İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

APPLICATION OF MOLECULAR BIOLOGY TECHNIQUES FOR MICROBIOLOGICAL ANALYSIS IN WASHING MACHINES

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MOLEKÜLER BİYOLOJİ TEKNİKLERİNİN ÇAMAŞIR MAKİNESİNDE MİKROBİYOLOJİK ANALİZLER İÇİN UYGULANMASI

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ABBREVIATIONS

EDTA	**:** Ethylene di-amine tetra-aceticacid
SDS	**:** Sodium dodecyl sulfate
THM	**:** Trihalomethane
PVC	**:** Polyvinylchloride
PE	**:** Polyethylene
PU	**:** Polyurethane
PP	**:** Polypropylene
PTFE	**:** Polytetrafluoroethylene
QS	**:** Quorum sensing
bp	**:** Base pair
LB	**:** Luria-Bertani
SOC	**:** Super optimal broth with catabolite repression
X-gal	**:** 5-Bromo-4-chloro-3-Indolyl-B-D-Galactopyranoside
TBE	**:** Tris-Borate-EDTA
DMF	**:** Di-methyl formamide
EtBr	**:** Ethidium bromide
UARR	**:** Universal amplified ribosomal region
PCR	**:** Polymerase chain reaction
BLAST	**:** Basic local alignment search tool
MEGA	**:** Molecular evolutionary genetics analysis
DD	**:** Detergent drawer
DH	**:** Discharge hose
TS	**:** Tub seal
ORF	**:** Open reading frame

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MOLEKÜLER BİYOLOJİ TEKNİKLERİNİN ÇAMAŞIR MAKİNESİNDE MİKROBİYOLOJİK ANALİZLER İÇİN UYGULANMASI

ÖZET

Son yıllarda biyofilm oluşumu, su sistemlerinde, medikal uygulamalarda ve evsel yüzeylerde sıkça rastlanılan bir problem haline gelmektedir. Sistem performansı ve yüzey etkilerine olan olumsuz etkilerinin yanı sıra, biyofilm mikroorganizmaları göz, kulak, mide, akciğer gibi çeşitli enfeksiyonlara da sebep olarak insan sağlığı için potansiyel risk teşkil etmektedir.

Bu çalışmanın amacı, çamaşır makinelerinde oluşan biyofilmin ökaryotik içeriğini belirlemek ve farklı ülkelerde düşük sıcaklıklarda çalıştırılan iki çamaşır makinesinin biyolojik çeşitliliğini karşılaştırmaktır.

Çalışmada, mikroorganizmaların tayini, 18s ribozomal RNA gen dizilimlerini hedefleyen polimeraz zincir reaksiyonu ve reaksiyonu takiben klonlama ve transformasyon ile gerçekleştirilmiştir. 18s ribozomal RNA gen dizilimleri taksonomik ve filogenetik analizlerde sıkça kullanılmaktadır.

Örnekler iki farklı çamaşır makinesine ait 3 farklı bölgeden (deterjan çekmecesi, körük ve ön kapak çevresi, kazan çıkış hortumu) toplanmıştır. Örneklerin toplandığı bölgeler; nem, havalandırma ve sürtünme gibi farklı çevresel özellikler ile birbirinden ayrılmaktadır. Toplanan örneklerden, kültür yöntemi kullanılmaksızın, genomik DNA izolasyonu gerçekleştirilmiştir. Daha sonra UARR PCR reaksiyonu gerçekleştirilmiş ve PCR ürünleri saflaştırıldıktan sonra TOPO TA klonlama vektörüne klonlanmıştır. Klonlama sonrasında elde edilen sekans analizi sonuçları BLAST veritabanında bulunan diğer 18s ribosomal RNA dizilimleri ile karşılaştırılarak tür tayini gerçekleştirilmiştir. Belirlenen türlerin; protozoa, mantar ve metazoa krallıklarına ait olduğu belirlenmiştir.

Farklı çevresel faktörler ve kullanıcıların yaşam farklılıkları nedeniyle, iki çamaşır makinesi arasında ciddi bir benzerlik görülmemiştir. Benzer özellik gösteren coğrafik koşullarda yaşayan kullanıcılardan alınan çamaşır makineleri üzerinde yapılacak çalışmalarla daha ayrıntılı karşılaştırmalar yapılabilecektir.

APPLICATION OF MOLECULAR BIOLOGY TECHNIQUES FOR MICROBIOLOGICAL ANALYSIS IN WASHING MACHINES

SUMMARY

In last years, biofilm formation become a serious problem in water systems, medical applications and house hold surfaces. In addition to its negative effects on system performance and surface properties, biofilm microorganisms are potential risks for human health causing several infections such as eye, ear, gastrointestinal, lung infections etc…

Purpose of this study is to identify eukaryotic sources of biofilm in the washing machines and compare the biodiversity of washing machines which were primarily operated at low temperature and in different countries.

