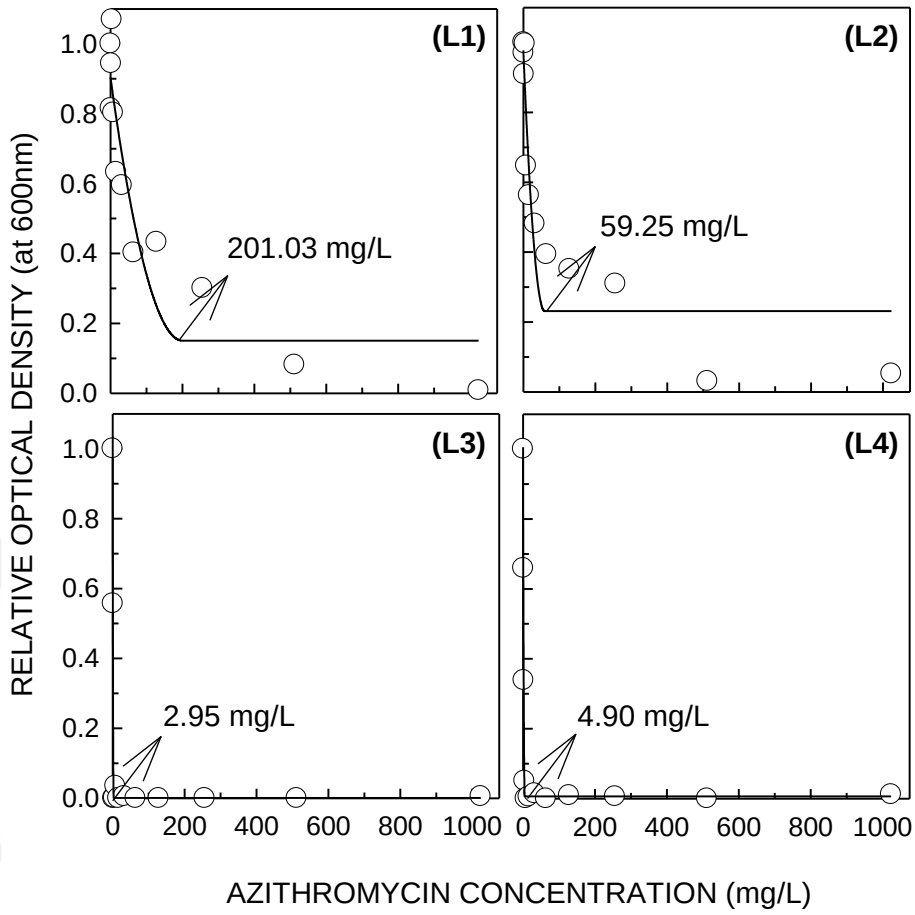

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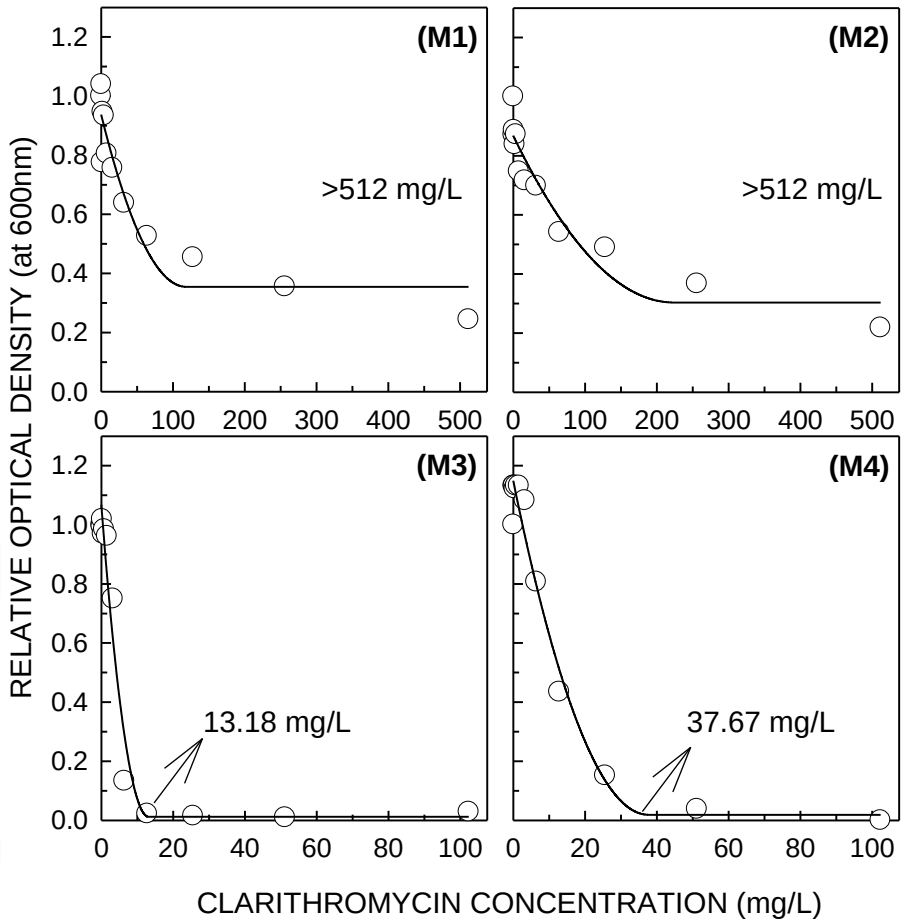

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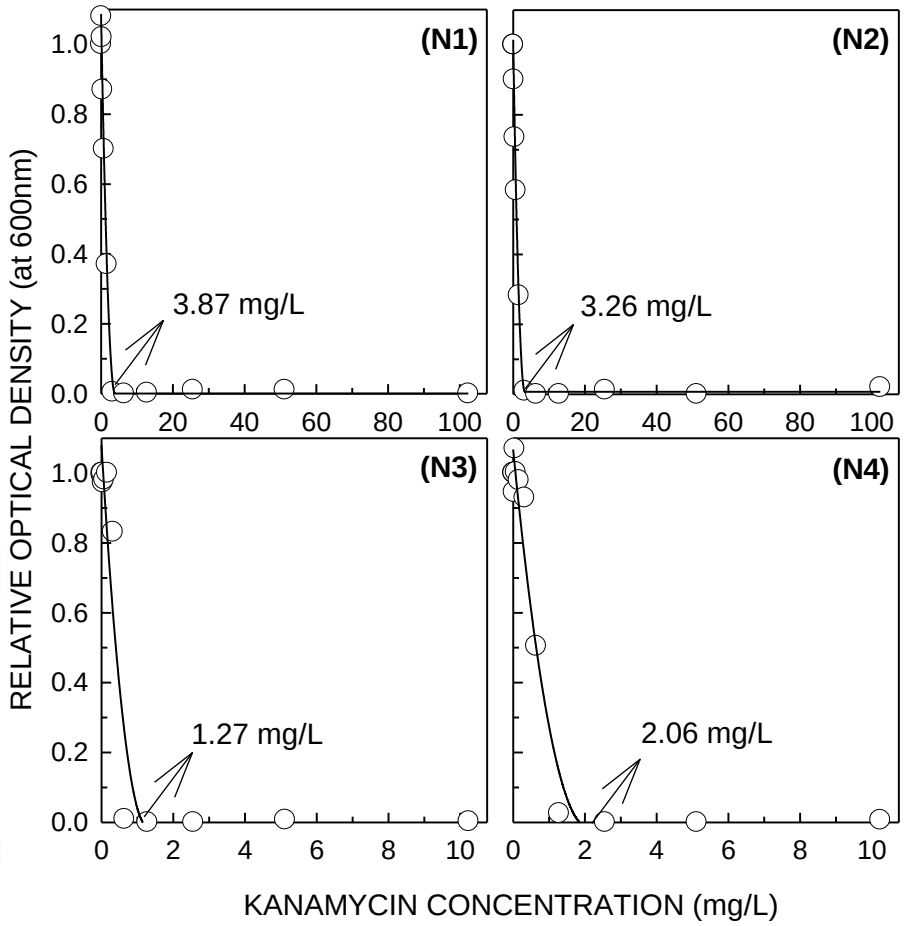

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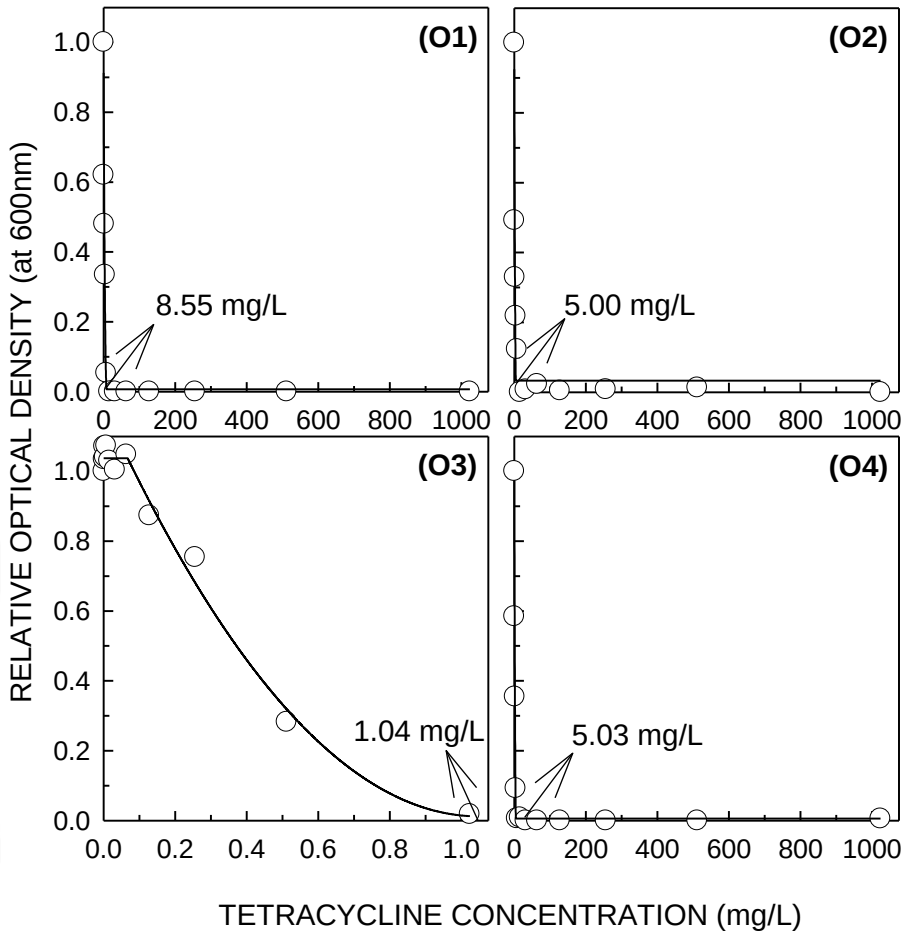

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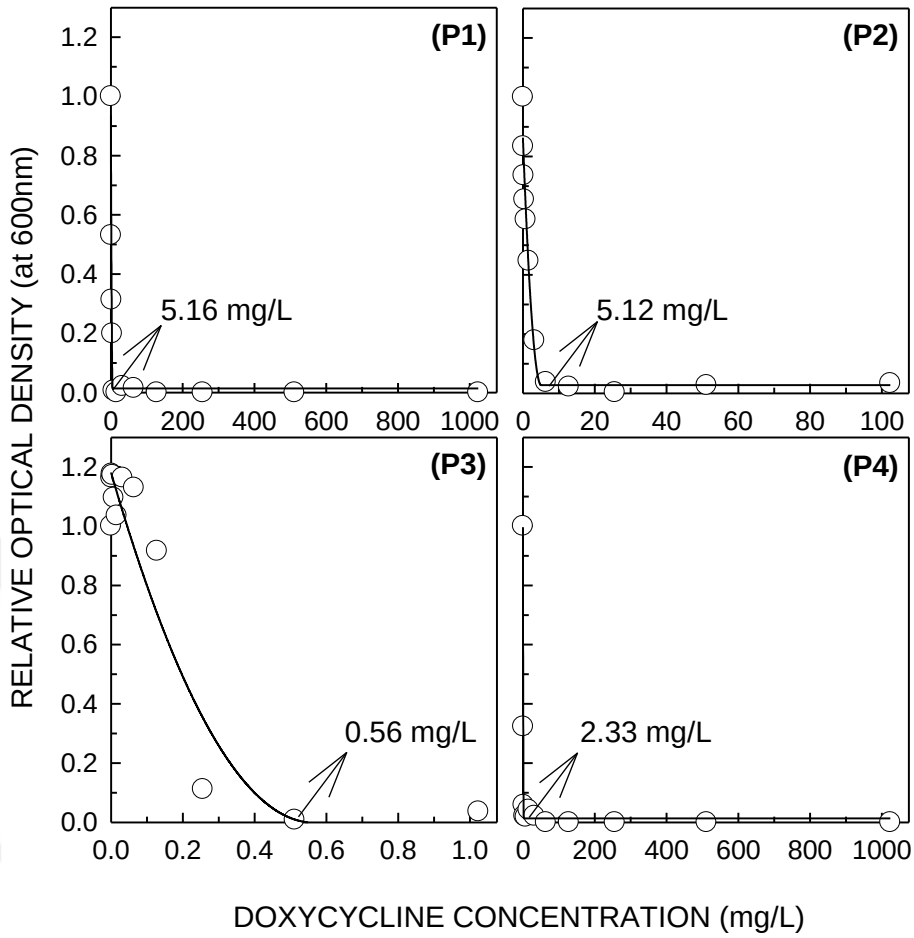

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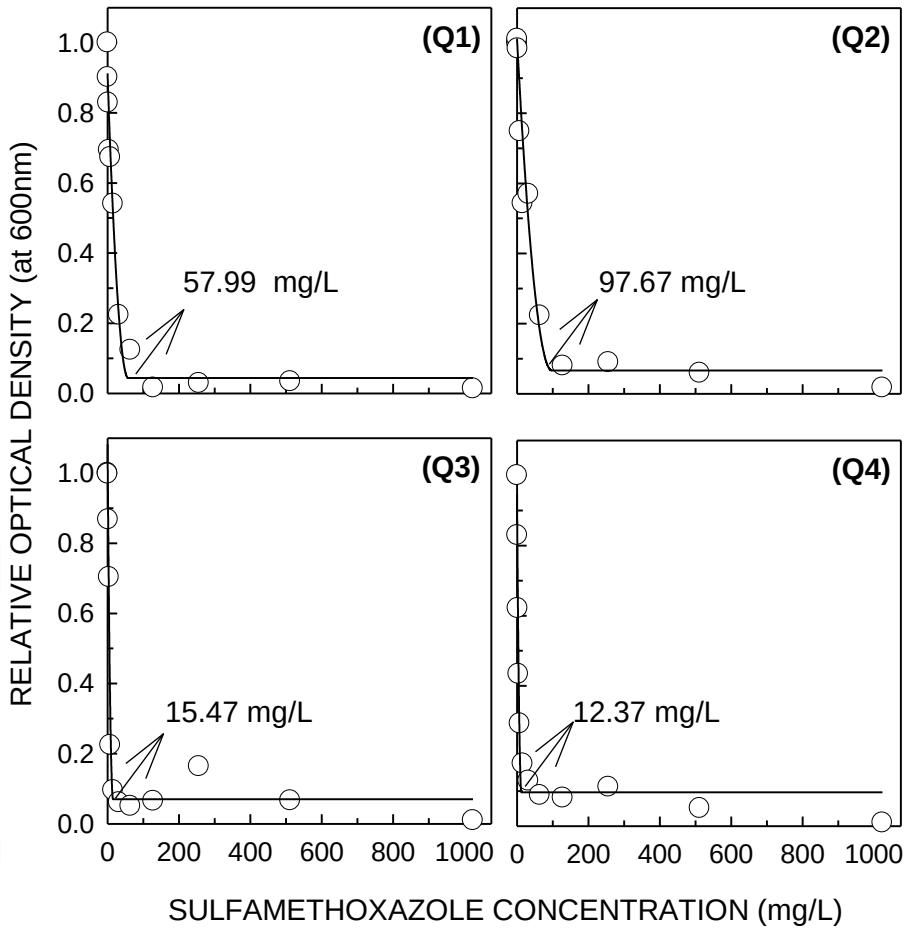

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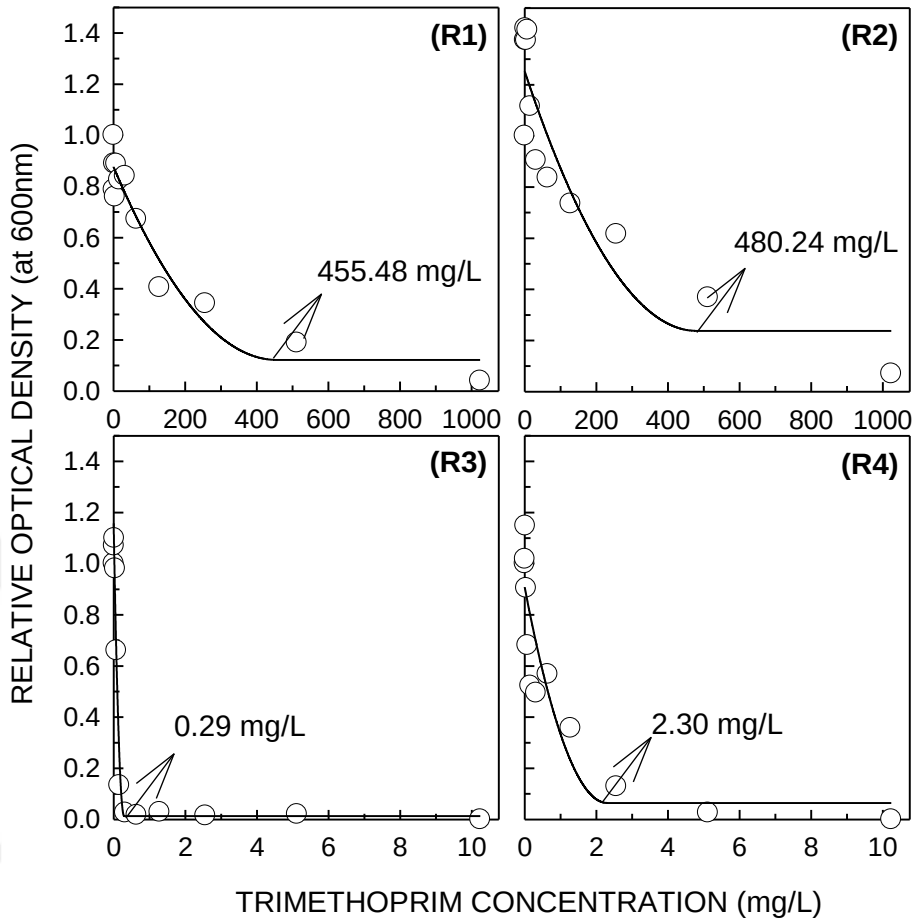


Figure 6.3. Continued.

In Figure 6.3. A1, the point that curved line turns to straight line correspond to 361.08 mg/L BAC concentration. That concentration is *Pseudomonas sp.* BIOMIG1 SEW's MIC for penicillin g. In Figure 6.3. A2, the MIC of *Pseudomonas sp.* BIOMIG1 AS is 602.64 mg/L. In Figure 6.3. A3, it is seen that after 44.52 mg /L penicillin g concentration relative optical density of *E.coli* was decreased sharply. In Figure 6.3. A4, it is seen that *Serratia marcescens'* relative optical density is not decreased with increasing penicillin g concentration. That's why the MIC of *Serratia marcescens* for penicillin g was reported as >1024 mg /L BAC concentration. Inhibitory effect of penicillin g on the growth of microorganisms in descending order as follows; *E.coli*, *Pseudomonas sp.* BIOMIG1 SEW, *Pseudomonas sp.* BIOMIG1 AS and *Serratia marcescens* (Figure 6.3. A). Inhibition effect

of amoxicillin on the growth of microorganisms followed same trend as for penicillin g (Figure 6.3. B).

Clindamycin did not show inhibitory effect on both *Pseudomonas sp.* BIOMIG1 SEW and *Pseudomonas sp.* BIOMIG1 AS (MIC >1024 mg/L) and its effect on the growth of microorganisms in descending order as follows; *Serratia marcescens, E.coli, Pseudomonas sp.* BIOMIG1 AS and *Pseudomonas sp.* BIOMIG1 SEW (Figure 6.3. C). Vancomycin also did not show inhibitory effect on both *Pseudomonas sp.* BIOMIG1 SEW and *Pseudomonas sp.* BIOMIG1 AS (MIC >1024 mg/L) like clindamycin (Figure 6.3. D).