In the study, identification of microorganisms were performed by polymerase chain reaction targeting 18s ribosomal RNA gene and followed by cloning and transformation. 18s ribosomal RNA gene sequences were used for taxonomic and phylogenetic analysis. Samples were collected from three different parts (detergent drawer, tub seal and discharge hose) of two washing machines. The parts differ from each other by the medium properties such as humidity, aeration and shearing effects. Genomic DNA isolation was performed for the each sample without cultivation. Next, UARR PCR was set and products were purified. Purified PCR products were cloned into TOPO TA cloning vector and plasmids were transformed into *E. Coli* TOP10 cells. Species were identified by the comparison of sequence analysis results of 18s ribosomal RNA of the cloned insert using BLAST tool. Species were found to be belonging to protozoa, fungi and metazoa kingdoms.

In the result, no significant similarity between two washing machines probably due to different environmental factors and life style of consumers'. For a more detailed comparison, washing machines from closer regions with similar geographic properties may be used in future studies.

1. INTRODUCTION

Efficient use of energy become critically important due to the global warming and limited natural resources all over the world. In order to decrease energy consumption of electric-powered household gadgets, white goods industry started to focus on systems with less energy and water requirements. Similarly; washing machine manufacturers develop new programs working in lower temperatures and shorter periods. New products, designed to wash in low temperatures, provide energy savings; but after a period of use, microbial formations occur in both visible and invisible parts of washing machines. These microbial formations, defined to be biofilm, cause complaints by consumers such as visual pollution and unpleasant odors. Biofilm complaints were first occured in American market where cold-wash programs for washing machines are very common.

Biofilm is known to cause several problems such as blockage in water systems, decreased heat transfer from heat exchangers, corrosion on metalic surfaces, contamination in food, biotechnology and medical industries and consequently threatening consumers' health. Microorganisms of the biofilm may establish host interaction with human resulting in many serious infections and cautions against biofilm are usually remain insufficient because of the improved resistance of biofilm microorganisms.

In this study, different parts of two washing machines, were investigated for the biofilm information. Purpose of the study is to identify the eukaryotic sources of biofilm using molecular biology techniques and compare the biological diversity of biofilms formed in two different washing machines in different conditions.

2. THEORETICAL INFORMATION

2.1. Biofilm

When bacterial cells contact with inert surfaces, they first attach to the surface by their external structures such as flagella, fimbriae and/or capsular components. When the cells remain attached on the surface they secrete sticky extra cellular substances forming a matrix gel. The matrix consists of mainly polysaccharides, besides of proteins, nucleic acids, lipids, mineral ions and various cellular debris. Several layers of cells embed in the matrix gel and the layer of cells within the matrix is called biofilm [1].

Microorganisms attach on a living or non-living surface, aggregate on their self produced-extracellular polymeric matrix and form biofilm layer. These sessile communities may be any microorganism such as bacteria, fungi, protozoa and any other microorganisms secreting extra cellular polysaccharides [2, 3].

Biofilm formation includes many complex and controlled steps. These steps may vary due to metabolic activity of the microorganism but the overall formation is similar for every species [4]. Aggregation on a metal surface, reversible and irreversible attachment and maturation steps are shown in Figure 2.1.

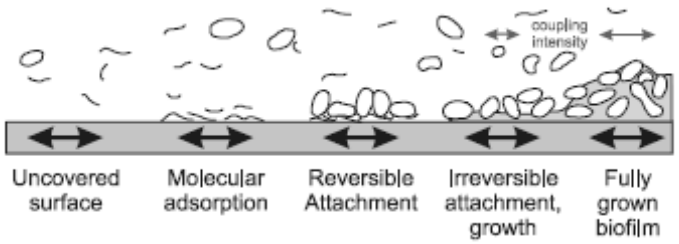


Figure 2.1. Steps of biofilm formation [4].

Biofilm formation starts with Brownian motion, van der Waals interactions, gravity, electrostatic and hydrophobic interactions take place in bacterial attachment. Irreversible attachment occurs when bacteria – surface interacts for a period. Irreversible attachment is followed by maturation, reproduction and secretion of

extracellular polysaccharides such as glucose, galactose, mannose and fructose. According to researchers, extracellular polysaccharides promotes different species attachment to the surface. In other words; it becomes possible for non-secreting microorganisms to attach to a pre-formed biofilm and increase in metabolic and physiological activities in the biofilm and variety of the biofilm is observed [2, 4].

2.2. Factors Effecting Biofilm Formation

The accumulation of microorganisms on the surfaces and the formation of biofilm depend on many factors prevailing in the system, such as temperature and hydraulics of the system, surface material and microbial occurrence in the water. Factors effecting biofilm formation can be classified into medium properties, surface properties and structure of microorganism.

2.2.1. Medium properties

One of the most important parameters effecting biofilm formation is humidity. Humid surfaces, aquatic mediums and soft tissue surfaces of living organisms are appropriate surfaces for biofilm formation. It is known that biofilm may occur in any temperature above +5°C. However biofilm community is determined by ambient temperature [5].

On living surfaces such as gingival and innards, pH of the medium is also very important for the content, biological activity and pathogenicity of biofilm. But in uncontrolled pH mediums thicker biofilm layers are observed. According to such studies, surface properties are more effective in non-living surfaces. According to similar studies polypropylene, polyethylene and polystyrene surfaces are appropriate for biofilm formation in different pH values [6, 7].

2.2.2. Surface properties

First step of biofilm development is the bacterial attachment to the surface and adherence of organic compounds. This is followed by matrix and biofilm formation. Attachment and adherence steps are significantly affected by surface properties such as roughness and charge distribution [8].

Surface roughness is a promoter parameter for biofilm formation. As rugosity (a parameter defined for surface roughness) of the material increases, biofilm formation

also increases. Activated carbon, having the highest rugosity among diatomite earth, sand and glass; has achieved the highest biofilm formation. Similarly, glass and diatomite earth, having lowest rugosity, achieved lowest biofilm formation [8].

Results of studies specific to microorganisms *Staphylococcus* and *Streptococcus sanguis* show that these microorganisms adhere more easily to a hydrophobic surface. However, correlation between hydrophobicity of surface and biofilm formation is not clear yet [9].

2.2.3. Structure of microorganism

Microorganism structure is also very important in biofilm development. Several virulence factors, exhibited by pathogenic bacteria associated with human infections, are also needed for biofilm development such as flagella and pilus [10].

Furthermore, extracellular polysaccharides secreted by microorganisms are also known to be effective in bacterial attachment but the mechanism is not clear yet.

2.3. Effects of Biofilm Formation

2.3.1. Effects on device

Biofilm layer, formed by bacterial aggregation and attachment on surface, leads to corrosion causing serious damage. *Pseudomonas*, *Hafnia alvei*, *Desulfovibrio desulfuricans* and *Bacillus* are genus known to cause corrosion on steel, iron and nickel surfaces [11]. Thick biofilm layers and metabolic activities running inside; make fluid flow more difficult and cause block in water pipes. Biofilm layer also acts like a barrier and affects heat transfer negatively [2, 4].

Although biofilm layer does not reach a thickness leading corrosion, it may become visible. So, biofilm formed on visible and available surfaces cause visual pollution and also bad odors [12].

2.3.2. Effects on human health

Studies concerning biofilm effects on human health are generally performed on water/systems (dentistry systems), prosthesis and implants. Biofilm formation and metabolic activities within may cause serious health risks due to the community. Most of these health risks are nosocomial infections of gastrointestine, eye and ear

etc. Nosocomial infections are especially common in hospitals, targeting patients with weaker immune system. Since microorganisms of biofilm has resistance to antibiotics, such contamination leads to longer treatment periods and disinfection problems [2].

People, just as in the case of water lines and domestic surfaces, also have surfaces for microorganisms to attach. Nevertheless, regenerative properties of people's living surfaces keep biofilm formation in a limited amount.

When microorganisms of a biofilm find themselves faced with a human host and establish the required interaction, the result can be a serious infection, even death. For example, pathogenicity of *Mycobacterium tuberculosis* comes out after long periods of interaction with its host [13].

Some of the common microorganisms forming biofilm on both living and non-living surfaces are listed in Table 2.1.

Table 2.1. Biofilm forming microorganisms [14].

Microorganism	Biofilm Surface
Staphylococcus aureus	Medical devices
Staphylococcus epidermidis	Medical devices
Pseudomonas aeruginosa	Lungs of cystic fibrosis patients
Escherichia coli	Urinary tract and digestive system
Streptococcus spp.	Teeth and gingiva
Actinomyces spp.	Teeth and gingiva
Lactobacillus spp.	Vaginal tissue, teeth and gingiva

2.4. Biofilm Formation on Washing Machines

Restoration of the fitness for use and the aesthetic properties of the textiles is the priority of laundering process; that is, removal of soil, stains and creases. During the usage period, visible soil and invisible microorganisms contaminate the textile products. It may become evident to consumers if present in too high amounts and under certain conditions in the form of biofilm on the inner surfaces of washing machines, unpleasant odors or visible mold growth on the textiles [15].

Another source of contamination in washing machines is the water line that is used for laundering and the air that we are breathing. Microorganisms, contaminating

washing machines by carriers such as water, air and textiles; can form biofilms in aquatic mediums.

Potential biofilm microorganisms, their sources and frequency on literature and health risks are listed in Table 2.2.

Table 2.2. Pathogens in washing machines [16 – 18].

Sources	Microorganisms	Frequency	Health Risks
Water Lines	*Legionella* sp.*	< 30 %	Legionnaires disease
	Mycobacterium sp.		
	Aeromonas sp.		
	*Pseudomonas aeruginosa**	< 30 %	Nosocomial infections
	E. coli		
	Cryptosporidium sp.		
	Helicobacter sp.		
Human Skin	*Staphylococcus epidermidis*	100%	
	*Staphylococcus aureus**	25%	
	*Streptococcus pyogenes**	< 5 %	Tonsillitis Pneumonia Endocarditis (heart disease)
	Corynebacteria	100%	Acnes
	Mycobacteria	25%	
Contaminated Textiles	*Klebsiella*		
	Enterobacter		
	Serretia		
	Staphylococcus spp.		
	Clostridium difficile		
	Cryptosporidium sp.		
	*Pseudomonas aeruginosa**		
	Rotavirus		
	Brucella spp.		
	Hepatitis C.		
	Mycobacterium fortuitum		

* Potential pathogens

30% of microorganisms coming from water lines are opportunistic pathogens causing health risks only if required conditions are provided. Not all of them but only a number of them are human pathogens. *Pseudomonas aeruginosa*, *Legionella pneumophila*, *nontuberculous mycobacteria* and *Acanthamoeba* are some of the water borne human pathogens. *Streptococcii* is the cause of bacteremia while species of *Acanthamoeba* cause eye infections [16, 17]. Potential risk of *Legionella,* present in water lines, is the Legionnaire's disease of which symptoms are very similar to pneumonia. Similar symptoms bring out diagnosis difficulties that may lead to death.