For fluoroquinolone group, macro dilution assays were repeated in between 0-10 mg/L antibiotic concentration for *Serratia marcescens, Pseudomonas sp.* BIOMIG1 AS and *Pseudomonas sp.* BIOMIG1 SEW and it was done in between 0-1 mg/L antibiotic concentration for *E.coli*, except from ciprofloxacin. For ciprofloxacin macro dilution assays were repeated in between 0-1 mg/L for all microorganisms since ciprofloxacin was very effective against all of the MOs. These experiments were repeated with low concentrations in order to detect MIC values precisely. When we were examine the effect of fluoroquinolone group of antibiotics; enrofloxacin (Figure 6.3. E), norfloxacin (Figure 6.3. F), ofloxacin (Figure 6.3. G), levofloxacin (Figure 6.3. H) and ciprofloxacin (Figure 6.3. I) on microorganisms, we can see same trends. The effect of fluoroquinolones against *Pseudomonas sp.* BIOMIG1 AS and *Pseudomonas sp.* BIOMIG1 SEW is less than *Serratia marcescens* and these antibiotics were more effective against *E.coli.*

Inhibition effect of chloramphenicol on the growth of microorganisms in descending order as follows; *Pseudomonas sp.* BIOMIG1 SEW, *Pseudomonas sp.* BIOMIG1 AS, *Serratia marcescens* and *E.coli* (Figure 6.3. J).

When we were examine the effect of macrolide group of antibiotics; erythromycin (Figure 6.3. K), azithromycin (Figure 6.3. L) and clarithromycin (Figure 6.3. M), microorganisms, we can see same trends. Macrolides more than 10 fold effective against *Serratia marcescens* and *E.coli* then it was effective against *Pseudomonas sp.* BIOMIG1s.

Since we had to use DMSO (Dimethyl sulfoxide) to dissolve clarithromycin, macro dilution assays for *Pseudomonas sp.* BIOMIG1s were done in between 0-512 mg/L antibiotic concentration. This was done in order to eliminate the inhibitory effect of DMSO on microorganisms.

For kanamycin, macro dilution assays were repeated in between 0-100 mg/L antibiotic concentration for *Pseudomonas sp.* BIOMIG1 AS and *Pseudomonas sp.* BIOMIG1 SEW and it was done in between 0-10 mg/L antibiotic concentration for *E.coli* and *Serratia marcescens.* These experiment were repeated with low concentrations in order to detect MIC values precisely. Inhibition effect of kanamycin on the growth of microorganisms in descending order as follows; *Pseudomonas sp.* BIOMIG1 SEW, *Pseudomonas sp.* BIOMIG1 AS, *Serratia marcescens* and *E.coli* (Figure 6.3. N).

For tetracycline group, macro dilution assay was repeated in between 0-10 mg/L antibiotic concentration for *E.coli.* These experiment were repeated with low concentrations in order to detect MIC values precisely. Inhibition effect of tetracycline and doxycycline on the growth of microorganisms in descending order as follows; *Pseudomonas sp.* BIOMIG1 SEW, *Pseudomonas sp.* BIOMIG1 AS, *Serratia marcescens* and *E.coli* (Figure 6.3. O and P).

Inhibition effect of sulfamethoxazole on the growth of microorganisms in descending order as follows; *Pseudomonas sp.* BIOMIG1 AS, *Pseudomonas sp.* BIOMIG1 SEW, *E.coli* and *Serratia marcescens* (Figure 6.3. Q).

For trimethoprim, macro dilution assay was repeated in between 0-10 mg/L antibiotic concentration for *E.coli.* These experiment were repeated with low concentrations in order to detect MIC values precisely. Inhibition effect of trimethoprim on the growth of microorganisms in descending order as follows; *Pseudomonas sp.* BIOMIG1 AS, *Pseudomonas sp.* BIOMIG1 SEW, *Serratia marcescens* and *E.coli* (Figure 6.3. R)

Table 6.1. MIC values of 4 microorganisms against 18 antibiotics according to macro dilution method.

MACRO DILUTION METHOD				
ANTIBIOTIC NAME	ISOLATES MIC(mg/L)			
	BIOMIG1 SEW	BIOMIG1 AS	*E.coli*	*Serratia marcescens*
Penicillin G	361.09	602.64	44.53	>1024
Amoxicillin	205.08	211.44	81.58	391.27
Ciprofloxacin	0.41	0.30	0.01	0.12
Enrofloxacin	0.90	0.94	0.01	0.25
Norfloxacin	0.93	1.20	0.04	0.27
Ofloxacin	2.19	2.74	0.03	0.47
Levofloxacin	1.24	1.28	0.02	0.26
Sulfamethoxazole	58.00	97.68	15.47	12.38
Trimethoprim	455.48	480.24	0.29	2.30
Tetracycline	8.56	5.01	1.05	5.04
Doxycycline	5.17	5.12	0.56	2.34
Clarithromycin	>512	>512	13.19	37.67
Erythromycin	218.57	237.59	19.82	56.06
Azithromycin	201.04	360.42	2.95	4.91
Clindamycin	>1024	>1024	424.90	227.28
Kanamycin	3.87	3.27	1.27	2.06
Vancomycin	>1024	>1024	206.29	742.37
Chloramphenicol	70.02	66.43	6.47	26.58

6.3.2. E-Test

The susceptibilities of each microorganism against 18 antibiotics were determined also by using E-Test method. The corresponding antibiotic concentration at the point that no cell growth observed on E-Test strips has been reported as the minimum inhibitory concentration of antibiotic (MIC). Since 256 mg/L was the highest antibiotic concentration present in the E-test strips, a homogeneous growth on MH agar has been reported as greater than 256 mg/L (> 256 mg/L). The photographs that were obtained by using BioRad EZ-DOC imager with Image Lab program were shown in Figure 6.4-6.7. The corresponding antibiotic concentration at the point that no cell growth observed on E-Test strips has been

reported as the minimum inhibitory concentration of antibiotic (MIC) and susceptibilities of each of the four microorganisms against 18 antibiotic were reported in terms of Minimum Inhibitory Concentration (MIC mg/L) in Table 6.2.

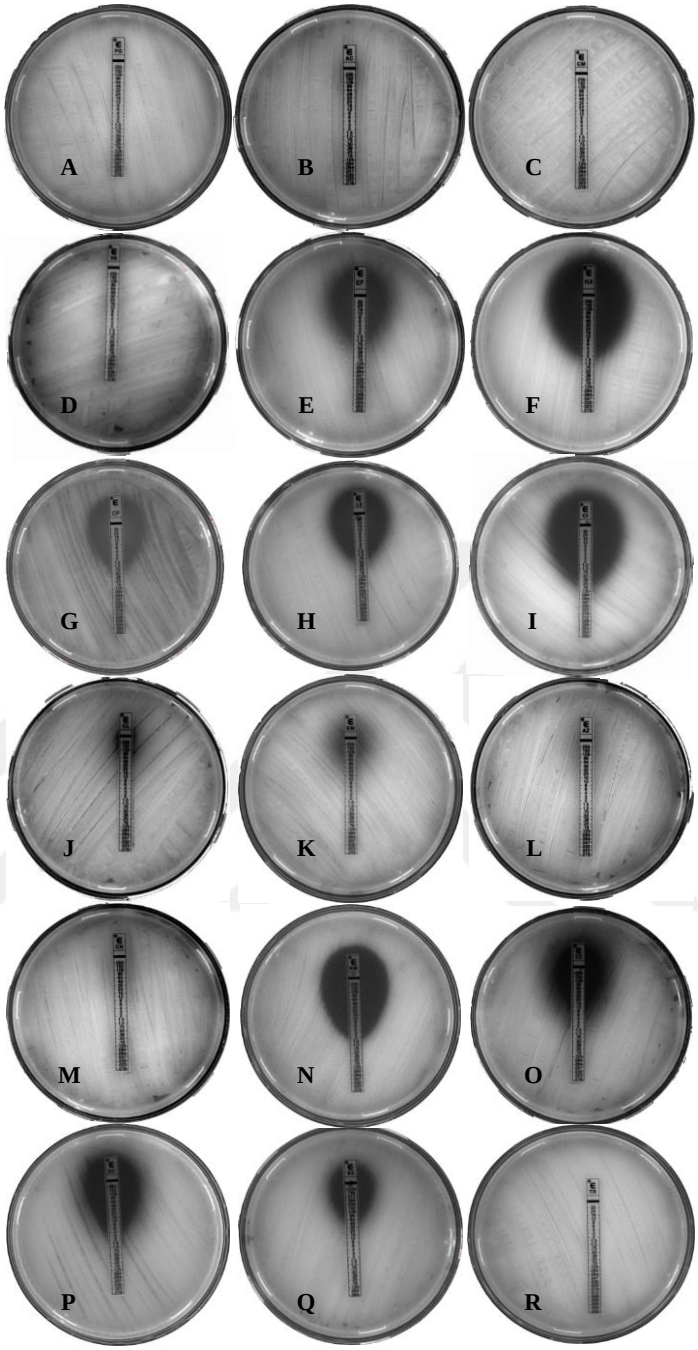


Figure 6.4. The pictures of E-test strips placed on MH agar for *Pseudomonas sp.* BIOMIG1 SEW (A) penicillin G, (B) amoxicillin, (C) clindaycin, (D) vancomycin, (E) enrofloxacin, (F) norfloxacin, (G) ofloxacin, (H) levofloxacin , (I) ciprofloxacin, (J) chloramphenicol, (K) erythromycin, (L) azithromycin, (M) clarithromycin, (N) kanamycin, (O) tetracycline, (P) doxycycline, (Q) sulfamethoxazole and (R) trimethoprim.

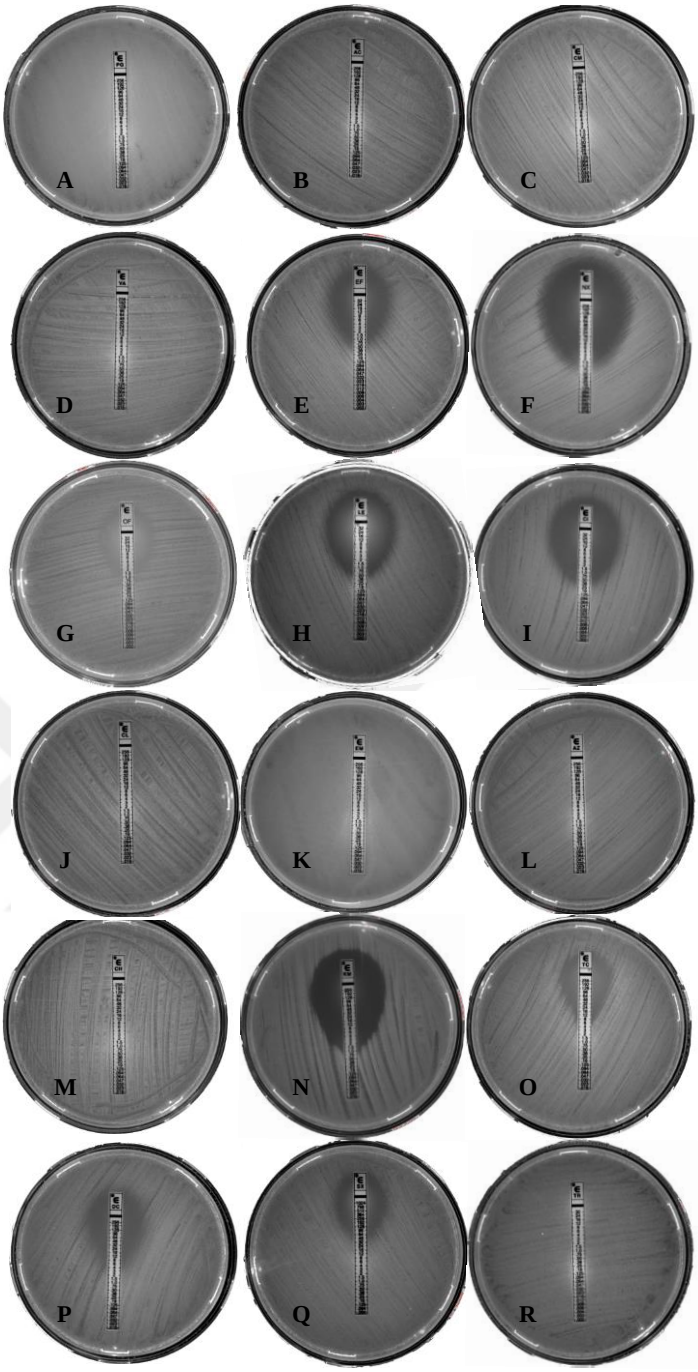


Figure 6.5. The pictures of E-test strips placed on MH agar for *Pseudomonas sp.* BIOMIG1 AS (A) penicillin g, (B) amoxicillin, (C) clindaycin, (D) vancomycin, (E) enrofloxacin, (F) norfloxacin, (G) ofloxacin, (H) levofloxacin , (I) ciprofloxacin, (J) chloramphenicol, (K) erythromycin, (L) azithromycin, (M) clarithromycin, (N) kanamycin, (O) tetracycline, (P) doxycycline, (Q) sulfamethoxazole and (R) trimethoprim.

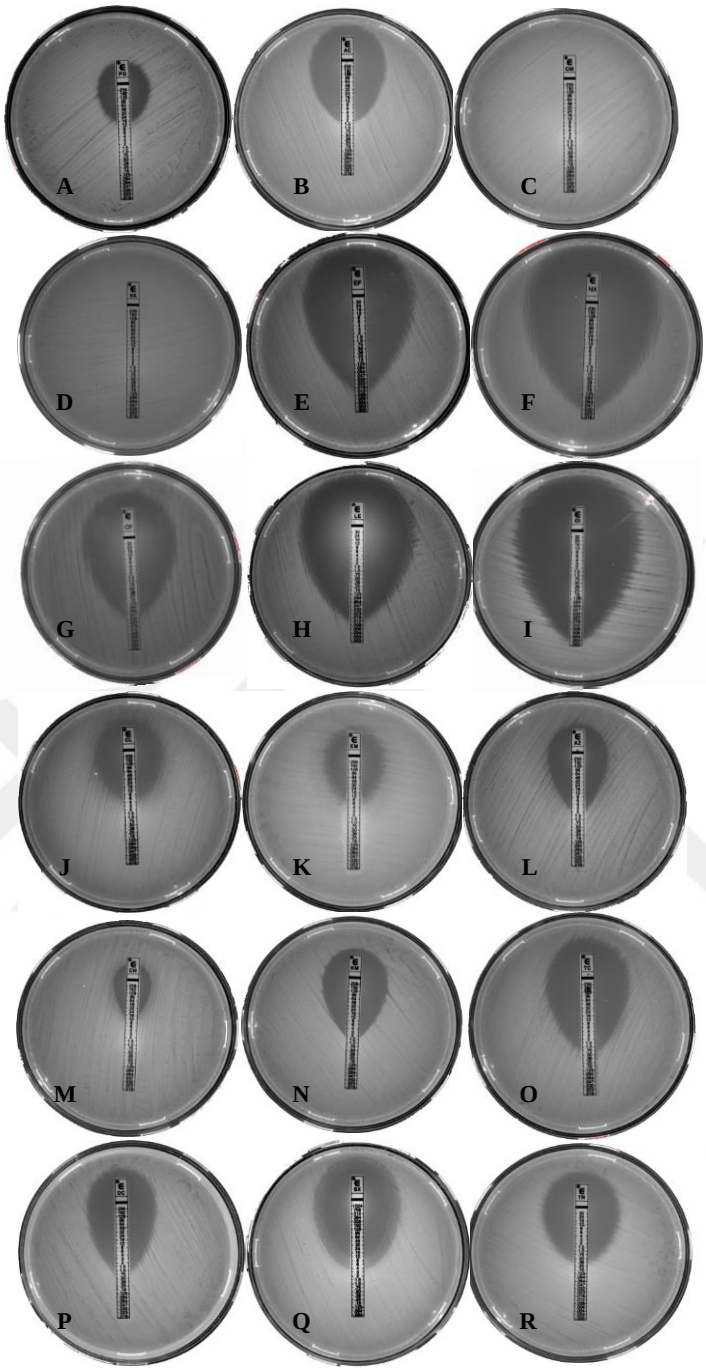


Figure 6.6. The pictures of E-test strips placed on MH agar for *E.coli* (A) penicillin g, (B) amoxicillin, (C) clindaycin, (D) vancomycin, (E) enrofloxacin, (F) norfloxacin, (G) ofloxacin, (H) levofloxacin , (I) ciprofloxacin, (J) chloramphenicol, (K) erythromycin, (L) azithromycin, (M) clarithromycin, (N) kanamycin, (O) tetracycline, (P) doxycycline, (Q) sulfamethoxazole and (R) trimethoprim.

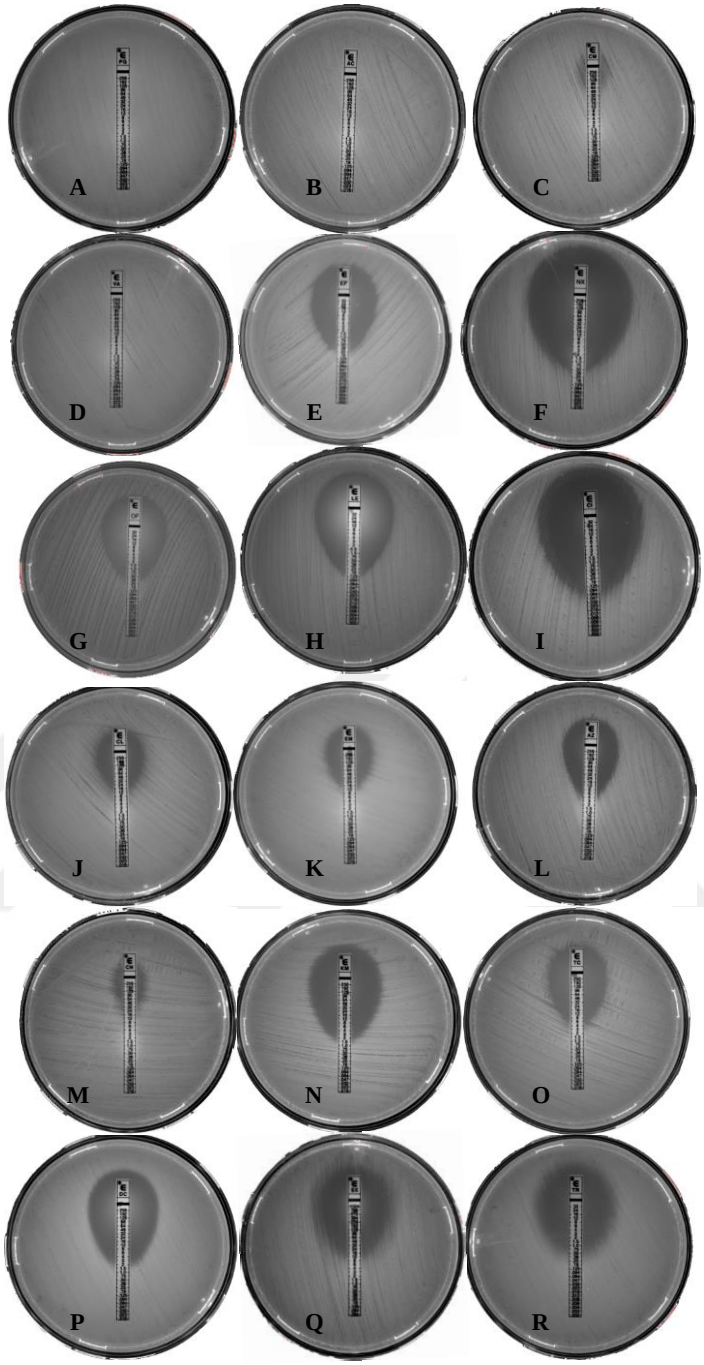


Figure 6.7. The pictures of E-test strips placed on MH agar for *Serratia marcescens* (A) penicillin g, (B) amoxicillin, (C) clindaycin, (D) vancomycin, (E) enrofloxacin, (F) norfloxacin, (G) ofloxacin, (H) levofloxacin , (I) ciprofloxacin, (J) chloramphenicol, (K) erythromycin, (L) azithromycin, (M) clarithromycin, (N) kanamycin, (O) tetracycline, (P) doxycycline, (Q) sulfamethoxazole and (R) trimethoprim.

MIC results obtained by using E-test method that were shown in Figure 6.4-6.7 were examined for each antibiotic for each MO and E-test results were compared with the results that were obtained by using macro dilution method in below paragraphs.

While penicillin group of antibiotics (penicillin g and amoxicillin) inhibited growth of *E.coli*, they did not show any inhibitory effect on other microorganisms. MIC values that were obtained from E-test method were slightly lower than results of macro dilution method.

While clindamycin inhibited growth of *Serratia marcescens* slightly, it did not show inhibitory effect on other microorganisms up to 256 mg/L antibiotic concentration. According to both macro dilution and E-test method, *Serratia marcescens* was found to be the most susceptible bacteria to clindamycin.

When we examined the effect of fluoroquinolone group of antibiotics; enrofloxacin, norfloxacin, ofloxacin, levofloxacin and ciprofloxacin on microorganisms, we can see same trends. The effect of fluoroquinolones against *Pseudomonas sp.* BIOMIG1 AS and *Pseudomonas sp.* BIOMIG1 SEW is less than *Serratia marcescens* and these antibiotics were more effective against *E.coli* same trend was found by using macro dilution method.

Inhibition effect of chloramphenicol on the growth of microorganisms in descending order has been found to be the same as macro dilution results; *Pseudomonas sp.* BIOMIG1 AS, *Pseudomonas sp.* BIOMIG1 SEW, *Serratia marcescens* and *E.coli.*

When we examined the effect of macrolide group of antibiotics; erythromycin, azithromycin and clarithromycin, microorganisms, we can see same trends that we saw in macro dilution method. Macrolides more than 10 fold effective against *Serratia marcescens* and *E.coli* then it was effective against *Pseudomonas sp.* BIOMIG1s.

Inhibition effect of kanamycin on the growth of microorganisms was almost the same for all microorganisms. . The MIC values were found to be very similar that has been found by using macro dilution method.

Inhibition effect of tetracycline and doxycycline on the growth of microorganisms in descending order has been found to be the same as macro dilution results; *Pseudomonas sp.* BIOMIG1 SEW, *Pseudomonas sp.* BIOMIG1 AS, *Serratia marcescens* and *E.coli*.

Inhibition effect of sulfamethoxazole on the growth of microorganisms in descending order as follows; *Pseudomonas sp.* BIOMIG1 AS, *Pseudomonas sp.* BIOMIG1 SEW, *Serratia marcescens* and *E.coli.*

While trimethoprim inhibited growth of *Serratia marcescens* and *E.coli*, it did not show any inhibitory effect on *Pseudomonas sp.* BIOMIG1 AS and *Pseudomonas sp.* BIOMIG1 SEW. The MIC values for *Serratia marcescens* and *E.coli* were found to be very similar that has been found by using macro dilution method.

Table 6.2. MIC values of 4 microorganisms against 18 antibiotics according to E-test method.

E-TEST METHOD				
ANTIBIOTIC NAME	ISOLATES MIC(mg/L)			
	BIOMIG 1-SEW	BIOMIG 1-AS	*E.coli*	*Serratia marcescens*
Penicillin G	>256	>256	16	>256
Amoxicillin	>256	>256	2	>256
Ciprofloxacin	0.25	0.38	0.003	0.064
Enrofloxacin	1	0.75	0.016	0.25
Norfloxacin	1.5	1	0.03	0.38
Ofloxacin	1	1.5	0.047	0.38
Levofloxacin	0.75	0.5	0.02	0.19
Sulfamethoxazole	48	64	8	12
Trimethoprim	>32	>32	0.38	0.75
Tetracycline	8	6	0.75	6
Doxycycline	4	4	0.75	2
Clarithromycin	>256	>256	16	48
Erythromycin	192	>256	8	16
Azithromycin	>256	>256	3	4
Clindamycin	>256	>256	>256	192
Kanamycin	2	1	1.5	1.5
Vancomycin	>256	>256	>256	>256
Chloramphenicol	192	>256	6	16

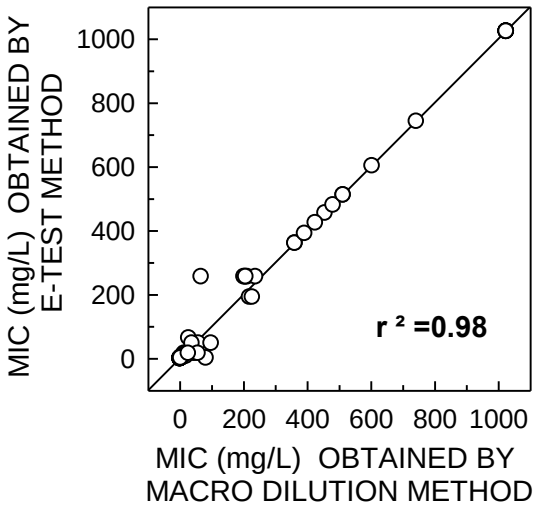


Figure 6.8. The comparison of MIC values obtained by macro dilution method and E-test method.

The MIC values that were obtained by E-test method were compared with the MIC values that were obtained by macro dilution method (Figure 6.8.). The r^2 value was found to be 0.98 which indicated that MIC values obtained by E-test method and macro dilution method is very similar.

6.3.2.1. Relative Tolerance of *Pseudomonas sp.* BIOMIG1 to Antibiotics

Results show that BIOMIG1s was around 10 fold resistant than BAC susceptible *E.coli* BIOMIG3 and more than 2 fold resistant than BAC resistant *Serratia marcescens* BIOMIG4 for penicillin g, amoxicillin, enrofloxacin, norfloxacin, ofloxacin, levofloxacin, ciprofloxacin, trimethoprim, erythromycin, azithromycin, clarithromycin and chloramphenicol. For the rest of the antibiotics; sulfamethoxazole, kanamycin, vancomycin, tetracycline, doxycycline and clindamycin, BIOMIG1s was more than 2 fold resistant than BAC susceptible *E.coli* BIOMIG3. BIOMIG1s were found to be the most resistant bacteria to all of the antibiotics except from amoxicillin and penicillin g. *Serratia marcescens* BIOMIG4 was found to be the most resistant bacteria to penicillin g and amoxicillin.

When the MIC results were examined according to antibiotic groups, it can be seen that microorganisms have higher MIC values for penicillin g, amoxicillin, clindamycin and vancomycin than they have for other antibiotics. Amoxicillin, penicillin g, clindamycin and vancomycin antibiotics were group together since all microorganism have high MIC values for these antibiotics. After them, macrolides, chloramphenicol, sulfonamides, kanamycin and tetracyclines have high MIC values respectively. Whereas all microorganisms show very low MIC values for fluoroquinolone group of antibiotics; ciprofloxacin, enrofloxacin, norfloxacin, ofloxacin and levofloxacin.

Moreover, the MIC values for *Pseudomonas sp.* BIOMIG1 SOIL, *Pseudomonas putida* BIOMIG2 VD and BIOMIG1^{N} were measured. *Pseudomonas sp.* BIOMIG1 SOIL is an ecotype of *Pseudomonas sp.* BIOMIG1 AS and SEW, they have same properties. The antibiotic susceptibility of *Pseudomonas sp.* BIOMIG1 SOIL was measured in order to see whether ecotypes of BIOMIG1 show the same susceptibility to antibiotics or not. BIOMIG1^{N} is a mutant *Pseudomonas sp.* BIOMIG1 SEW, it lost its ability to degrade BACs and the MIC value for BACs is 600 mg/L. The antibiotic susceptibility of mutant BIOMIG1^{N} was measured in order to check whether BAC degrading enzymes of *Pseudomonas sp.* BIOMIG1 effect its antibiotic resistance or not. The *Pseudomonas putida* BIOMIG2 VD was isolated from Vileda® samples used to clean home with BAC containing surface cleaners. It was found to be genetically very close to BIOMIG1. The MIC of the *Pseudomonas putida* BIOMIG2 VD for BACs was 125 mg/L. The antibiotic susceptibilities of mutant *Pseudomonas putida* BIOMIG2 VD were measured in order to check whether genetic similarity plays a role on antibiotic resistance or not.

The photographs were obtained by using BioRad EZ-DOC imager with Image Lab program shown in Figure 6.9-6.11. The susceptibilities of *Pseudomonas sp.* BIOMIG1 SOIL, *Pseudomonas putida* BIOMIG2 VD and BIOMIG1^{N} microorganisms against 18 antibiotics were reported in terms of Minimum Inhibitory Concentration (MIC mg/L) in Table 6.3.

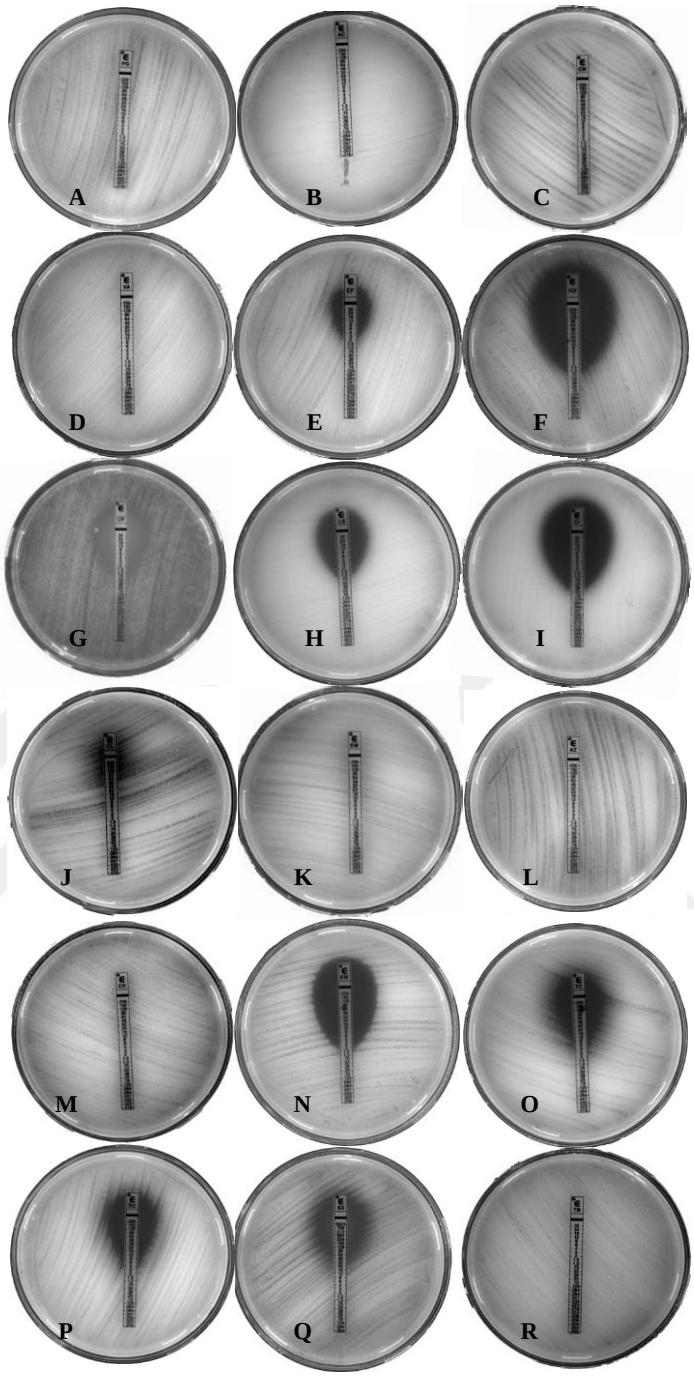


Figure 6.9. The pictures of E-test strips placed on MH agar for BIOMIG1^{N} (A) penicillin g, (B) amoxicillin, (C) clindaycin, (D) vancomycin, (E) enrofloxacin, (F) norfloxacin, (G) ofloxacin, (H) levofloxacin , (I) ciprofloxacin, (J) chloramphenicol, (K) erythromycin, (L) azithromycin, (M) clarithromycin, (N) kanamycin, (O) tetracycline, (P) doxycycline, (Q) sulfamethoxazole and (R) trimethoprim.

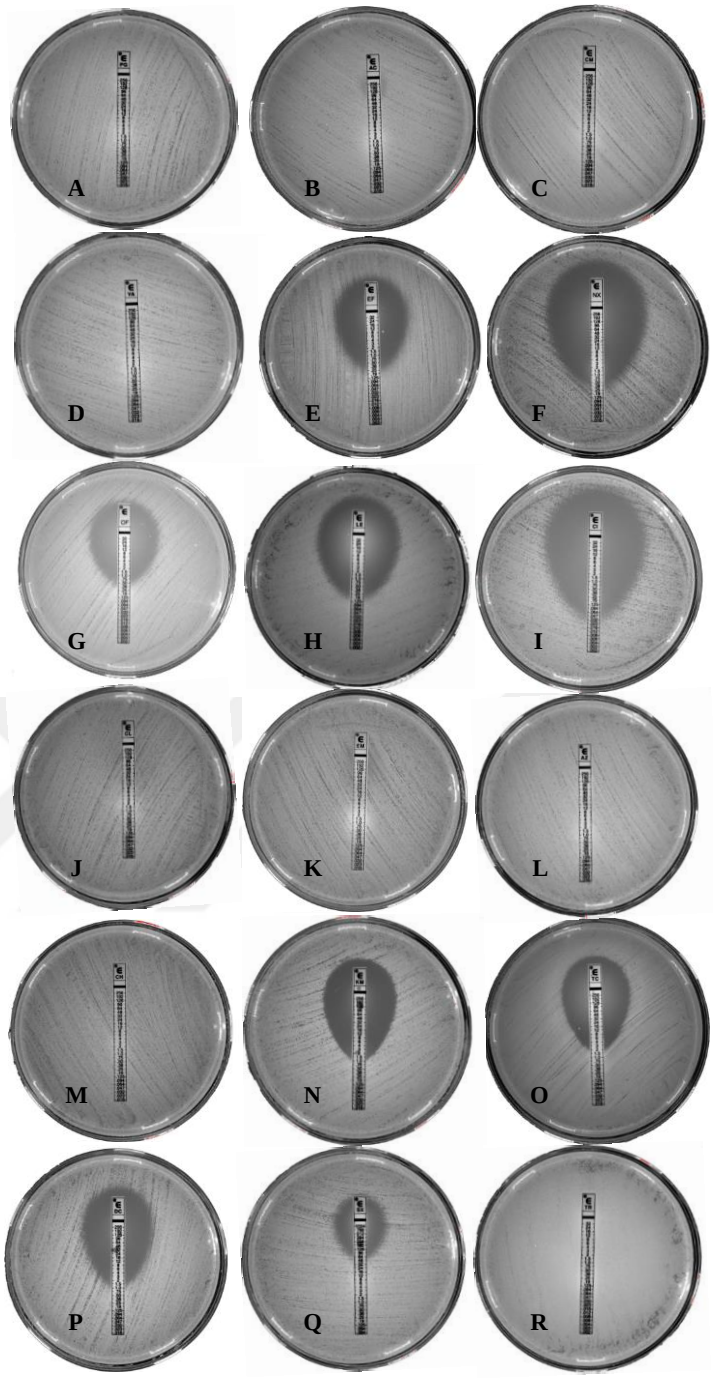


Figure 6.10. The pictures of E-test strips placed on MH agar for *Pseudomonas putida* BIOMIG2 VD (A) penicillin g, (B) amoxicillin, (C) clindaycin, (D) vancomycin, (E) enrofloxacin, (F) norfloxacin, (G) ofloxacin, (H) levofloxacin , (I) ciprofloxacin, (J) chloramphenicol, (K) erythromycin, (L) azithromycin, (M) clarithromycin, (N) kanamycin, (O) tetracycline, (P) doxycycline, (Q) sulfamethoxazole and (R) trimethoprim.

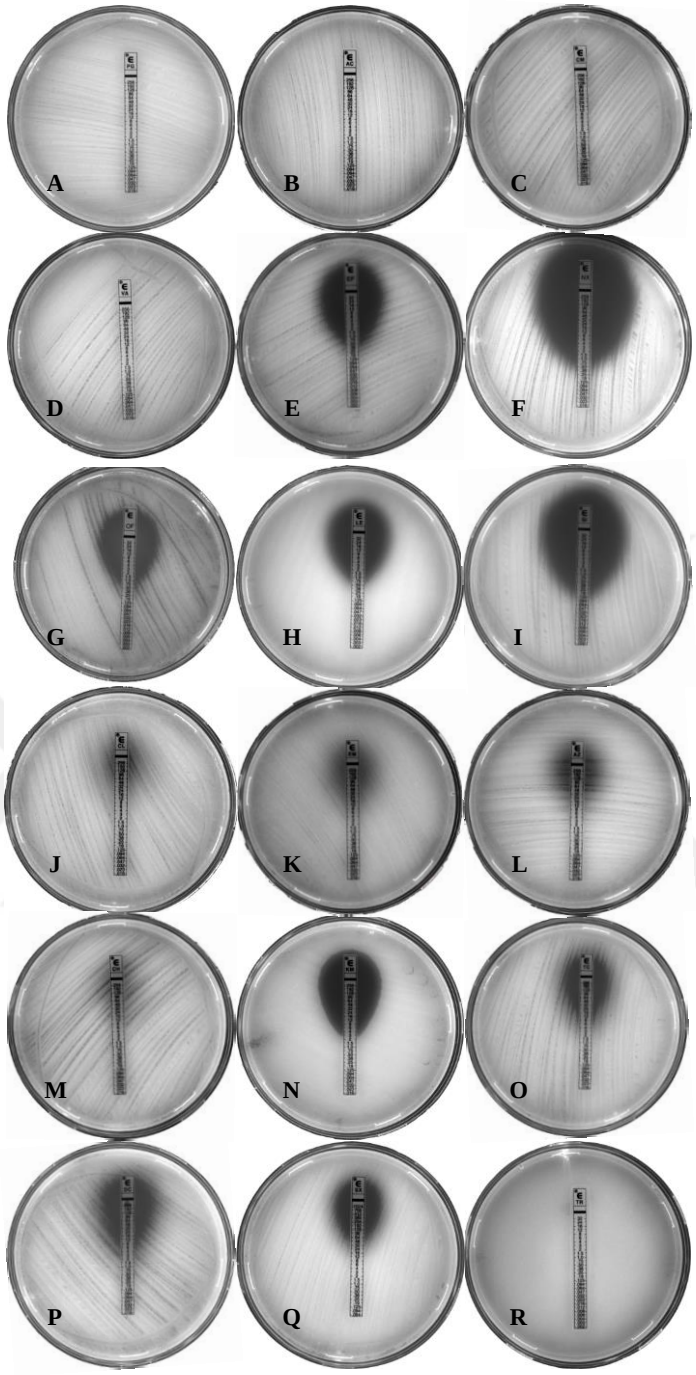


Figure 6.11. The pictures of E-test strips placed on MH agar for *Pseudomonas sp.* BIOMIG1 SOIL (A) penicillin g, (B) amoxicillin, (C) clindaycin, (D) vancomycin, (E) enrofloxacin, (F) norfloxacin, (G) ofloxacin, (H) levofloxacin , (I) ciprofloxacin, (J) chloramphenicol, (K) erythromycin, (L) azithromycin, (M) clarithromycin, (N) kanamycin, (O) tetracycline, (P) doxycycline, (Q) sulfamethoxazole and (R) trimethoprim.

Table 6.3. MIC values of 5 microorganisms against 18 antibiotics according to E-test method.

E-TEST METHOD					
ANTIBIOTIC NAME	ISOLATES MIC(mg/L)				
	BIOMIG1 SEW	BIOMIG1 AS	BIOMIG1 SOIL	BIOMIG1^{N}	BIOMIG2 VD
Penicillin G	>256	>256	>256	>256	>256
Amoxicillin	>256	>256	>256	>256	>256
Ciprofloxacin	0.25	0.38	0.25	0.38	0.047
Enrofloxacin	1	0.75	0.5	2	0.75
Norfloxacin	1.5	1	0.5	1	1
Ofloxacin	1	1.5	0.75	1	0.5
Levofloxacin	0.75	0.5	0.75	1	0.5
Sulfamethoxazole	48	64	64	32	64
Trimethoprim	>32	>32	>32	>32	>32
Tetracycline	8	6	12	8	6
Doxycycline	4	4	6	4	4
Clarithromycin	>256	>256	256	>256	>256
Erythromycin	192	>256	192	>256	>256
Azithromycin	>256	>256	128	>256	>256
Clindamycin	>256	>256	>256	>256	>256
Kanamycin	2	1	3	2	1
Vancomycin	>256	>256	>256	>256	>256
Chloramphenicol	192	>256	192	64	>256

The MIC values of most antibiotics against *Pseudomonas sp.* BIOMIG1 SOIL, AS, SEW, *Pseudomonas putida* BIOMIG2 VD and BIOMIG1^{N} were close to each other. But for sulfamethoxazole and chloramphenicol BIOMIG1^{N} had lower MIC values than BIOMIG1. With these findings we can speculate that there can be a relationship between BAC biodegradation and antibiotic degradation since BIOMIG1^{N} lost its enzymes which are responsible from BAC degradation. The resistance difference in between BIOMIG1^{N} and BIOMIG1 indicate that catabolic enzymes of BIOMIG1 which involve in BAC degradation may also facilitate utilization of sulfamethoxazole and chloramphenicol. Several microorganisms were reported in the literature as chloramphenicol and sulfamethoxazole degrader. Jiang et al. (2014) reported that Pseudomonas psychrophila HA-4 was able to

degrade sulfamethoxazole. They isolated HA-4 from activated sludge by using culture enrichment method and they proposed that HA-4 could biodegrade sulfamethoxazole totally. Aniline, 3-amino-5-mehtylisoxazole, 4-aminothiophenol and sulfanilamide were identified as sulfamethoxazole metabolites and they indicated initial step of biodegradation either start from N-C bond cleavage or S-N bond cleavage. Moreover, according to Tao et al. (2012), estDL136 (chloramphenicol acetate esterase gene) which was expressed in *E.coli* was responsible from hydrolysis of chloramphenicol to *p*-nitrophenylserinol. Loughlin et al. (2002) reported that adaptation *of Pseudomonas aeruginosa* to BACs cause increasing resistance against chloramphenicol. *sulI* is a mutated gene which encodes resistance to sulfonamides always coexist with qacΔE gene which is a QAC resistance gene (Shluter et al., 2007). In other In other words, presence of the *sulI* gene in an integron indirectly confers QAC resistance (Hall et al., 1998).