2.5. Biofilm Treatment Methods

Inhibition of biofilm formation and removal of biofilm is critically important because of effects to device performance, unpleasant odors and visual pollution and most important of all potential health risks.

Studies on biomaterials indicate that attachment of microorganism and biofilm formation may take some hours as well as couple minutes. So for effective treatment; complex and continuous applications should be preferred rather than basic cleaning methods [10].

Treatment methods against biofilm can be classified as mechanical, chemical and biological methods.

2.5.1. Mechanical methods

Mechanical methods aim removal of biofilm cells and resuspending them in the water system. However, these methods require dismantlement of the system which makes it impossible to use in some cases. Utility knife, swabbing and stomacher are examples of mechanical applications, which have no use in surfaces of white goods or water distribution systems [19].

Besides these methods, ultrasound applications have been more common and applicable. It is well established that ultrasound generates sufficient cavitational bubble activity to remove biofilms from metal, glass, ceramic and plastic surfaces [20].

After the successful development and utilization of an ultrasonic apparatus to detach biofilm from food processing equipment in order to achieve the efficient of cleaning protocols, a new study was performed for the removal of biofilm in internal or curved food contact surfaces such as internal surfaces of pipes and dead ends. A transducer made of barium titanate, 3 mm in diameters, is used to generate frequency of 40 kHz and operate for 10 s (two times at 5 s each). For increasing the efficiency of biofilm removal, ultrasound was applied also in combination with EDTA solution and enzyme mixtures [20]. Results of the study carried out on two biofilms formed by *E. coli* and *Staphylococcus aureus* is listed in Table 2.3.

Table 2.3. Biofilm removal by ultrasound [20].

Combination of Application	Biofilm Removal (%)	
	E.Coli	*S. Aureus*
Ultrasound	49	39
Ultrasound + EDTA Solution	58	89
Ultrasound + EDTA Solution + Amyloglucosidase	42	100
Ultrasound + EDTA Solution + Trypsin + Lysozyme	27	100
Ultrasound + EDTA Solution + Trypsin + Protease	50	79
Ultrasound + EDTA Solution + Papain + Lysozyme	54	30
Ultrasound + EDTA Solution + Protease + Papain	75	70

The ultrasonic treatment is reported to be effective in detaching bacterial cells into water but it is also reported that as biofilm is getting aged and thicker, ultrasound remains insufficient [5].

2.5.2. Chemical methods

Disturbance of medium by disinfectants, surface coatings, ozonation or addition of chemicals in any form is classified as chemical methods.

2.5.2.1. Disinfectants

Chlorine and ozone are some of the most common disinfectants used in pool, drinking and waste water treatment systems. Ozone (O_3), is antibacterial chemical used for removal of microorganisms. Recently, it has been used as an alternative to chloride. But according to studies, ozonation increases biodegradable organic carbon in water which can be used as energy source by microorganisms. As a result, biofilm formation is more rapid in surfaces exposed to ozonated water [5].

Other disinfectants are listed in Table 2.4 with required concentration and their efficiency against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.*

Table 2.4. Biofilm removal by chemicals [21].

Chemical	Concentration	Removal (%)
NaCl	0.3 M	40 - 69
$CaCl_2$	0.21 M	48
MgCl	0.21 M	23
EDTA	0.01 M	26
SDS	1000 mg/L	63 - 91
Sucrose	0.47 M	8
Urea	2 M	73
NH_2Cl	5 - 100 mg/L	56 - 70
Other Applications		
pH change from 6.4 to 2.9		16
pH change from 6.4 to 11.2		47

The biggest advantage of disinfectants is being not specific to microorganism. Successful removal is possible if applied in sufficient concentration not depending on structure and species of microorganisms. Disinfectants are economic compared to enzymatic applications. Besides these advantages, disadvantages of disinfections are listed as following.

- Chemicals are dangerous molecules and difficult to work with because of being corrosive, toxic, carcinogen, mutagen etc…
- Disinfectants used in water treatment may cause by-products. Cl_2 and ozone applications are known to cause trihalomethanes (THM).
- By-products do not only change water quality negatively causing unpleasant odor and taste but they also can be dangerous on their own [21, 22].

2.5.2.2. Surface coatings

Surface coatings are used for improving surface resistance, isolation, easy-to-clean properties, antibacterial surface and more. Nano-coatings, ion exchange coatings with copper and silver, zeolites etc. have been widely used for hygiene and antibacterial properties. Besides these common and well known techniques, furanone also become popular recently. Microbial aggregation and attachment is prevented on polyvinylchloride (PVC), polyethylene (PE), polyurethane (PU), polypropylene (PP), polytetrafluoroethylene (PTFE – Teflon) and silicone surfaces coated with furanone [9].

2.5.3. Biological methods

Methods using biologically produced molecules (enzymes, antibiotics, hormones etc.) or targeting microbial communication are classified as biological methods.