6.4. Summary

The lowest MIC values were reported for quinolones followed by tetracylines, aminoglycosides, sulfonamides, chloramphenicol, macrolides, whereas penicillins, lincosamides, and glycopeptides were less effective antibiotics on the tested bacteria. Results showed that *E.coli* which is susceptible to BACs is also the most susceptible bacteria to almost all antibiotics except to clindamycin and sulfamethoxazole. *Serratia marcescens* is more resistant to antibiotics than *E.coli* but it showed less resistance than *Pseudomonas sp.* BIOMIG1 which can also degrade BACs. Results show that BIOMIG1 was around 10 fold resistant than BAC susceptible *E.coli* BIOMIG3 and more than 2 fold resistant than BAC resistant *Serratia marcescens* sp. BIOMIG4 for 13 antibiotics out of 18. Comparison of MICs of each antibiotic for each bacteria suggests that BAC resistance may favor resistance particularly to tetracyclines, macrolides, penicillins and glycopeptides. *Pseudomonas sp.* strains (BIOMIG1 SEW and AS), which possess the highest resistance to all antibiotics were not only highly resistant to BACs but also they can biodegrade BACs. Given the fact that many antibiotics are biodegradable by certain microorganisms; Above findings suggest that catabolic enzymes which involve in BAC degradation may also degrade antibiotics. The hypothesis of strain BIOMIG1 may subsist on antibiotics was tested in the following steps of this study.

7. ANTIBIOTIC BIOTRANSFORMATION POTENTIAL OF BACTERIA RESISTANT TO BACs

7.1. Introduction

Extensive and often inappropriate use of antibiotics lead to their release into the environment and is believed to be a contributory factor in the development and dissemination of antibiotic resistance (Scenihr, 2009). In addition, presence of different kinds of antibiotics in commercial use and their concurrent use by patients resulted in evolution of multidrug resistant bacteria. For instance, ESCAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) - a group of pathogenic bacteria that evokes many nosocomial infectious- is the prominent bacteria that evolves resistance mechanisms to multiple-drugs (Rice, 2010). As a result, antimicrobial resistance is a major health threat that the human society is facing today, and many authorities try to find a solution for this growing problem (Hegstad et al., 2010; Tezel and Pavlostathis, 2012a).

Forsberg et al. (2012) showed that antimicrobial resistance genes present in soil bacteria are similar to many clinical pathogens proving that these genes are transferred to pathogens from natural bacteria. Given the fact that most of the antibiotic resistant genes have an environmental origin, controlling the release of antibiotics into the environment may reduce the evolution and dissemination of antibiotic resistance both in the environment and medical settings. Antibiotic biodegradation is one of the most effective way to reduce antibiotic concentrations in both wastewater treatment plants and environment. There are several examples in the literature that microorganisms can biodegrade antibiotics. For example β-Lactamase or penicillinase enzymes cause cleavage of penicillin group of antibiotics (Bust et al., 1995; Li et al., 2008; Al-Ahmad et al., 1999; Langin et al., 2009; Maulin et al., 1986; Dougherty et al., 1980). Johnsen et al. (1977) isolated a strain of *Pseudomonas fluorescens* which can biotransform penicillin g by using penicillinase enzyme to benzylpenicilloic acid and benzylpenicillenic acid. *Pseudomonas cepacia* and

Pseudomonas fluorescens strains were able to use benzylpenicilin as a carbon source (Beckman et al., 1979; Johnsen, 1977). Eichhorn et al. (2005) reported bacteria in the nitrifying activated sludge sample can biotransform trimethoprim to α-hydroxytrimethoprim and protonated form of trimethoprim. Also bacteria present in nitrifying activated sludge was reported that it can biotransform trimethoprim (Khunjar et al., 2011; Perez et al., 2004). It was reported that sulfamethoxazole can be biodegraded in activated sludge process (Perez et al., 2005; Deng et al., 2016). Malek et al. (1961) reported *Streptomyes sp.* as chloramphenicol degrader and Tao et al. (2012) reported *E.coli* strain which has estDL136 gene as chloramphenicol degrader. *Pseudomonas psychrophila* HA-4 which is genetically close to *Pseudomonas sp.* BIOMIG1 was reported as sulfamethoxazole degrader (Jiang et al., 2014). Langin et al. (2009) reported that bacteria present in active sludge can break the β-Lactam ring of amoxicillin. Dantas et al. (2008) found bacterial isolates from soil subsist on different antibiotics such as chloramphenicol, penicillin g, vancomycin, carbenicillin, ciprofloxacin, mafenide, kanamycin, sisomicin, amikacin, trimethoprim, D-cycloserine, gentamicin, dicloxacillin, nalidixic acid, thiamphenicol, levofloxacin, sulfamethizole, sulfisoxazole. After checking phylogenetic profiles of these isolates, they found that half of the isolates that subsist on antibiotics belongs to *Burkholderiales* and *Pseudomonadales* order. They also found the phylogenetic distribution of isolated species show similar trends within same antibiotic groups. The resulting phylogenetic distribution of bacteria that subsist on chloramphenicol belongs to *Burkholderiales, Pseudomonadales* and *Rhodospirillales* order. The resulting phylogenetic distribution of bacteria that subsist on penicillin belongs to *Burkholderiales* order. The resulting phylogenetic distribution of bacteria that subsist on vancomycin and trimethoprim belongs to *Burkholderiales, Pseudomonadales* and *Rhizobiales* order. When we checked the phylogenetic relationship of *Pseudomonas sp.* BIOMIG1 and for each bacteria that can subsist on vancomycin, trimethoprim and chloramphenicol, we found that *Pseudomonas sp.* BIOMIG1 clustered with antibiotic degrading isolates (Figure 2.6-2.8.). Although Dantas et al. (2008) also reported some isolated that can degrade ciprofloxacin, levofloxacin, kanaymcin antibiotics, we did not focus on these antibiotics. Since *Pseudomonas sp.* BIOMIG1 was found to be susceptible to these antibiotics and also the bacteria that can utilize these antibiotics did not belong to *Pseudomonadales* order.

With the light of the above mentioned information, we can speculate that *Pseudomonas sp.* BIOMIG1 may subsist on vancomycin, chloramphenicol and trimethoprim antibiotics. Also, we know that *Pseudomonas sp.* BIOMIG1 is able to degrade BACs and it has high MIC values for penicillin g, amoxicillin and clindamycin. Also in previous chapter, *Pseudomonas sp.* BIOMIG1 found to be resistant to penicillin g, amoxicillin and clindamycin. Moreover, when the antibiotic MIC results of *Pseudomonas sp.* BIOMIG1 and mutant BIOMIG1^{N} (mutant BIOMIG1 which lost its ability to degrade BACs) were compared, it was found that BIOMIG1 found 2 fold more resistant to sulfamethoxazole and chloramphenicol. This is supporting the idea that BIOMIG1 may subsist on these antibiotics.

Therefore this chapter of the study focused to elucidate the biodegradation potential of penicillin g, amoxicillin, clindamycin, vancomycin, sulfamethoxazole, chloramphenicol and trimethoprim by *Pseudomonas sp.* BIOMIG1.

7.2. Materials and Methods

7.2.1. Antibiotic Biotransformation Using Modified E-test Method

The antibiotic biodegradation potential of each microorganism against selected antibiotics was determined using newly developed E-Test method. A similar E-test method, was used to determine the MIC values of the antibiotics, and to determine antibiotic degradation potential. In this method, instead of using Muller Hinton agar plate, agar plates which are prepared with MSM medium were used. Since there was no carbon source in the preparation of MSM agar plates, E-test antibiotic strips were the only carbon source and existence of any growth is assumed to be due to the degradation of the antibiotic.

According to the E-test method protocol, a single colony of each microorganism was transferred into a sterile falcon tube containing 5 mL Mueller-Hinton broth. Colonies were taken from CHROM®Agar Orientation plates prepared as described in 4.2.2. After microorganisms were grown overnight in Mueller-Hinton broth, cultures were transferred into sterile microcentrifuge tubes and centrifuged at 10, 000 rpm for 10 min. The supernatant

was discarded, the pellet was suspended in 85% saline solution and this solution was further diluted with 85% saline solution to a turbidity comparable to that of a 0.5 McFarland turbidity standard (c.a. 0.5 x 10^8 CFU/mL). Optimally, within 15 minutes after adjusting turbidity of inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swap was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This removed excess inoculum from the swab. The dried surface of a MSM agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, by rotating the plate approximately 60 ° each time to ensure an even distribution of inoculum. Once the agar plate was completely dry, the antibiotic strip (E-Test strip) was applied aseptically with the help of sterile tweezer and plates were incubated at 22 °C.

7.2.2. Batch Antibiotic Biotransformation in Liquid Medium

The antibiotic degradation potential of each microorganism against selected antibiotics was determined using liquid culture method.

A single colony of each microorganism was transferred into sterile falcon tube containing 5 mL LB broth. Colonies were taken from CHROM®Agar Orientation plates prepared as described in 4.2.2. Then, falcon tubes were placed on an orbital shaker and agitated at 130 rpm. After microorganisms were grown overnight in LB broth, cultures were transferred into sterile microcentrifuge tubes and centrifuged at 10, 000 rpm for 10 min. The supernatant was discarded, the pellet was suspend in 85% saline solution and the solution was further diluted with 85% saline solution to a turbidity comparable to that of a 0.5 McFarland turbidity standard (c.a. 0.5 x 10^8 CFU/mL).

A sufficient number of 100 mL Erlenmeyer flasks were taken and 35 mL DI water was put into each of the Erlenmeyer flasks. They were autoclaved. Then, 5 mL of 10X MSM was put in each Erlenmeyer flask. A 1 mL culture which was prepared as in 7.2.2. was placed into Erlenmeyer flask (batch reactors) and diluted with addition of 4 mL DI water. Control reactors were set up as above but without inoculum.

For reactors that were prepared with microorganisms which were able to degrade BAC, necessary amounts of BAC were added to reach 50 mg/L BAC concentration. This step was done in order to activate the enzymes responsible for BAC degradation. The content in each Erlenmeyer flask was agitated on an orbital shaker at 130 rpm at room temperature until all BACs were consumed. As soon as BACs were consumed, 5 mL of antibiotic solution with necessary concentrations (around MIC/2 of each antibiotic for the microorganism) was added both to batch reactors and to control reactors.

For reactors that were prepared with microorganism which cannot degrade BAC, a 5 mL of antibiotic solution with necessary concentration (around MIC/2 of each antibiotic for the microorganism) was added both to batch reactors and to control reactors.

All of the reactors were placed on an orbital shaker and agitated at 130 rpm at 22 °C. Samples were taken three times a week and antibiotic concentrations were measured with HPLC method as described in 4.2.2.

7.3. Results and Discussion

7.3.1. Antibiotic Biotransformation Potential of Bacteria with Modified E-test Method

A new E-test method was developed and tested to find out the antibiotic biotransformation potential of bacteria against selected antibiotics. In this new method, agar plates were prepared with MSM medium instead of Muller Hinton agar. Agar plates prepared with MSM did not contain any carbon source. E-test antibiotics strips placed on agar were used as the sole carbon source and any bacterial growth on plates was assumed to be due to the antibiotic degradation. But *Pseudomonas sp.* BIOMIG1 grew on MSM agar plates even without E-test strips present. Experiments were repeated with single carbon source medium (SCS) (Dantas et al., 2008) instead of MSM medium, however results were the same, BIOMIG1 growth was observed without E-test strips present.

Since agar consist of agarose and agaropectin, we suspected that *Pseudomonas sp.* BIOMIG1 may have been degrading agaropectin to survive. To test this idea, experiments were repeated with pure agarose instead of agar, yet the results did not change. We have reached the conclusion that *Pseudomonas sp.* BIOMIG1 is most likely to degrade agarose, therefore a negative control could not be obtained. Bacteria will always grow on agar plate. Since negative control could not be obtained, it was decided to experiment with liquid culture method to test antibiotic biodegradation potential (Figure 7.1.).

Figure 7.1. The growth profile of *Pseudomonas sp.* BIOMIG1 SEW.

7.3.2. Antibiotic Biotransformation Potential of Bacteria in Liquid Medium

According to Dantas et al. (2008) that bacteria belonging to *Pseudomonadales* order, can degrade chloramphenicol, vancomycin, trimethoprim, carbenicillin, dicloxacillin and mafenide (Figure 7.2.). Since did not have dicloxacillin, carbenicillin and mafenide, we were just able to test biodegradation potential of *Pseudomonas sp.* BIMOG1 against chloramphenicol, vancomycin, and trimethoprim. Amoxicillin, sulfamethoxazole, clindamycin and penicillin g antibiotics also were selected since MIC values for these antibiotics against *Pseudomonas sp.* BIOMIG1 were very high.

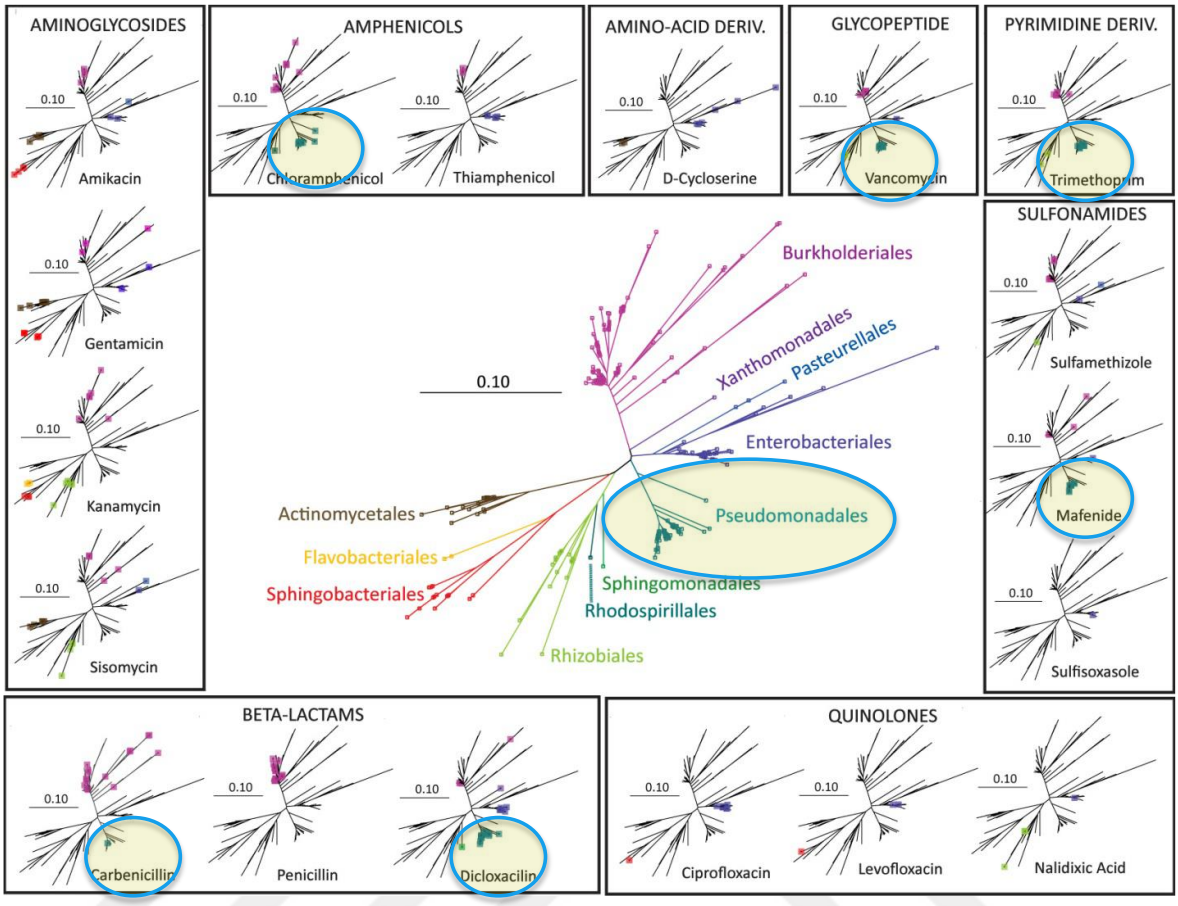


Figure 7.2. Phylogenetic distribution of bacteria subsist on antibiotics (Dantas et al, 2008).

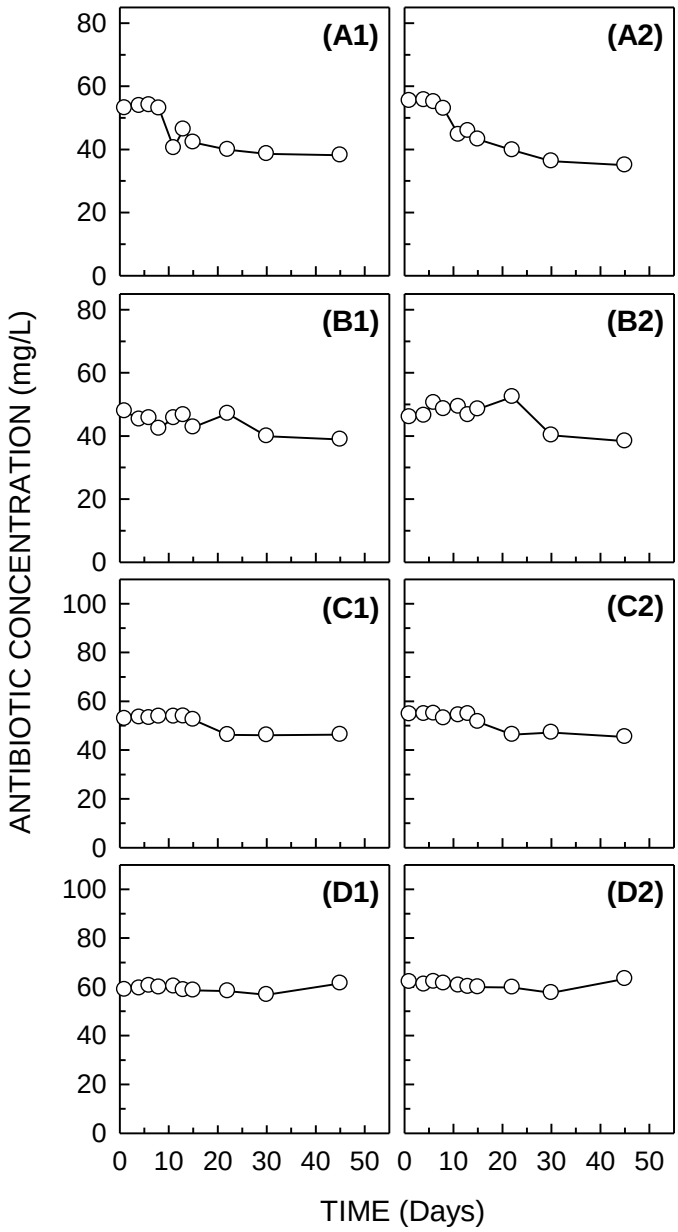


Figure 7.3. Profile of (A) chloramphenicol, (B) vancomycin, (C) sulfamethoxazole, (D) trimethoprim, (E) amoxicillin and (F) penicillin g and (G) clindamycin utilization in (1) control and (2) *Pseudomonas sp.* BIOMIG1 SEW.

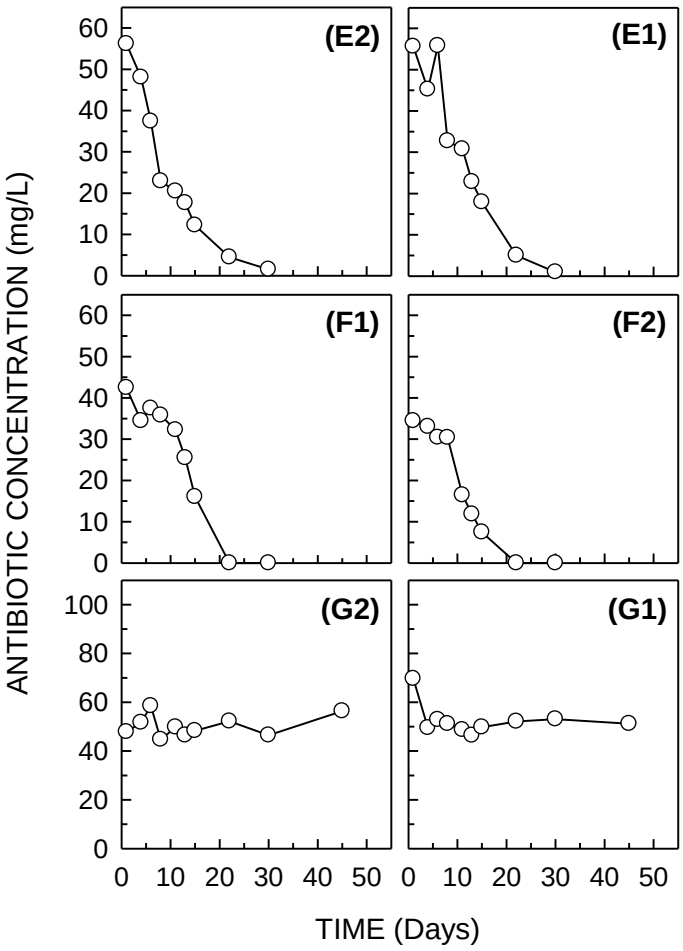


Figure 7.3. Continued.

The results of the HPLC analyses of samples taken from the *Pseudomonas sp.* BIOMIG1 reactors and control reactors without any culture were shown in above Figure 7.3. When we analyzed the results, we saw that the antibiotic concentration of sulfamethoxazole, trimethoprim and clindamycin reactors did not change in both control and *Pseudomonas sp.* BIOMIG1 reactors during in the incubation course (Figure 7.3. C, D and G). Thereby, we come to a conclusion that *Pseudomonas sp.* BIOMIG1 could not degrade sulfamethoxazole, trimethoprim and clindamycin. Although chloramphenicol and vancomycin concentration were decreased on average 20% *Pseudomonas sp.* BIOMIG1 reactors, the same decrease was seen in control reactors too (Figure 7.3. A and B). Therefore decrease in the antibiotic concentration in *Pseudomonas sp.* BIOMIG1 reactors could not be attributed to

biotransformation. In Figure 7.3. E and F, it was seen that the concentration of penicillin group of antibiotics (amoxicillin and penicillin g) was zero after one month in both *Pseudomonas sp.* BIOMIG1 and control reactors. Since the rate of antibiotic concentration decrease was same both in *Pseudomonas sp.* BIOMIG1 and in control reactors for each antibiotic, it could not be a result of any possible biotransformation of these antibiotics by *Pseudomonas sp.* BIOMIG1. Disappearance of these antibiotics can be a result of β-Lactam ring being chemically unstable and opens easily with change in pH and temperature. (Clarke et al., 1949; Hou and Poole, 1971; Bush et al., 1995).

After finding out that *Pseudomonas sp.* BIOMIG1 SEW could not biodegrade 7 antibiotics that were tested, the antibiotic degradation potential of soil bacteria was investigated. The experimental set up was prepared as described in 7.2.2. But this time, instead of adding pure culture, 5 gram of soil sample was added to soil reactors. Since Dantas et al. (2008) reported that they were isolated hundreds of bacteria from soil which can use antibiotics as carbon source, we wanted to checked whether the bacteria present in soil in the university is able to degrade antibiotics or not. The results of the HPLC analyses of samples taken from the soil reactors and their control reactors without any culture shown in Figure 7.4.

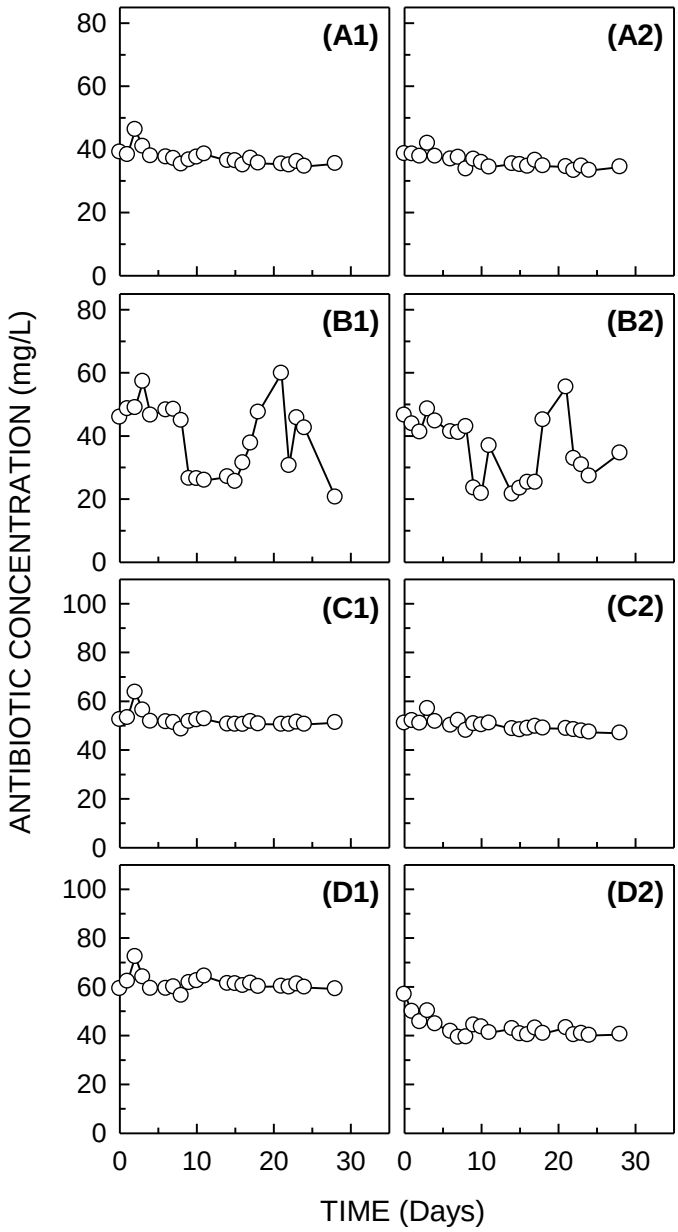


Figure 7.4. Profile of (A) chloramphenicol, (B) vancomycin, (C) sulfamethoxazole, (D) trimethoprim, (E) amoxicillin and (F) penicillin g and (G) clindamycin utilization in (1) control and (2) sample taken from soil.

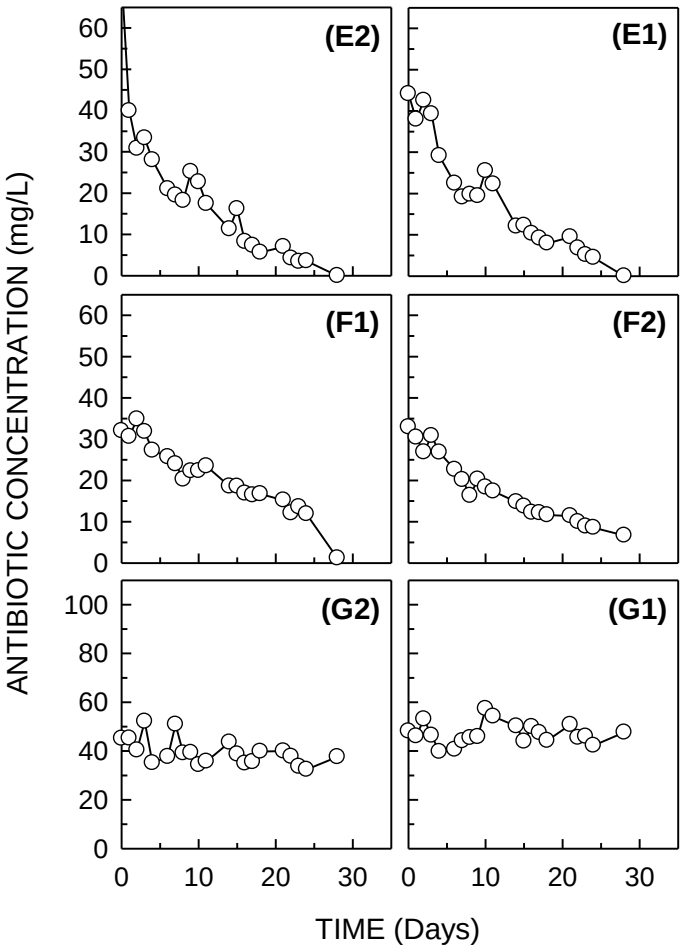


Figure 7.4. Continued.

When we analyzed the results shown in Figure 7.4., we saw that the antibiotic concentration of chloramphenicol, sulfamethoxazole, trimethoprim and clindamycin reactors did not change in both control and soil reactors during in the incubation course (Figure 7.4. A, C, D and G). Thereby, we come to a conclusion that bacteria present in the soil could not degrade chloramphenicol, sulfamethoxazole, trimethoprim and clindamycin. Although vancomycin concentration was fluctuated in soil reactors, the same fluctuation was seen in control reactor too (Figure 7.4. B). This fluctuation may come from absorption and desorption of vancomycin to surface of the reactor bottles but it could not attributed to biotransformation. In Figure 7.4. E and F, it was seen that the concentration of penicillin group of antibiotics (amoxicillin and penicillin g) got zero after twenty-eight days in both

soil and control reactors. Since the rate of antibiotic concentration decrease was the same in both soil and control reactors for each antibiotic, it could not be come from biotransformation of these antibiotics by bacteria present in soil. However it may happened because of β-Lactam ring is chemically unstable and β-Lactam ring cleave easily with change in pH and heat. (Clarke et al., 1949; Hou and Poole, 1971; Bush et al., 1995).

Pseudomonas putida BIOMIG2 VD, *Alcaligenes sp.* BIOMIG7, *Serratia marcescens* BIOMIG4, are bacteria that were isolated in our laboratories. The MIC values of sulfamethoxazole and chloramphenicol against *Pseudomonas sp.* BIOMIG1 SOIL, AS, SEW, *Pseudomonas putida* BIOMIG2 VD were found to be higher than the MIC values of BIOMIG1^{N}. Because BIOMIG1^{N} lost its enzymes which are responsible from BAC degradation, higher MIC value of *Pseudomonas putida* BIOMIG2 VD may come from biodegradation of these antibiotics. Thereby biodegradation potential of *Pseudomonas putida* BIOMIG2 VD against chloramphenicol and sulfamethoxazole were tested. When *Alcaligenes sp.* BIOMIG7 were compared to bacteria isolated in Dantas et al. (2008) study, it was found that it is geneticly closer to the bacteria which degrade vancomycin and trimethoprim (Figure 7.5 and 7.6). Thereby biodegradation potential of *Alcaligenes sp.* BIOMIG7 against vancomycin and trimethoprim were tested. Since the MIC values of *Serratia marcescens* for penicillin g and amoxicillin were so high, the penicillin g and amoxicillin biodegradation potential of *Serratia marcescens* were tested to find out whether these high MIC values may be a result of biodegradation or the R factor present in plasmid of *Serratia marcescens* which contain resistance genes against several antibiotics (http://microblog.me.uk/taxonomy/term/1?page=26).

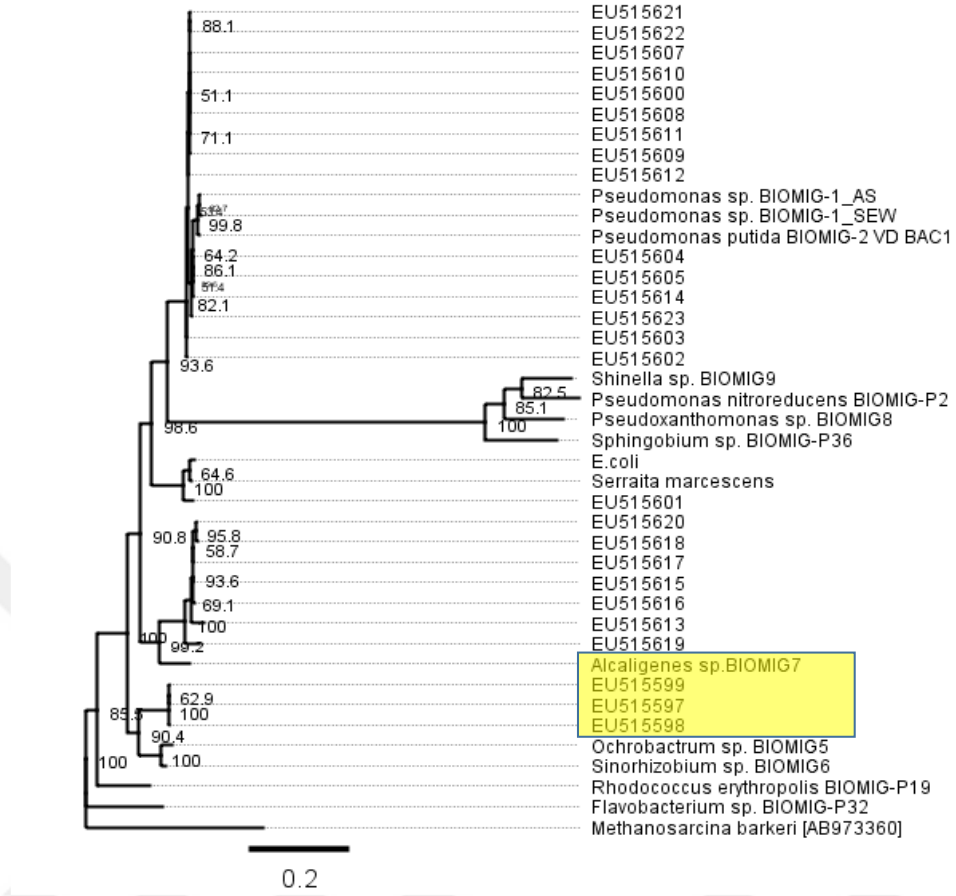


Figure 7.5. Phylogenetic tree of relationships of bacteria isolated in our lab, determined by maximum likelihood followed by neighbor joining tree building method and Hasegawa-Kishino-Yano genetic distance model, relative to bacteria reported as vancomycin degraders according to Dantas et al. (2008). Bootstrap values represents 100 replicates. The scale bar represents 0.2 substitution per nucleotide position. *M. barkeri* (AB973360) was used as the out group. (Branches were shown in yellow, when the bacteria isolated in our lab clustered with bacteria which can degrade vancomycin).

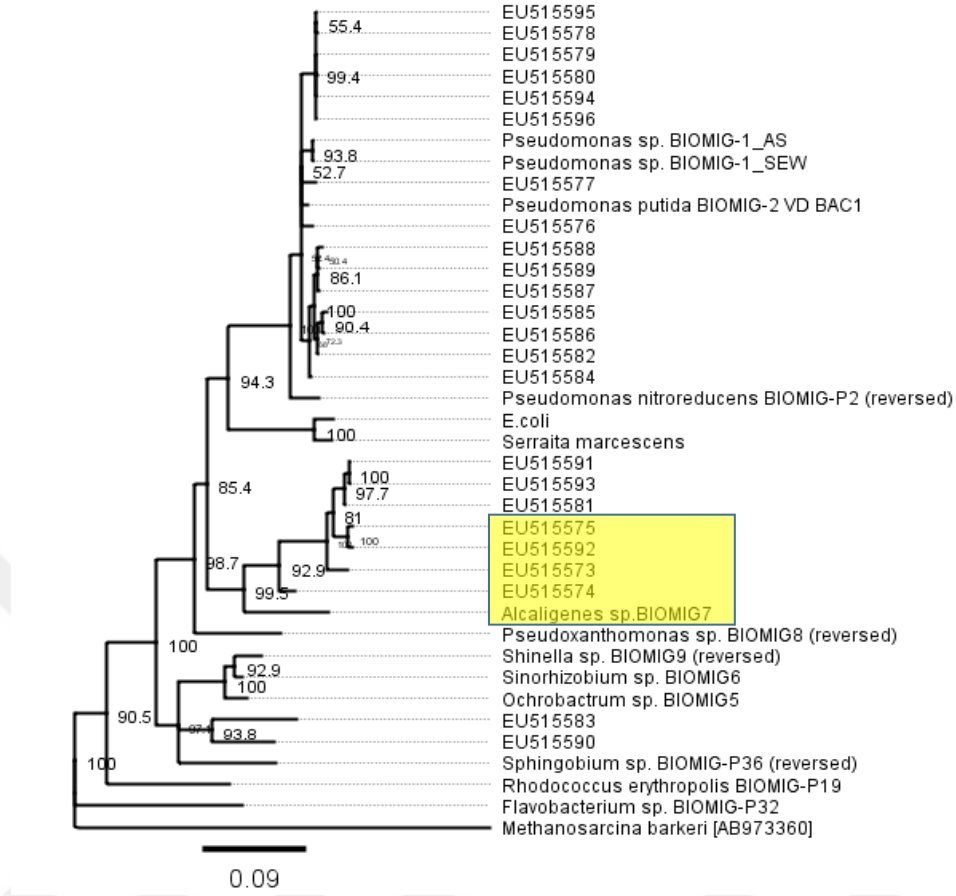


Figure 7.6. Phylogenetic tree of relationships of bacteria isolated in our lab, determined by maximum likelihood followed by neighbor joining tree building method and Hasegawa-Kishino-Yano genetic distance model, relative to bacteria reported as trimethoprim degraders according to Dantas et al. (2008). Bootstrap values represents 100 replicates. The scale bar represents 0.09 substitution per nucleotide position. *M. barkeri* (AB973360) was used as the out group. (Branches were shown in yellow, when the bacteria isolated in our lab clustered with bacteria which can degrade trimethoprim).

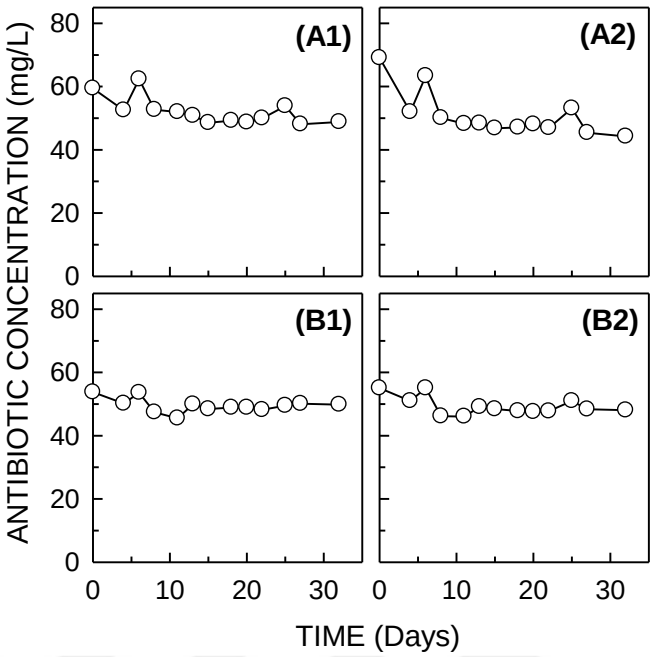


Figure 7.7. Profile of (A) chloramphenicol, (B) sulfamethoxazole, utilization in (1) control and (2) *Pseudomonas putida* BIOMIG2 VD.

The results of the HPLC analyses of samples taken from the *Pseudomonas putida* BIOMIG2 VD reactors and control reactors without any culture were shown in above Figure 7.7. When we analyzed the results, we saw that the antibiotic concentration of chloramphenicol and sulfamethoxazole reactors were decreased on average 10% at the beginning of incubation course in *Pseudomonas putida* BIOMIG2 VD reactors, but the same decrease was seen in control reactors (Figure 7.7. A and B). Therefore decrease in the antibiotic concentration in *Pseudomonas putida* BIOMIG2 VD reactors could not attributed to biotransformation. It may come from may come from absorption of chloramphenicol and sulfamethoxazole to surface of the reactor bottles.

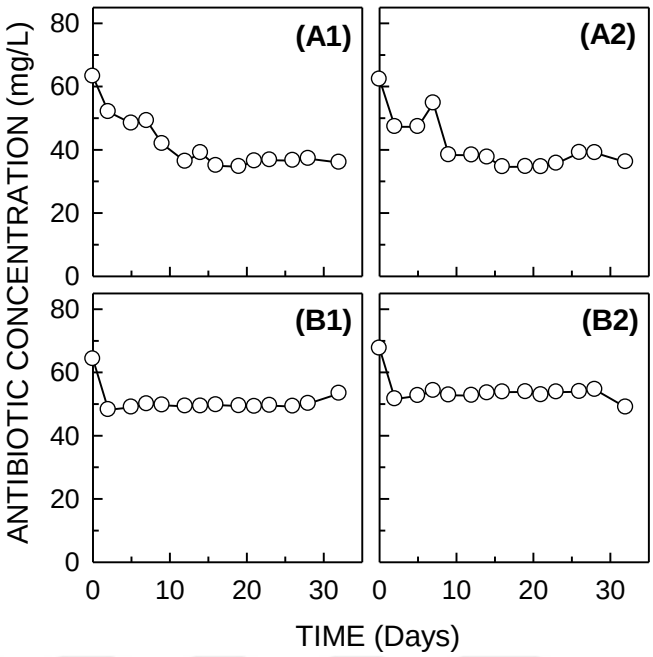


Figure 7.8. Profile of (A) vancomycin, (B) trimethoprim, utilization in (1) control and (2) *Alcaligenes sp*. BIOMIG7.