2.5.3.1. Antibiotics

Antibiotics are biomolecules produced by microorganisms to defend themselves against other microorganisms. But it is possible for them to develop resistance to antibiotics rapidly. In addition to this, biofilm microorganisms are more resistant to antibiotics than planctonic microorganisms [23]. Since generation of stronger and more resistant species is possible by antibiotic applications, they are not used biofilm treatment in white goods or domestic surfaces.

The only biofilm removal application of antibiotics is against living surfaces such as tissue and organs together with non-living surfaces such as prosthesis and implants. A study was carried out about antibiotics used for therapeutic purposes and their efficiency against biofilm community. According to this study planctonic *E. Coli* is reported to be sensitive to enrofloxacine, gentamicine, oxitetracycline and trimetoprim/sulphaoxine while biofilm cells are only sensitive to enrofloxacine and gentamicine. Similarly, planctonic *Pseudomonas aeruginosa* is sensitive to enrofloxacine, erythromycine and oxitetracycline while biofilm cells are sensitive only to enrofloxacine [23]. These results prove improved resistance of biofilm microorganisms against antibiotics.

Potential reasons for improved resistance against antibiotics are listed as following.

- Polymeric matrix may prevent antibiotic to diffuse in the biofilm.
- Antibiotics may react with external layer of biofilm and microorganisms are not affected by this.
- Matrix may consist of enzymes to degrade antibiotics.
- Phenotype of microorganisms in external and internal layers may be different [2].

2.5.3.2. Enzymes

Biofilm is a heterogeneous structure with a polymeric matrix consists of a variety of microorganisms and various polysaccharides secreted by them. Enzymes can be used

for degradation of biofilm but due to substrate-specific nature of enzymes and heterogeneity of the extracellular polysaccharides in the biofilm, a mixture of enzyme activities may be necessary for a sufficient degradation of bacterial biofilm [24].

There are applications using mixtures of hydrolase and oxidoreductase enzymes. Hydrolase enzymes such as protease, amylase and esterase are used for degradation of polysaccharides within the matrix while oxidoreductase enzymes such as oxidase, peroxidase and laccase are used for killing microorganisms [24, 25].

Degradation of extracellular polysaccharides present in biofilm is possible by enzyme applications. Polysaccharides differ from species to species forming biofilm. Biofilm formed by *Pseudomonas fluorescens* consists of gum Arabic and pectin polysaccharides. Fungal strains, which are able to use these polysaccharides as carbon source can be used for biofilm treatment. Strains of *Aspergillus niger, Trichoderme viride* and *Penicillium* spp. are able to produce enzymes which are able to degrade gum Arabic and pectin polysaccharides and they are used in industrial treatment purposes. Different enzymes can be applied from different points of biofilm for the optimization of removal process. Optimum activity for the enzymes is given at 25-40ºC [1]. Fungal strains, carbon source and efficiency against *Pseudomonas fluorescens* biofilm on glass is listed in Table 2.5.

Table 2.5. Biofilm removal by fungal strains [1].

Culture Medium		Time (days)	Biofilm Removal (%)
Strain	Carbon Source		
A. Niger	*Gum arabic*	14	65±5
A. Niger	Alginate	9	38±7
A. Niger	Xylane	8	19±6
A. Niger	Pectin	1	60±5
T. Viride	Pectin	1	84±2
Penicillium H18	Alginate	8	45±5

2.5.3.3. Quorum Sensing inhibition

Biofilm microorganisms differ from planctonic microorganisms in two aspects;

- Biofilm microorganisms are strongly resistant to disinfection and antibiotics by the help of extracellular matrix.

- Biofilm microorganisms are in communication with each other by their special signal molecules. The mechanism, called Quorum Sensing (QS) is critically important in biofilm formation.

Traditional methods, used for preventing biofilm formation in industrial applications and medical areas, remain insufficient due to the improved resistance of biofilm against treatment methods. Consequently, alternative methods have been studied for biofilm treatment and many researches have been focused on inhibition of microbial communication. QS inhibitors, targeting signal molecules and blocking microbial communication, directly prevent biofilm formation instead of blocking bacterial growth [26-28].

2.6. Microorganism Identification Methods

Accurate and rapid identification of microorganisms are very important especially in diagnosis and therapeutics of diseases, contamination of food products and military applications. Molecular and genetic analysis methods have been used for identification [14].

Cultivation; is one of the oldest methods used for microbial identification. Principle of cultivation is to grow samples in an appropriate culture media. The method is selective and not rapid [14].

Biosensors are also used for selective and rapid identification of microorganisms recently. Method is quite insensible to any contamination [14].

Immunoassays, taking advantage of sensitivity and specifity of antigen and antibodies, are also used for identification of microorganisms [14].

In this study, 18S ribosomal DNA cloning procedure has been chosen for the identification of biofilm community.

3. MATERIALS & METHODS

3.1. Materials

3.1.1. Bacterial strains

3.1.1.1. *E. Coli* TOP10 strains

F- mcrAΔ (mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 recA1 araD139 Δ (araleu) 7697 galU galK rpsL (StrR) endA1 nupG (One Shot TOP10 Electrocompotent cells, Catalog #C4040-10, Invitrogen) strain is supplied in cloning 18s ribosomal RNA gene sequences.

3.1.1.2. *E. Coli* DH5αTM – T1R strains

F- φ80*lac*Z ΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 *ton*A, (Catalog #12297-016, Invitrogen), which are electrocompotent cells, was used for cloning 18s ribosomal RNA gen fragments.

3.1.2. Cloning Vector

3.1.2.1. pCR®2.1.-TOPO® vector

pCR®2.1.-TOPO® vector (given in Appendix A) was purchased from Invitrogen. Vector was linearised for cloning from 3'- Thymidine (T) end and it is bound to Topoisomerase I enzyme covalently (Catalog #K4560-40, Invitrogen).

3.1.3. Enzymes

3.1.3.1. Platinum® Taq DNA Polymerase High Fidelity

Platinum® Taq DNA Polymerase High Fidelity enzyme is a mixture of recombinant, Pyrococcus species GB-D polymerase with proofreading property and Platinum®. It is possible to clone template DNAs up to 12 kb with high fidelity with proofreading enzyme and Taq DNA polymerase, while Taq Antibody makes it possible to work with high temperatures for Taq Polymerase (Catalog #11304-011, Invitrogen).

3.1.3.2. Fast-Start *Taq* DNA Polymerase

Fast-Start *Taq* DNA polymerase enzyme (Catalog #2 158 264, Roche) enhances product amplification by keeping primer dymer formation at minimal. It was used together with Platinum *Taq* DNA polyermase High Fidelity in UARR PCR.

3.1.4. DNA Molecular weight markers

DNA molecular weight standard markers were purchased from Fermentas Company.

3.1.5. Oligonucleotides

Oligonucleotides, listed in the following, is synthesized by IONTEK company using Applied Biosystems 308A DNA synthesizer.

EUKA	5′-AACCTGGTTGATCCTGCCAGT 3’
EUKB	5′-TGATCCTTCTGCAGGTTCACCTAC 3’
M13-F	5’ GTAAAACGACGGCCAG 3’
M13-R	5’ CAGGAAACAGCTATGAC 3’

3.1.6. Culture media

3.1.6.1. LB (Luria-Bertani) culture

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen) were dissolved in distilled water up to 1 lt and the pH was adjusted to 7.0 with 10 M NaOH and sterilized by autoclaving for 15 min. under 2 atm at 121°C.

3.1.6.2. LB Agar culture

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g bactoagar (Acumedia) were dissolved in distilled water up to 1 lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized by autoclaving.

3.1.6.3. SOC Medium

20 g trypton, 5 g yeast extract and 0.5 g NaCl were resolved in deionised water. 10 mL 250 mM KCl was added to the mixture and pH was adjusted to 7.0 by NaOH.

Solution volume was completed to 1 l by distilled water and sterilized. 10 mM $MgCl_2$ and 20 mM glucose were added just before usage.

3.1.7. Stock Solutions

3.1.7.1. Ampicilline stock

100 mg/ml ampicilline sodium salt was dissolved in deionized water, filter-sterilized and stored in dark at -20°C.

3.1.7.2. X-gal Stock solution

40 mg/mL X-gal (5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside) was dissolved in dimethyl formamide (DMF). Solution was stored in dark at -20°C.

3.1.7.3. Glycerol stock

80 mL glycerol (Riedel-de-Haen) and 20 ml distilled water were mixed to have 80 % (w/v) solution. It was sterilized for 15 minutes under 1.5 atm at 121°C.

3.1.8. Buffer solutions

3.1.8.1. Sodium acetate buffer

3 M sodium acetate (Riedel-de-Haen) was dissolved in 65 mL distilled water and pH was adjusted to 5.6. Solution volume was completed to 100 mL.

3.1.8.2. 5X TBE Buffer solution

54 g of Tris base, 27.5 g boric acid and 20 ml 0.5 M EDTA at pH 8.0 were dissolved in 1 liter deionized water, its pH was titrated to 8.3 and sterilized for 15 min. under 1.5 atm at 121°C.

3.1.9. Laboratory equipments

Laboratory equipments are listed in Appendix B.

3.2. Methods

3.2.1. Sample collection and genomic DNA isolation

Samples were collected from two washing machines which were primarily operated in different conditions.

3.2.1.1. Preparation of samples

Study was performed synchronously for three domains of washing machines. Biofilm samples were collected from detergent drawer, tub seal and discharge hose of each washing machine by a sterile spatula.

3.2.1.2. Genomic DNA isolation

6 samples, collected from the machines, were homogenized in a porcelain bowl by liquid nitrogen. Genomic DNA Isolation was performed by MoBio UltraClean™ Microbial DNA Isolation Kit (Catalog #12224-50). Microbial DNA Isolation kit, by mechanical degradation, enhances genomic DNA isolation of eukaryote species which have thicker cell walls compared to bacteria's.

- **Genomic DNA isolation procedure**

1. Samples, scraped from surfaces, were collected in microfuge tubes separately.
2. 30 mg of samples were transferred to clean microfuge tubes.
3. Each sample was dissolved in 300µL MicroBead solution and vortexed. Dissolved cells were transferred to MicroBead tubes.
4. 50 µL MD1 solution was added to MicroBead tubes.
5. Samples were vortexed for 10 minutes at maximum rate.
6. MicroBead tubes were centrifuged for 30 sec at 10.000 g and pellets were formed.
7. Supernatants were transferred to collection tubes with a volume 2 mL.
8. 100 µL MD2 solution was added to collection tubes and vortexed for 5 sec. Following vortex, tubes were incubated on ice for 5 min.
9. Collection tubes were centrifuged for 1 min at 10.000 g at room temperature.