The results of the HPLC analyses of samples taken from *Alcaligenes sp*. BIOMIG7 reactors and control reactors without any culture were shown in above Figure 7.8. When we analyzed the results, we saw that the antibiotic concentration of vancomycin and trimethoprim reactors were decreased on average 30% at the beginning of incubation course in *Alcaligenes sp*. BIOMIG7 reactors, but the same decrease was seen in control reactors (Figure 7.8. A and B). Therefore decrease in the antibiotic concentration in *Alcaligenes sp*. BIOMIG7 reactors could not attributed to biotransformation. It may come from may come from absorption of vancomycin and trimethoprim to surface of the reactor bottles.

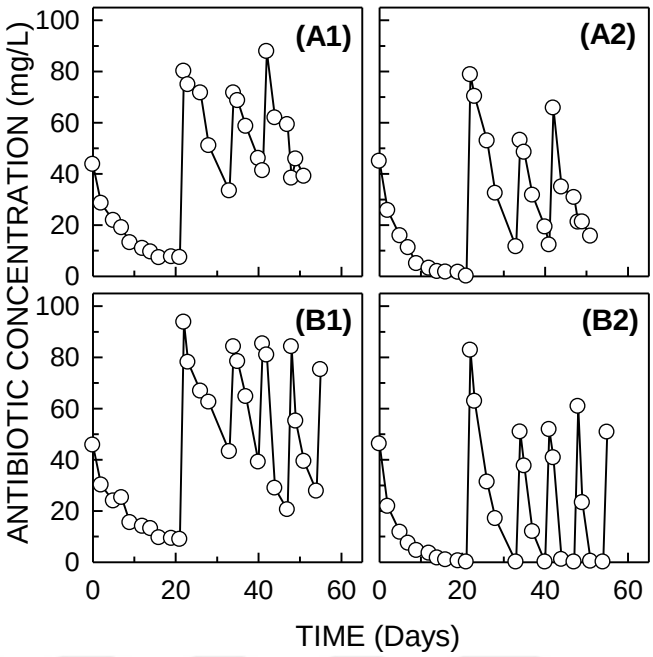


Figure 7.9. Profile of (A) amoxicillin, (B) penicillin g, utilization in (1) control and (2) *Serratia marcescens.*

The results of the HPLC analyses of samples taken from the reactors and their control reactors without any culture shown in the above Figure 7.9. In the reactors containing *Serratia marcescens,* it was observed that penicillin g and amoxicillin concentration got zero in reactors containing *Serratia marcescens* after twenty days of incubation. After that, both control and *Serratia marcescens* reactors were fed with amoxicillin and penicillin g antibiotics. This time antibiotic concentrations got zero around ten days. Reactors were fed with antibiotics whenever their concentration dropped to zero in *Serratia marcescens* reactors. The rate of antibiotic concentration decrease in *Serratia marcescens* reactors were 50% faster than it was in control reactors. While the antibiotic concentration decrease in control reactors most probably come from physicochemical cleavage of β-Lactam ring since β-Lactam ring was sensitive to pH and heat changes. In the *Serratia marcescens* reactors, the antibiotic concentration decreased because of both physicochemical and biological cleavage of β-Lactam ring.

7.3.3. Biotransformation of Beta-lactams by *Serratia Marcescens*

To find out penicillin g and amoxicillin degradation products by *Serratia marcescens*, liquid medium experiments of *Serratia marcescens* were performed. This time besides penicillin g and amoxicillin concentrations, concentrations of their metabolites were monitored too.

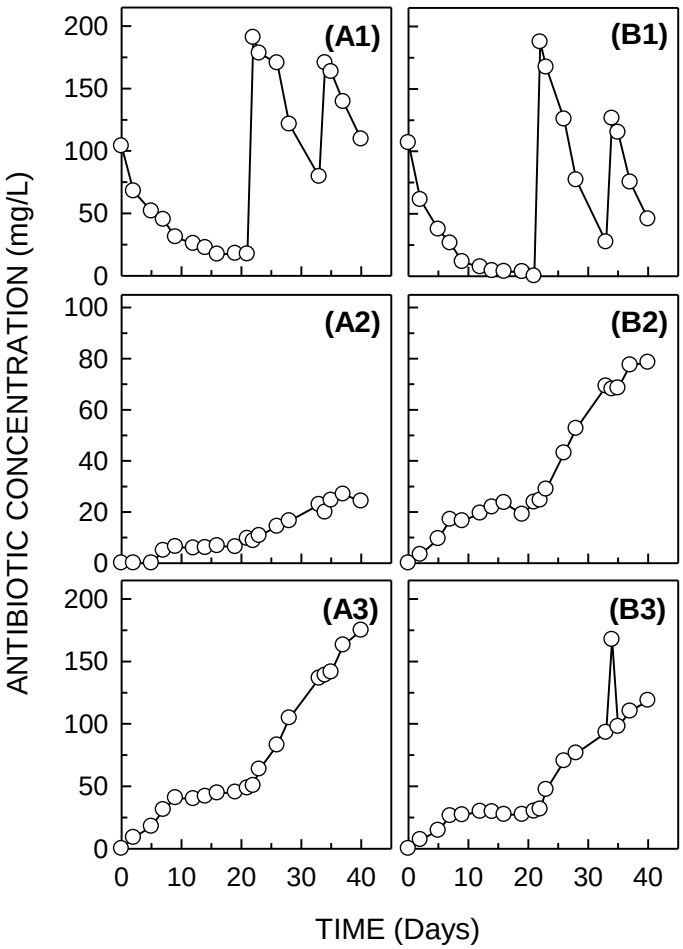


Figure 7.10. (1) Amoxicillin utilization, (2) its metabolite A and (3) its metabolite B in (A) control and (B) *Serratia marcescens.*

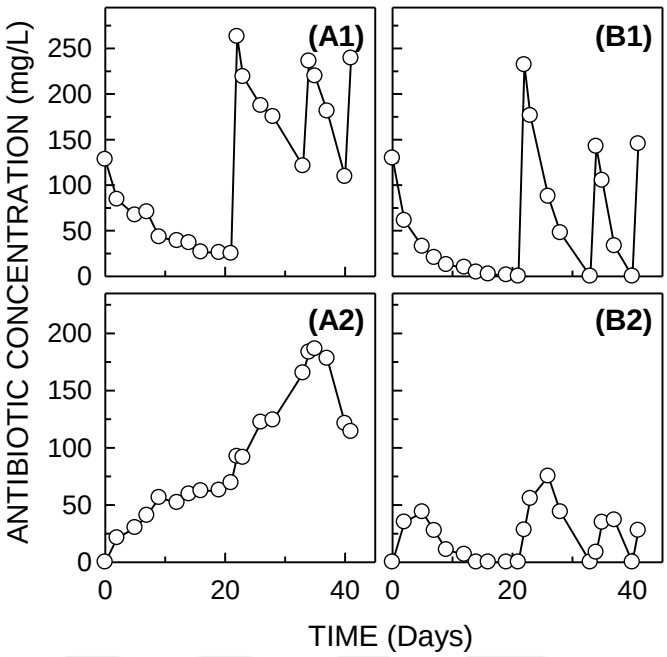


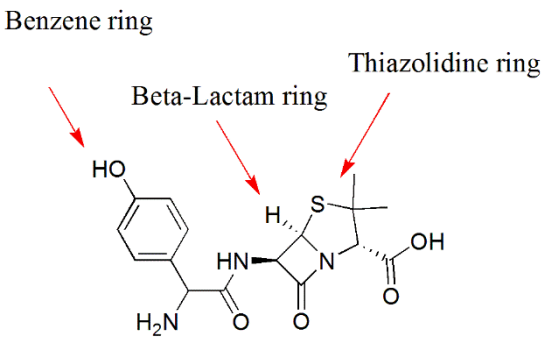
Figure 7.11. (1) Penicillin g utilization, (2) its metabolite A and in (A) control and (B) *Serratia marcescens.*

During amoxicillin degradation, two degradation products were observed both in control and *Serratia marcescens* reactor at 4.6 min (metabolite A) and 7.4 min (metabolite B) (Figure 7.10.). In control reactor amoxicillin was first converted to the degradation product seen at 7.4 min (metabolite B) then amoxicillin was converted very slowly to other degradation product seen at 4.6 min (metabolite A). While in *Serratia marcescens* reactor, amoxicillin was converted mainly to the degradation product seen at 4.6 min (metabolite A).

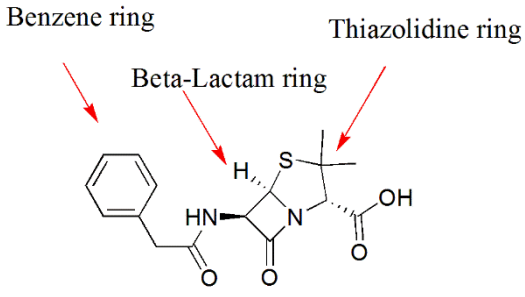
During penicillin g degradation a single degradation product was observed both in control reactor and *Serratia marcescens* reactor at 11 min (metabolite A) (Figure 7.11.). Despite in control reactor degradation product concentration was increasing while penicillin g concentration was decreasing, in *Serratia marcescens* reactor at first metabolite concentration was increased with decreasing penicillin g concentration but then dropped to zero and no other metabolites were observed. This observation supports the idea that *Serratia*

marcescens completely catabolize penicillin g, whereas in control reactor penicillin g was transformed to one degradation product which then accumulated.

Penicillin g and amoxicillin belong to penicillin group antibiotics and both have β-Lactam ring (Figure 7.12.). β-Lactam ring is chemically unstable since it is sensitive to pH and heat (Clarke et al., 1949; Hou and Poole, 1971; Bush et al., 1995). That's why β-Lactam ring can open physicochemically. Also β-Lactam ring can open with enzymes released from resistant bacteria (Bush et al., 1995), upon opening of β-Lactam ring penicillins lose their antibiotic properties (Hou and Poole, 1971). Nevertheless, De weck (1962) and Klaus et al. (1973) reported that penicillin degradation products also cause allergic reactions in patients. Also, according to Li et al. (2008) penicillin g degradation occurs both in wastewater treatment plant and after the discharge of penicillin g into surface water. Yet in their study it was found that penicillin g degradation products were persistent. Another research reported penicillin g biodegraded only to some degree (27%) by wastewater bacteria (Al-Ahmad et al., 1999). Dantas et al. (2008) were reported that bacteria belongs to *Burkholderiales* order can biodegrade penicillin group of antibiotics (penicillin g, dicloxacillin and carbenicillin). They also proposed penicillin g degradation pathway. They reported that penicillin g was first converted to benzylpenicilloic acid then benzylpenilloic acid and then totally catabolized to CO_2 by Peni-S2N-M1LLLSSL-2 strain belongs to *Burkholderiales* order. Langin et al. (2009) tested the biodegradability of amoxicillin by using both closed bottle test and Zahn-Wellens test. While β-Lactam ring of amoxicillin was cleaved abiotically in both tests, full mineralization of amoxicillin by bacteria present in active sludge was observed just in Zahn-Wellens test.



Amoxicillin Structure



Penicillin G Structure

Figure 7.12. Penicillin g and amoxicillin structures.

Considering above mentioned research results and our findings, the biodegradation pathway for amoxicillin and penicillin g were predicted by using UMBBD (University of Minnesota Biocatalysis/Biodegradation Database) web page. (Figure 7.13 and 7.14.).

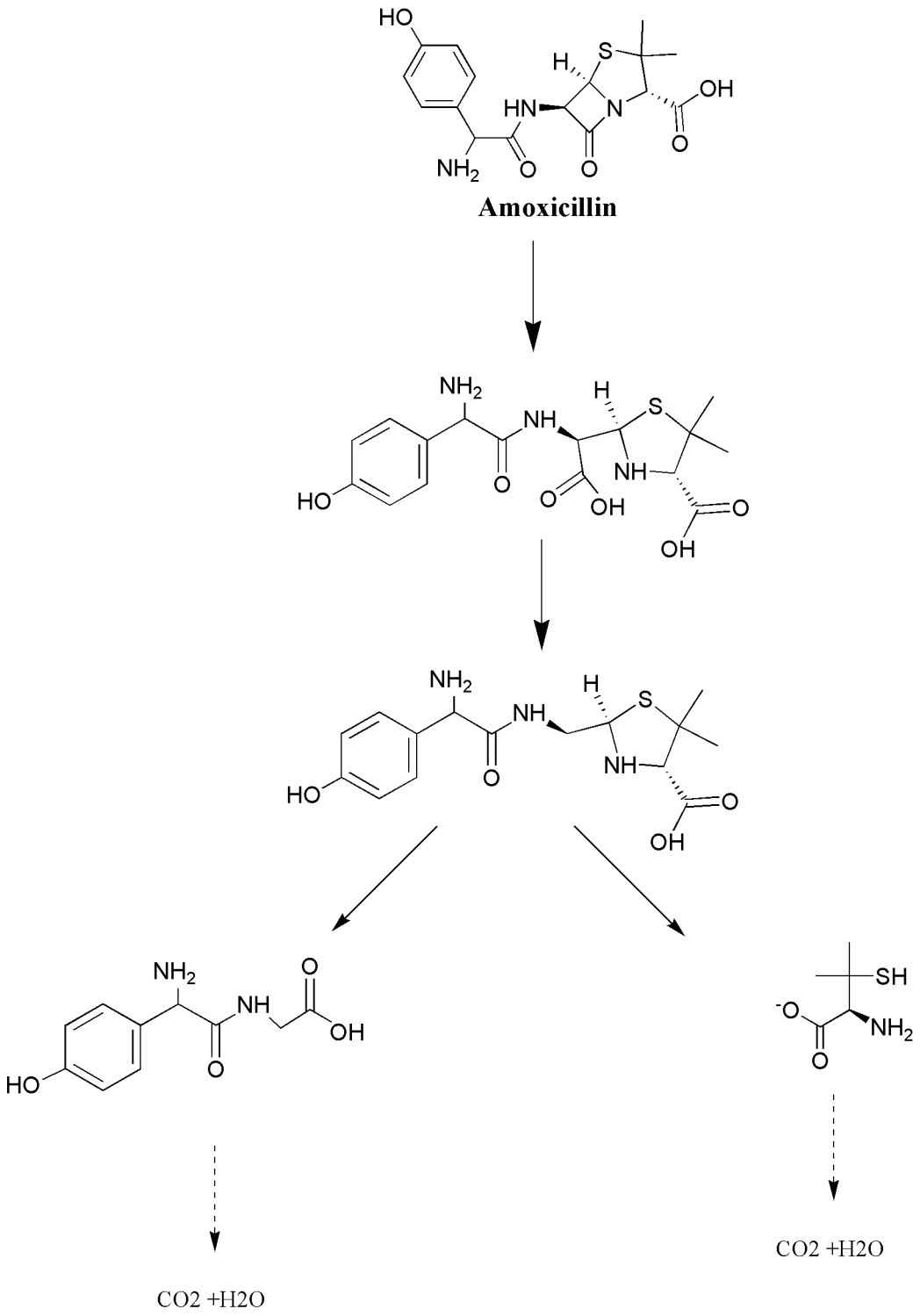


Figure 7.13. Predicted pathyway of amoxicillin biodegradation by *Serratia marcescens.*

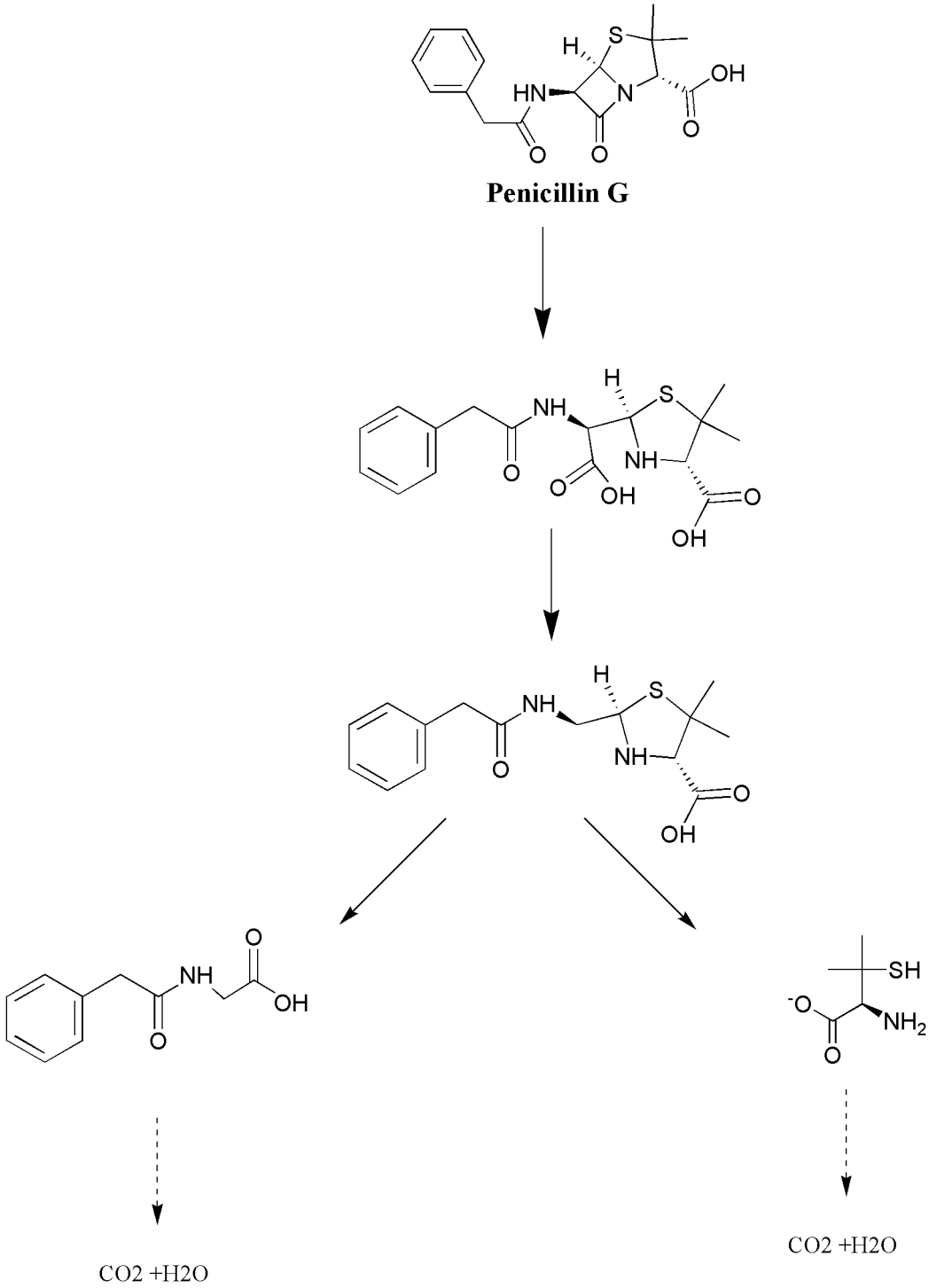


Figure 7.14. Predicted pathyway of penicillin g biodegradation by *Serratia marcescens.*

7.4. Summary

According to our results, *Serratia marcescens* BIOMIG4 was found to be a novel bacteria that can biotransform amoxicillin and degrade penicillin g. Total mineralization of penicillin g by *Serratia marcescens* BIOMIG4 has the great potential to become a treatment biotechnology to control penicillin g pollution and penicillin related allergy dissemination.

8. THE ROLE OF BIOTRANSFORMATION ON BIOCIDE RESISTANCE IN A CO-CULTURE OF *PSEUDOMONAS SP.* BIOMIG1 AND *E.COLI*

8.1. Introduction

Since QACs maintain biocidal properties in a wide pH range, they are extensively used in domestic, industrial, agricultural and medicinal applications as antimicrobial agents (McDonnell and Pretzer, 2001). They used as fabric softeners, surfactants, emulsifiers, disinfectants, pesticides, corrosion inhibitors and personal care products (Garcia et al., 1999; Steichen, 2001; Patrauchan and Oriel, 2003). Also they are added to dishwashing liquids, hand soaps, window cleaners, floor cleaners, baby care products, disinfectant sprays, air fresheners and so on (Hegstad et al., 2010). The active agent of most disinfectants available in the market is benzalkonium chlorides (BACs), the most extensively used QACs. Domestos®, Lysol® and Dixi® are extensively used commercial surface cleaners containing BACs as an active agent. BAC concentration is changing from 5% to 0.1% in these surface cleaners while the application concentration is changing from 500 mg/L to 50 mg/L but BAC concentration on the surface of floors is less than those values after application. Also BAC degrading microorganisms create BAC gradients in which susceptible microorganisms survive or even develop BAC resistance and proliferate.

It is a fact that extensive use biocides as disinfectants cause biocide resistance. Lots of bacteria reported as biocide degraders but the effect of presence of biocide degraders to biocide susceptible microorganism is not clear. Given the fact that the strain BIOMIG1 is commonly found in environmental biological systems, it is possible that we have these bacteria in our homes. Since it can degrade the active agent of surface cleaners, presence of BIOMIG1 at home may decrease the efficiency of surface cleaners. In other words, we may not be effectively cleaning our homes with such surface cleaners since BIOMIG1 may create BAC gradient which enable BAC susceptible microorganisms to survive.

This chapter of the study is focusing on the biodegradation of commercial surface cleaners, Dixi® and Domestos®, by *Pseudomonas sp.* BIOMIG1. The susceptibility of *E.coli* BIOMIG3 was tested against both synthetic BACs mixture, Dixi® and Domestos®. But the aim of the study in this chapter is to understand the influence of BAC degrading bacteria presence to the effectiveness of Domestos®, one of the most widely used commercial disinfectants. In this research *E.coli:* BAC susceptible bacteria (BAC MIC: 16 mg/L) and *Pseudomonas sp.* BIOMIG1: BAC resistant and BAC degrading bacteria were used in co-culture.

8.2. Materials and Methods

8.2.1. Domestos and Dixi

Dixi® and Domestos® are two commercial disinfectants that are being used for surface cleaning. Domestos is produced by Unilever Company and it is composed of less than 5% cationic active material (Benzalkonium chloride, phosphate and perfume). It is written on its label that it contains 1.7 g benzalkonium chloride (BAC) per liter. Dixi is produced by Henkel Company and it is composed of less than 5% nonionic active material, cationic active material and alcohol. It is written on its label that it contains 5 g of quaternary ammonium compounds (QACs) (benzyl-C12-16-alkyldimethyl chlorides) per liter.

BAC concentration in Dixi® and Domestos® were measured with HPLC method for BACs as described in 4.2.2. As it is printed on their labels, it was found that Domestos® contains 16,753 mg/L and Dixi® contains 5,006 mg/L total BACs. According to the instruction by the label, recommended application concentration for Domestos® is 335 mg/L and for Dixi® is 90 mg/L.

BACs distribution in Domestos® is as follows: 72% Dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl, $C_{21}H_{38}NCl$, 340 g/mole), 24.6% tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl, $C_{23}H_{42}NCl$, 368 g/mole) and 3.4% hexadecyl benzyl dimethyl ammonium chloride (C_{16}BDMA-Cl, $C_{25}H_{46}NCl$, 396.1 g/mole).

BACs distribution in Dixi® is as follows: 75% Dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl, $C_{21}H_{38}NCl$, 340 g/mole), 25% tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl, $C_{23}H_{42}NCl$, 368 g/mole).

BACs distribution in synthetic BACs mixture that we prepared is as follows: 40% Dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl, $C_{21}H_{38}NCl$, 340 g/mole), 50% tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl, $C_{23}H_{42}NCl$, 368 g/mole) and 10% hexadecyl benzyl dimethyl ammonium chloride (C_{16}BDMA-Cl, $C_{25}H_{46}NCl$, 396.1 g/mole).

8.2.2. BAC Biodegradation Assays by *Pseudomonas sp.* BIOMIG1

The BACs degradation potential of *Pseudomonas sp.* BIOMIG1 against Dixi®, Domestos® (commercial disinfectants containing BACs) and a synthetic BACs mixture was determined by using liquid culture method. A single colony of *Pseudomonas sp.* BIOMIG1 was transferred into sterile falcon tube containing 5 mL LB-BAC broth. Colonies were taken from CHROM®Agar Orientation plates prepared as described in 4.2.2. Then, falcon tubes were placed on an orbital shaker and agitated at 130 rpm. After microorganism were grown overnight in LB-BAC broth, cultures were transferred into sterile microcentrifuge tubes and centrifuged at 10, 000 rpm for 10 min. The supernatant was discarded, the pellet was suspend in 85% saline solution and further diluted with 85% saline solution to a turbidity comparable to that of a 0.5 McFarland turbidity standard (c.a. 0.5 x 10^8 CFU/mL). This suspension was further diluted 1:10 with 85 % saline solution.

A sufficient number of 50 mL falcon tubes were taken and 5 mL MSM was put into each of the falcon tube. A 1 mL aliquot of the diluted culture sample was transferred to culture tubes containing 9 mL MSM and a range of BAC concentrations ranging from 1 to 500 mg/L (Content is diluted by factor 2) (Figure 8.1.). A control set was prepared as described above but excluding the culture. The content in each falcon tube was agitated on an orbital shaker at 130 rpm at room temperature until all BACs were utilized.

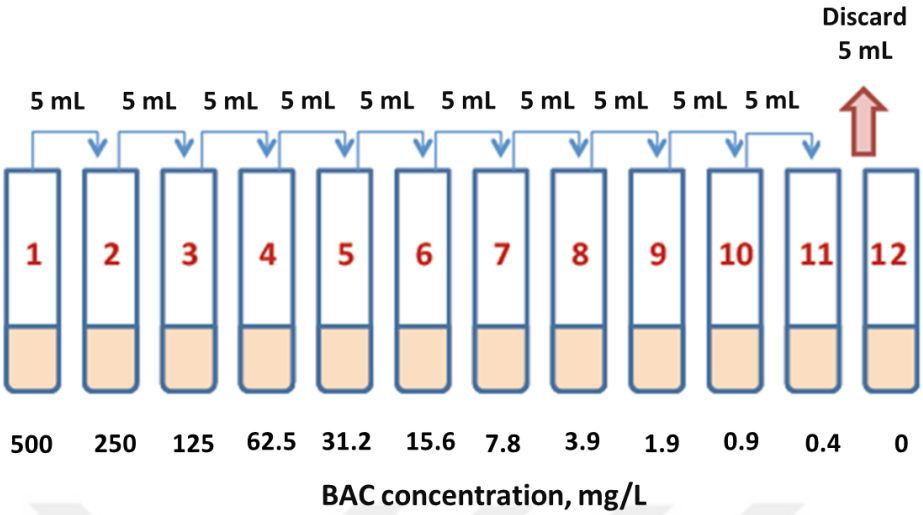


Figure 8.1. Preparation of biodegradation testing tubes using serial dilution.

8.2.3. Mono-culture and Co-culture Susceptibility Assays

a. Mono Culture Susceptibility Assays: The susceptibility of *E.coli* against Dixi®, Domestos® and a synthetic BACs mixture was determined using macro dilution assay as described by Clinical and Laboratory Standards Institute (CLSI 2006). A single colony of each microorganism was transferred into sterile falcon tube containing 5 mL Mueller-Hinton broth. Colonies were taken from CHROMAgar™ ECC Agar plates prepared as described in 4.4.2. After microorganism were grown overnight in Mueller-Hinton broth, they were diluted in Mueller-Hinton broth to a turbidity comparable to that of a 0.5 McFarland turbidity standard (c.a. 0.5 x 10^8 CFU/mL). This suspension was further diluted 1:100 with Mueller-Hinton broth. A 1 mL of the diluted culture sample was transferred to culture tubes containing 1 mL broth and a range of BAC concentrations ranging from 1 to 500 mg/L (Content is diluted by factor 2) (Figure 8.2.). The tubes were incubated at room temperature (22 °C) for 24 hours and the growth was measured using a UV/Vis spectrometer at 600 nm.

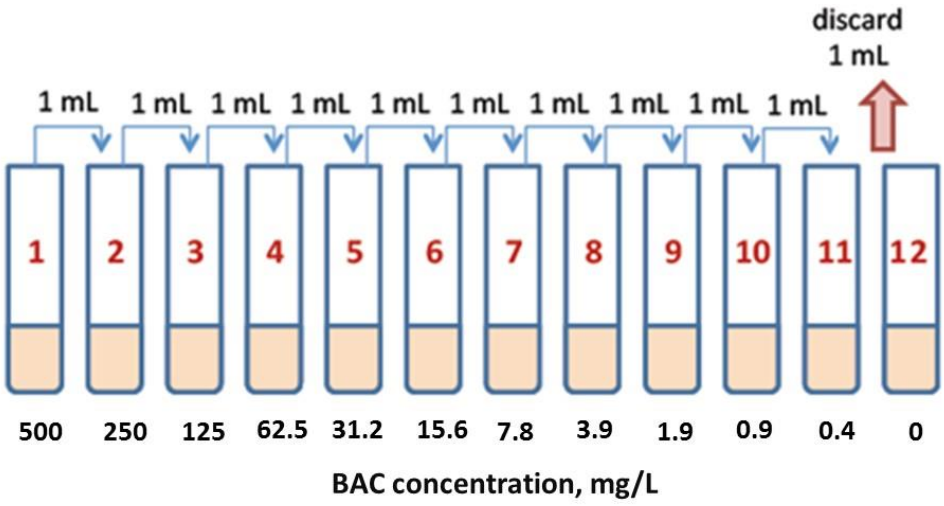


Figure 8.2. Preparation of susceptibility test tubes using serial dilution.

<u>b. Co-culture Susceptibility Assays:</u> Co-culture susceptibility assay was done against Domestos® with Pseudomonas sp. BIOMIG1 and E.coli using liquid culture method. *E.coli* and *Pseudomonas sp.* BIOMIG1 cultures were prepared same as in BAC biodegradation assays performed with *Pseudomonas sp.* BIOMIG1.

A sufficient number of 50 mL falcon tubes were taken and 5 mL 2X MSM was put into each of the falcon tubes. Content was diluted by factor 2 (a range of QAC concentrations from 1 to 125 mg/L). 1 mL of a 10,000 mg/L maltose solution was added to the each falcon tube to have a 1,000 mg/L final concentration. This procedure was repeated four times. These four sets were labeled as co-culture, control *E.coli*, control *Pseudomonas sp.* BIOMIG1 and negative control. For co-culture set, 1 mL of *E.coli* and 1 mL of *Pseudomonas sp*. BIOMIG1 culture were added to falcon tubes and total volume of each falcon tube was adjusted to 10 mL by addition of necessary amount of DI water. For control *E.coli* set, 1 mL of *E.coli* culture was added to falcon tubes and total volume of each falcon tube was adjusted to 10 mL by addition of a necessary amount of DI water. For control *Pseudomonas sp.* BIOMIG1 set, 1 mL of *Pseudomonas sp.* BIOMIG1 culture was added to falcon tubes and total volume of each falcon tube was adjusted to 10 mL by addition of necessary amount of DI water. For negative control set, volume of each falcon tubes was adjusted to 10 mL by addition of

necessary amount of DI water without putting any culture in it. The content in each falcon tube was agitated on an orbital shaker at 130 rpm at room temperature. Growth was measured daily using a UV/Vis spectrometer at 600 nm wavelength and BACs concentration was measured using HPLC method for BACs.

8.3. Results and Discussion

8.3.1.1. Biodegradation of BACs in Commercial Disinfectants by *Pseudomonas sp.* BIOMIG1

Biodegradation pattern of Dixi® and Domestos® (commercial disinfectants containing BACs) and synthetic BACs mixture by *Pseudomonas sp.* BIOMIG1 was determined (Figure 8.3-5.). While BACs concentration did not change in the control reactors having no culture, 100% of the BACs was utilized within 20 days in all culture series having different initial concentration of synthetic BACs mixture (Figure 8.3.). Also *Pseudomonas sp.* BIOMIG1 utilized 100% of the BACs within one week in culture series having initial synthetic BACs mixture concentration up to 62.5 mg/L.

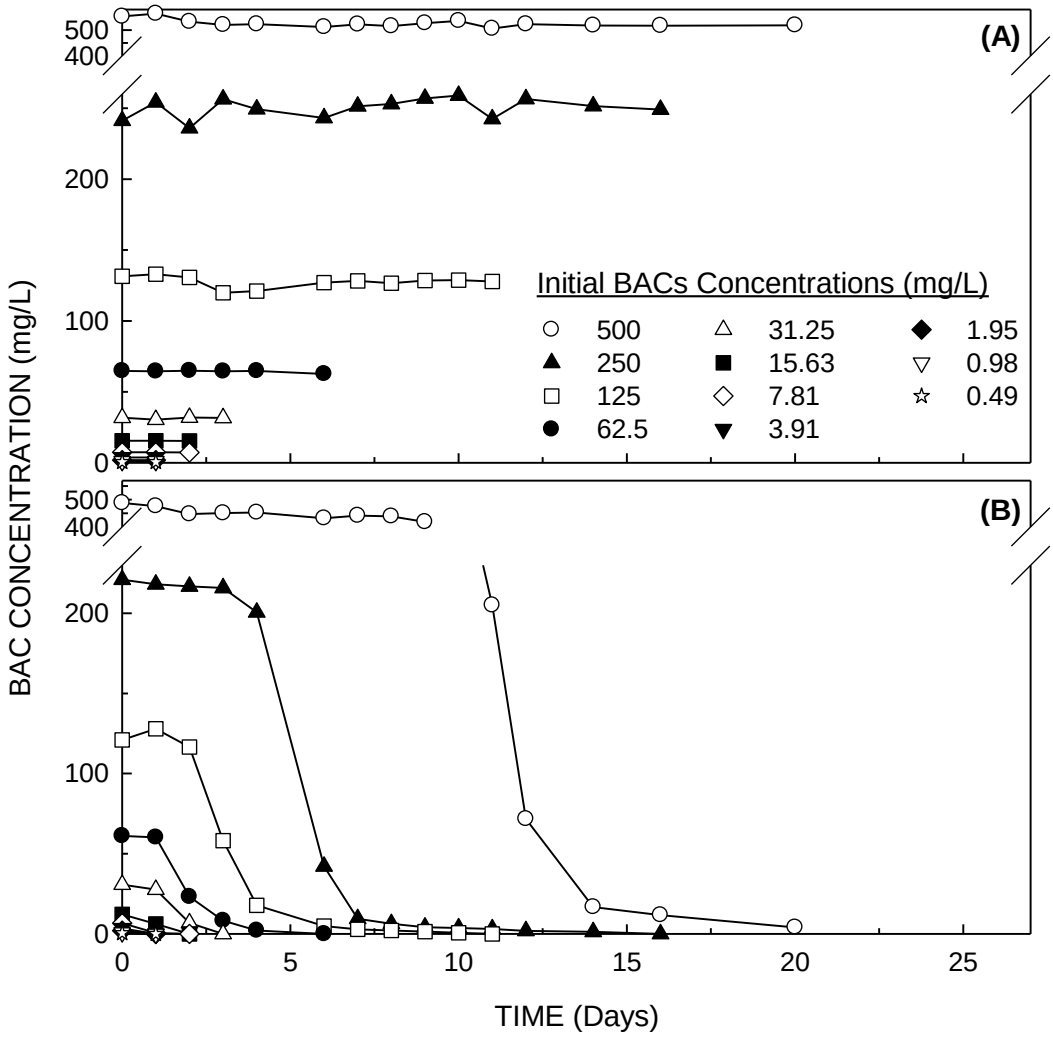


Figure 8.3. Utilization of synthetic BACs mixture (A) Control (B) *Pseudomonas sp.* BIOMIG1.

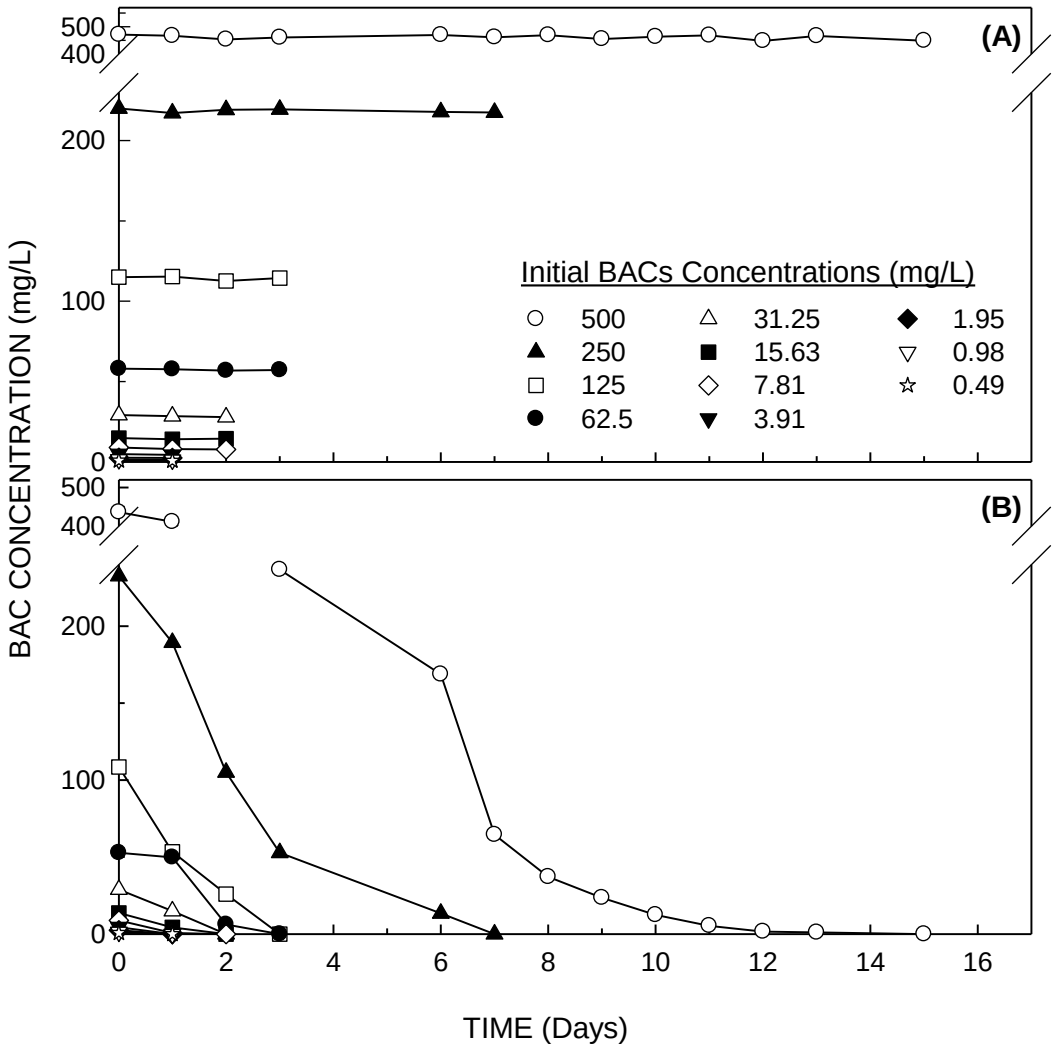


Figure 8.4. Utilization of Dixi® (A) Control (B) *Pseudomonas sp.* BIOMIG1.

While BACs concentration did not change in the control reactors having no culture, 100% of the BACs was utilized within 15 days in all culture series having different initial concentration of BACs present in Dixi® (Figure 8.4.). Also *Pseudomonas sp.* BIOMIG1 utilized 100% of the BACs within one week in culture series having initial synthetic BACs mixture concentration up to 250 mg /L.

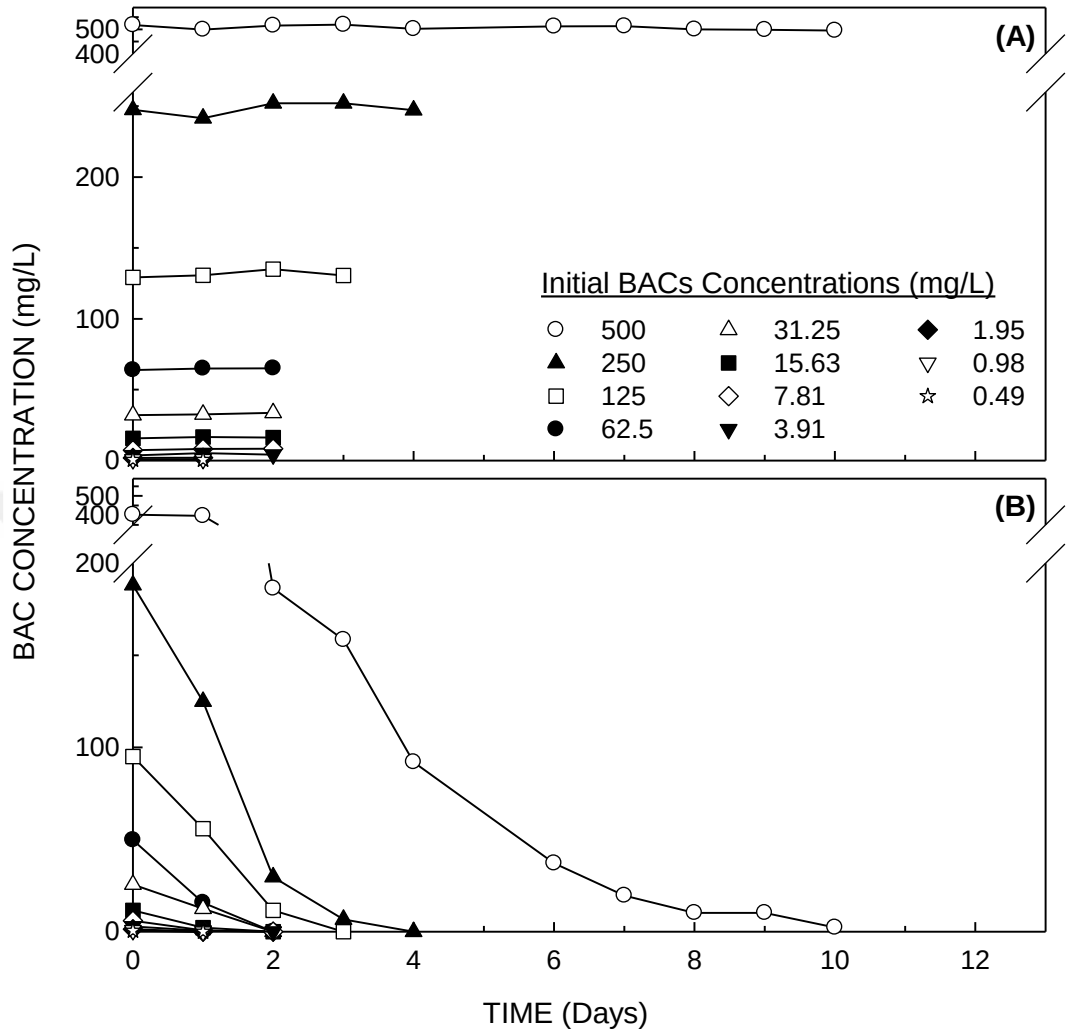


Figure 8.5. Utilization of Domestos® (A) Control (B) *Pseudomonas sp.* BIOMIG1.