10. All of the supernatants were transferred to new/clean collection tubes.
11. 900 µL MD3 solution was added to supernatants and vortexed for 5 sec.
12. 700 µL for each sample was transferred to spin filters and centrifuged for 30 sec at room temperature. Liquid, collected in collection tube, was discarded and supernatants were transferred over filters. The tubes with filters were centrifuged for 30 sec at 10.000 g. This step was repeated until supernatants are over.
13. 300 µL MD4 solution was added over the filter and centrifuged for 30 sec at 10.000 g.
14. Liquid in the collection tube was discarded and empty filters were centrifuged for 1 min at 10.000 g.
15. Used collection tubes were discarded and spin filters were transferred to clean 2 mL-tubes.
16. 50 µL MD5 solution was added to filter and centrifuged for 30 sec at 10.000 g.
17. Spin filters were discarded and 2 mL-tubes were stored at -20ºC until usage.

In order to see isolated genomic DNAs, agarose gel electrophoresis was performed. 1% (w/v) gel was prepared with 0.5 g agarose dissolved in 50 mL TBE buffer. Mixture was heated until boiling and cooled to 50-60ºC. 3 µL ethidium bromide (EtBr) was added to cooled mixture and poured to electrophoresis plate. 5 µL from each sample were loaded to gel with 1 µL 6x loading dye. Results, given in Figure 3.1., were visualized by transilluminator.

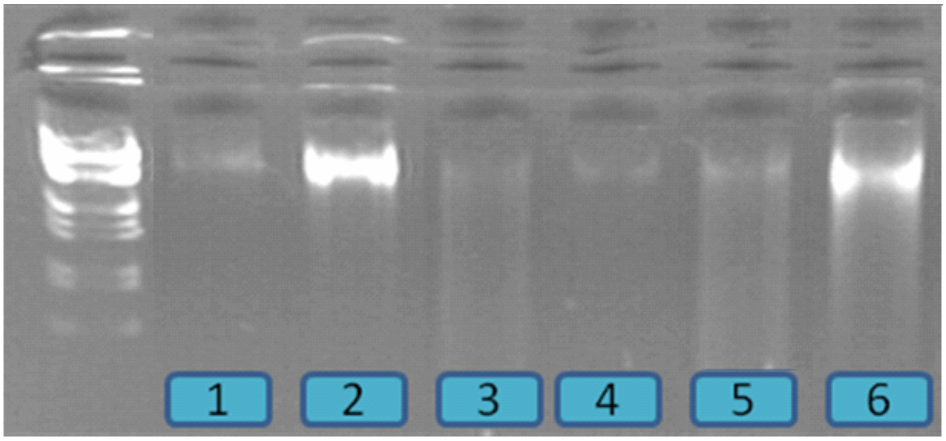


Figure 3.1. Agarose gel after genomic DNA isolation; 1.Machine A-Detergent Drawer, 2.Machine A-Tub Seal, 3.Machine A-Discharge Hose, 4.Machine B-Detergent Drawer, 5.Machine B-Tub Seal, 6.Machine B-Discharge Hose

3.2.2. Cloning

In this step 18s ribosomal RNA region of the genomic DNAs were multiplied using specific primers to given region. Contaminations were removed from PCR products by agarose gel extraction and cloned into plasmids for sequence analysis.

3.2.2.1. Universal Amplified Ribosomal Region PCR

After genomic DNA isolation, PCR reaction was set up with primers EukA and EukB. Roche Taq DNA Polymerase dNTPack was used for PCR reaction. Chemicals, listed in Table 3.1., were all mixed in separate tubes for each sample and placed into PCR device.

Table 3.1. Chemicals used for UARR PCR

Chemical	Amount
10X PCR Buffer	5 µL
10 mM dNTP	1 µL
Forward Primer EukA	1 µL
Reverse Primer EukB	1 µL
Taq Polymerase : Pfu Polymerase (9:1)	1 µL
25 mM $MgCl_2$	8 µL
Template DNA	30 ng (≈1 µL)
Water	to 50 µL
Total volume	50 µL

Device was first heated up 95ºC and held for 4 min. Then temperature was set to 95ºC, 57ºC and 72ºC for 30 sec, 50 sec and 70 sec, respectively. Last three step was

repeated for 35 cycles and then, temperature was hold at 72ºC for 20 sec. Finally temperature was fixed at 10ºC until samples were taken. Reaction condition is briefly given in Table 3.2.

Table 3.2. PCR reaction conditions

Temperature	Duration	
95ºC	4 min	
95ºC	30 sec	35 cycle
57ºC	50 sec	
72ºC	70 sec	
72ºC	20 sec	
10ºC	∞	

3.2.2.2. Gel extraction

PCR products were controlled by agarose gel electrophoresis and bands, corresponding to approximately 1800 bp, were cut and transferred to 2 mL tubes. Extraction was performed by QIAGEN – QIAquick Gel Extraction Kit (Catalog #28604, Qiagen).