While BACs concentration did not change in the control reactors having no culture, 100% of the BACs was utilized within 10 days in all culture series having different initial concentration of BACs present in Domestos® (Figure 8.5.). Also *Pseudomonas sp.* BIOMIG1 utilized 100% of the BACs within one week in culture series having initial synthetic BACs mixture concentration up to 500 mg /L.

According the results obtained by using liquid culture method, Domestos® biodegradation rate of *Pseudomonas sp.* BIOMIG1 is higher than Dixi® followed by synthetic BACs mixture. Different biodegradation rates may because of BAC distribution differences in Domestos®, Dixi® and our synthetic BACs mixture. It was recently reported that *Pseudomonas sp.* BIOMIG1 biodegrade C_{14}BDMA-Cl faster than C_{12}BDMA-Cl followed by C_{16}BDMA-Cl (Yılmaz. 2014).

8.3.2. Susceptibility of *E. coli* to BACs in Commercial Disinfectants

The MIC values for *E.coli* against commercial disinfectant Domestos® and Dixi® and a synthetic BACs mixture were measured by using macro dilution method and they found to be around 16 mg/L for all of them.

8.3.3. Susceptibility of *E.coli* to BACs in the Presence of *Pseudomonas sp.* BIOMIG1

During co-culture susceptibility assays turbidity and BACs concentrations were monitored daily (Figure 8.6-9.). In negative control series having different initial BACs concentration, no turbidity were observed and BACs concentration did not change during the assay (Figure 8.6.). This showed that there was no contamination in the medium and BAC concentration did not change with time in the absence of bacteria.

In control *E.coli*, significant turbidity was measured in tubes having 0, 0.48, 0.96, 1.98 and 3.91 mg/L initial BAC concentrations (Figure 8.7. A-E), while tubes having initial BAC concentration higher than 3.91 mg/L turbidity was not observed (Figure 8.7. F-J). This suggests that *E.coli* can only grow BACs present in Domestos® up to 3.91 mg/L and after that concentration they died (Figure 8.7.). Also no change was observed in BAC concentration in any control *E.coli* tubes (Figure 8.7.).

No growth was observed in control *Pseudomonas sp.* BIOMIG1 series having zero initial BACs concentration (Figure 8.8. A). This trend is same for all control *Pseudomonas sp.* BIOMIG1 series having different initial BACs concentration (Figure 8.9. B-J). However,

BACs were degraded by *Pseudomonas sp.* BIOMIG1 depending on the initial BACs concentration (Figure 8.8.). This was showing that the growth of *Pseudomonas sp.* BIOMIG1 does not contribute turbidity. Also, *Pseudomonas sp.* BIOMIG1 utilized 100% of the BACs within 12 days in all culture series having different initial BACs concentrations.

When we compare the turbidity of control *E.coli* with zero BACs conteration (Figure 8.7. A) and control *Pseudomonas sp.* BIOMIG1 with zero BAC conteration (Figure 8.8. A), it was observed that while the growth of *E.coli* was contributing to turbidity, the growth of BIOMIG1 did not contribute to turbidity. So we can suggest that any turbidity in co-culture reactors will come from *E.coli.*

The turbidity results of co-culture series (Figure 8.9. A-E), were followed same trend with control *E.coli* series (Figure 8.7. A-E) having initial BACs concentrations up to 3.91 mg/L. On the other hand, in co-culture tubes having initial BACs concentration more than 3.91 mg/L, it was observed that the turbidity increased by decreasing BACs concentration (Figure 8.9. E-J). Given the fact that *Pseudomonas sp.* BIOMIG1 did not contribute to turbidity, the turbidity in these tubes was assumed to come from growth of *E.coli* (Figure 8.9. E-J). So it can be suggested that the presence of *Pseudomonas sp.* BIOMIG1 enable BAC susceptible *E.coli* to live up to 125 mg/L initial BACs concentration while *E.coli* can only tolerate 3.91 mg /L initial BACs concentration alone.

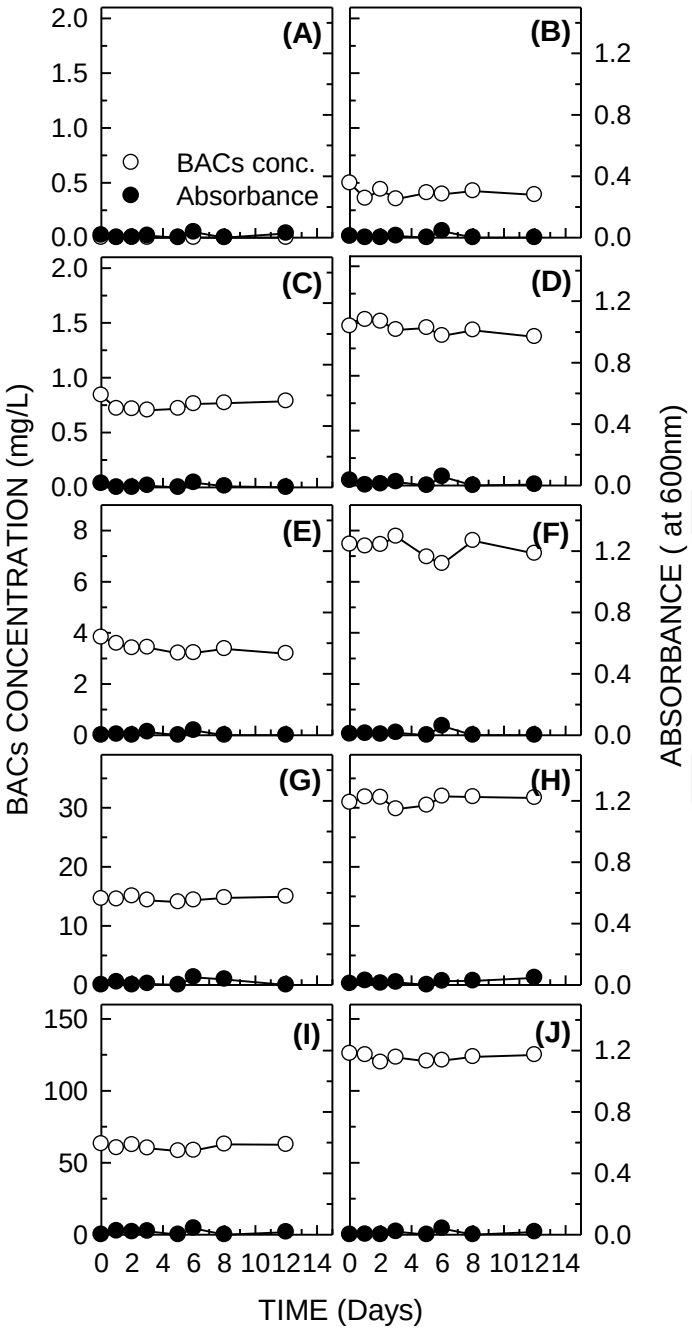


Figure 8.6. Profile of BAC concentration in control at (A) 0, (B) 0.48, (C) 0.97, (D) 1.95, (E) 3.9, (F) 7.8, (G) 15.62, (H) 31.25, (I) 62.5, (J) 125 mg /L initial BACs concentrations.

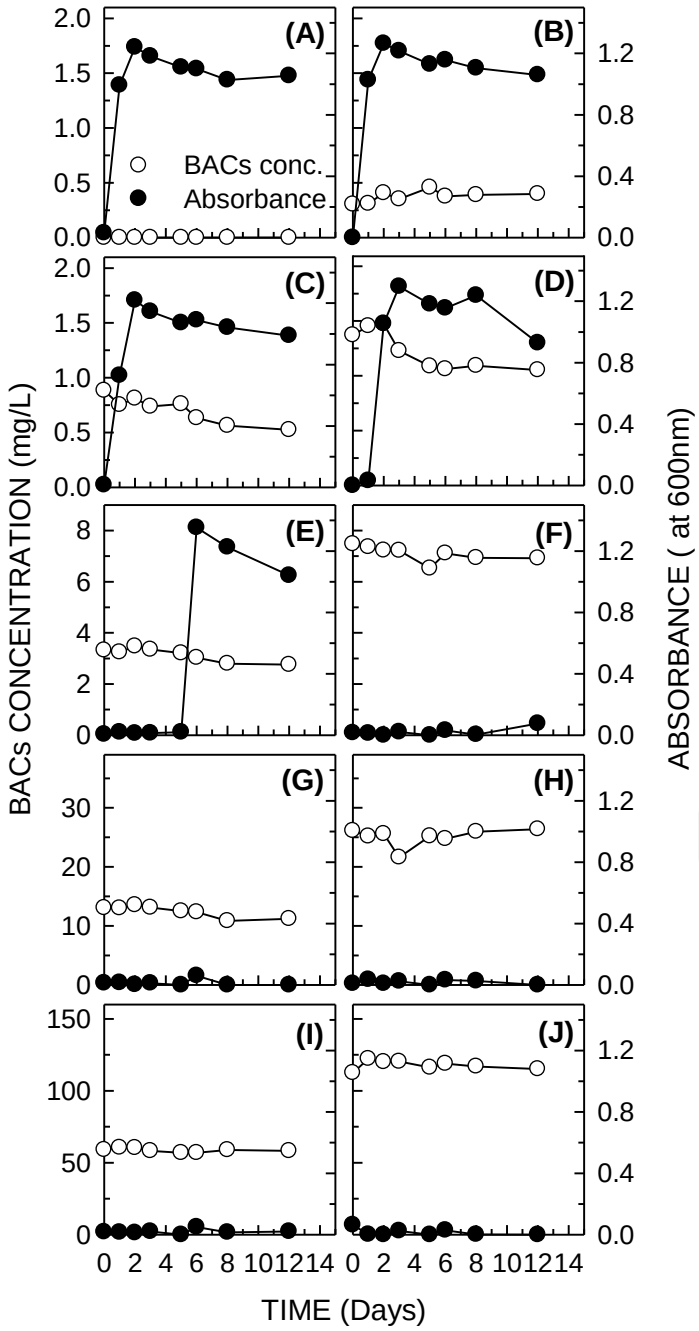


Figure 8.7. Profile of BAC utilization in *E.coli* and growth of *E.coli* at (A) 0, (B) 0.48, (C) 0.97, (D) 1.95, (E) 3.9, (F) 7.8, (G) 15.62, (H) 31.25, (I) 62.5, (J) 125 mg /L initial BACs concentrations.

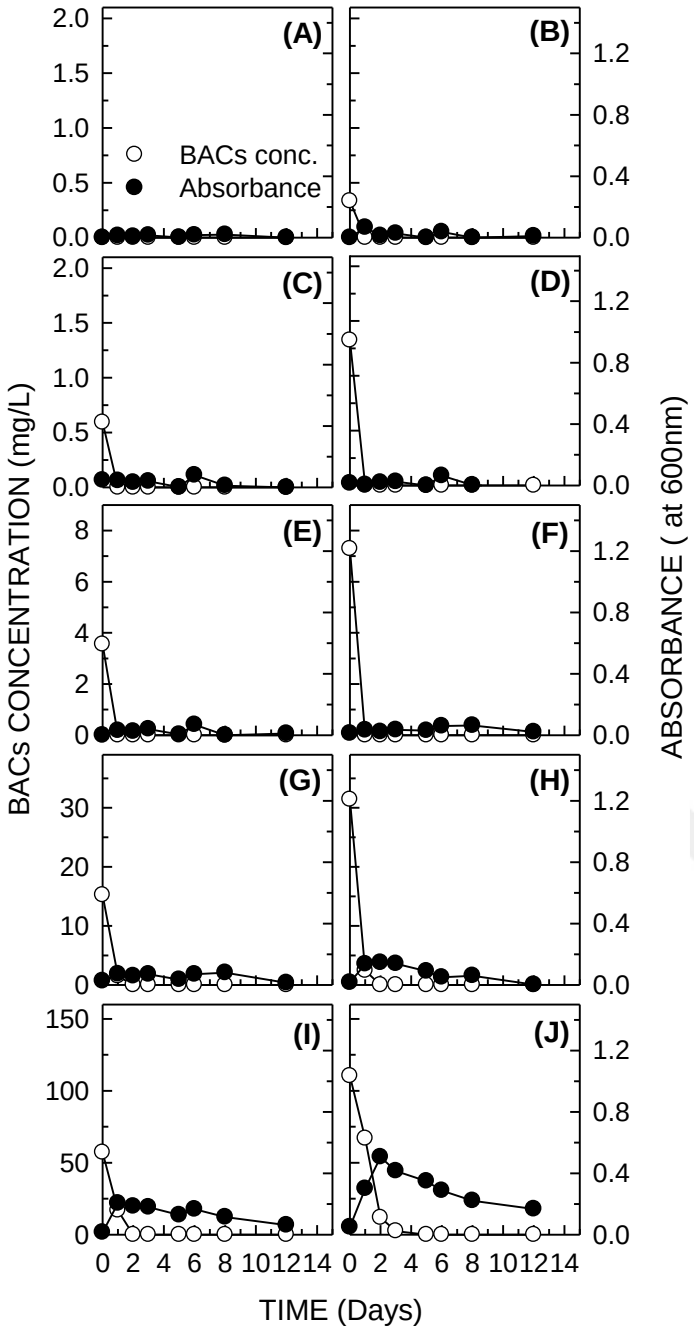


Figure 8.8. Profile of BAC utilization in *Pseudomonas sp.* BIOMIG1 SEW and growth of *Pseudomonas sp.* BIOMIG1 SEW at (A) 0, (B) 0.48, (C) 0.97, (D) 1.95, (E) 3.9, (F) 7.8, (G) 15.62, (H) 31.25, (I) 62.5, (J) 125 mg /L initial BACs concentrations.

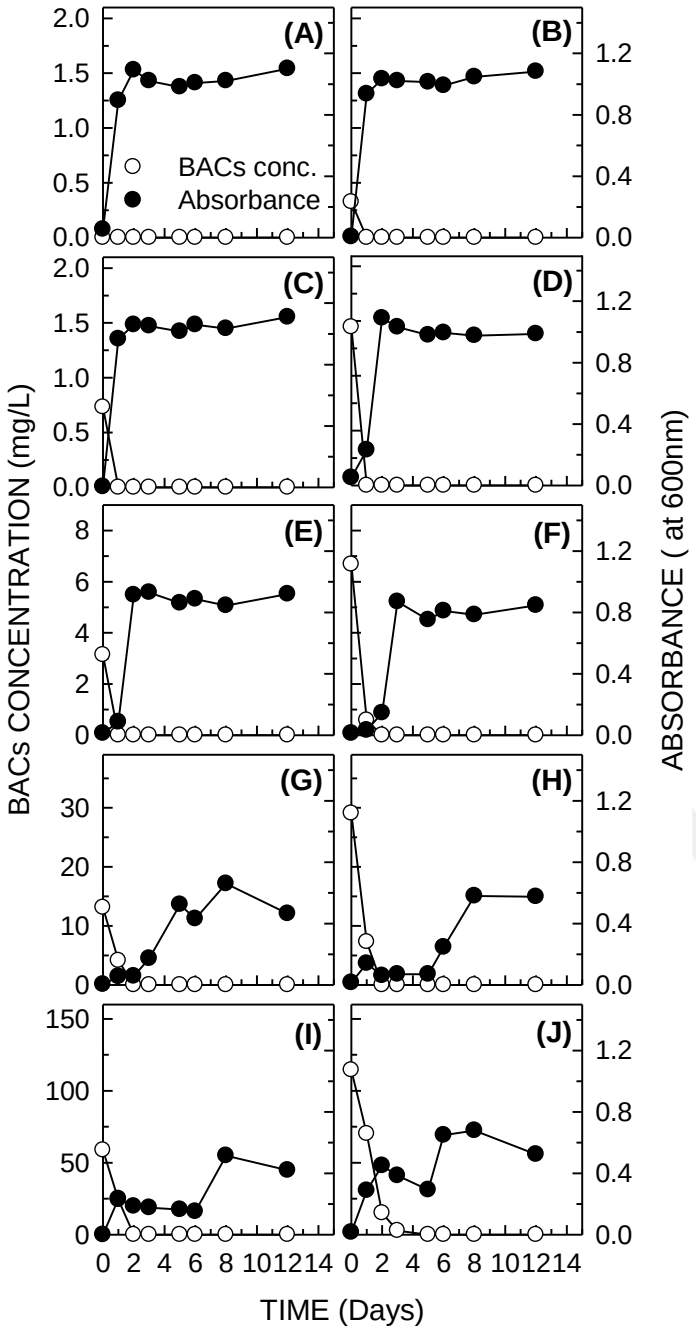


Figure 8.9. Profile of BAC utilization in co-culture and the growth of *E.coli* at (A) 0, (B) 0.48, (C) 0.97, (D) 1.95, (E) 3.9, (F) 7.8, (G) 15.62, (H) 31.25, (I) 62.5, (J) 125 mg /L initial BACs concentrations.

8.4. Summary

According to our results *E.coli* can survive initial BAC concentrations that are up to 125 mg/L in the presence of *Pseudomonas sp.* BIOMIG1, while it can survive only 3.91 mg/L initial BAC concentration without the presence of *Pseudomonas sp.* BIOMIG1. These findings suggest that *Pseudomonas sp.* BIOMIG1 protects BACs susceptible *E.coli* from disinfectant.

9. CONCLUSIONS

During the course of this thesis research, an activated sludge ecotype of *Pseudomonas sp.* BIOMIG1, which was resistant to BACs and can degrade BACs, was isolated from a sample taken from Paşaköy Municipal Wastewater Treatment Plant, Istanbul. Tolerance of *Pseudomonas sp.* BIOMIG1 AS as well as *Pseudomonas sp.* BIOMIG1 SEW, *E.coli* and *Serratia marcescens* to 18 antibiotics, which belong to different antibiotic groups with distinct mode of actions, was determined in terms of MIC by using macro dilution and E-test methods. MIC values of 18 antibiotics obtained for BIOMIG1 ecotypes were compared to MICs obtained for *E. coli* which was a BAC susceptible bacteria and *Serratia marcescens* which was a BAC tolerant bacteria but cannot degrade BACs. Results showed that *Pseudomonas sp.* BIOMIG1s were more resistance to 16 out of 18 tested antibiotics than the other bacteria tested. Furthermore, it was found that BIOMIG1 exhibits an approximately 10 fold resistance compared to *E.coli* and more than 2 fold resistance compared to *Serratia marcescens* against 13 out of 18 antibiotics. In addition, BAC resistant *Serratia marcescens* was more tolerant to antibiotics compared to BAC susceptible *E.coli*. These results may suggest that BAC resistance mechanisms also favor antibiotic resistance.

Moreover, biodegradation potential of 7 antibiotics by BAC tolerant *Pseudomonas sp.* BIOMIG1 and *Serratia marcescens* was investigated. However, none of the tested seven antibiotics; clindamycin, vancomycin, chloramphenicol, sulfamethoxazole, trimethoprim, amoxicillin and penicillin g, were degraded by BIOMIG1 suggesting that although BIOMIG1 can degrade BACs using a novel Rieske-type oxygenase, this enzyme did not facilitate biodegradation of these antibiotics. On the other hand, *Serratia marcescens* which cannot degrade BACs, degraded amoxicillin and penicillin g antibiotics of which *Serratia marcescens* are more resistant than *Pseudomonas sp.* BIOMIG1.

In the last part of the study, survival of *E. coli*, which is a BAC susceptible bacteria, in a commercial disinfectant formulation containing BACs in the presence of BIOMIG1 was monitored. Results showed that presence of BIOMIG1 facilitated the survival and growth of *E. coli* upto 125 mg/L BACs which was 30 times higher than its MIC.

In conclusion, BAC degrading BIOMIG1, which is abundant in the environment, is antibiotic resistant. Given the fact that it promotes the survival of susceptible pathogens in commercial BAC disinfectants at concentrations that are supposed to be kill those pathogens, it not only decreases the efficacy of disinfectants but also does create habitable environments in human contact settings for development and dissemination of antimicrobial resistance.

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