- **Gel extraction procedure**

1. DNA fragments, corresponding to approximately 1800 bp, were excised from the agarose gel with a clean, sharp scalpel. Gel slices were weighed in a colorless tube separately for each sample.
2. 3 volumes of Buffer QG was added to 1 volume of gel (100 mg ≈ 100 mL).
3. Samples were incubated at 50ºC for 10 min and mixed by vortexing every 2-3 min during the incubation.
4. After the gel slice was totally dissolved, 1 gel volume of isopropanol was added to the sample and mixed by inverting the tube several times.
5. Samples were applied to the MinElute column and centrifuged for 1 min at 13.000 rpm.
6. Flow through was discarded from collection tubes.
7. 500 µL Buffer QG was added to the column and centrifuged for 1 min at 13.000 rpm.

8. Flow through was discarded from collection tubes.
9. 750 µL Buffer PE was added to the column and centrifuged for 1 min at 13.000 rpm.
10. Flow through was discarded from collection tubes and empty MinElute column was centrifuged for 1 min at 13.000 rpm.
11. MinElute column was placed into a clean 1.5 mL microcentrifuge tube and 10 µL Buffer EB was added to the center of the membrane. Column was incubated for 1 minute and then centrifuged for 1 min at 13.000 rpm at room temperature.
12. DNA extracts were stored -20ºC until usage.

Steps of the procedure are briefly given in Figure 3.2.

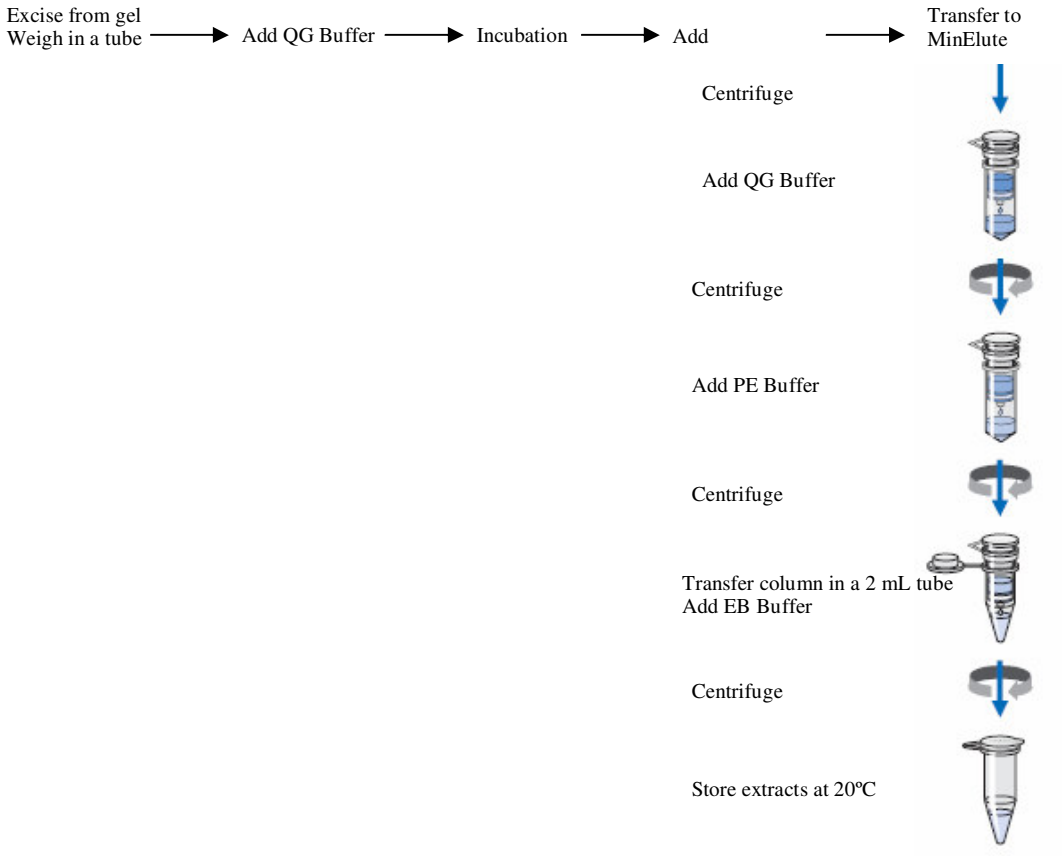


Figure 3.2. Gel extraction procedure

3.2.2.3. Cloning and transformation

TOPO TA Cloning® Kit was used for cloning and transformation. Chemicals used for cloning procedure are given in Table 3.3. All the chemicals were mixed in a 0.5 mL tube and the mixture was incubated for 30 min at room temperature. After incubation, transformation procedure was proceeded.

Table 3.3. Chemicals used for cloning

Chemicals	Amount
Salt solution	1 µL
TOPO TA cloning vector	1 µL
PCR product (extracted DNA)	0.5 µg ≈ 2µL
Water	to 6 µL

- **Transformation procedure**

1. 2 µL of the TOPO® Cloning reaction was added into a vial of One Shot® Chemically Competent *E. coli* and mixed gently. Mixture was incubated on ice for 5 minutes.
2. Solution was transferred to a 0.1 cm electroporator cuvette avoiding bubble formation. Voltage was set to 1800 and current was applied.
3. 250 µL of room temperature S.O.C. medium was added immediately.
4. Solution was transferred to 15 ml snap-cap tube (e.g. Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance genes.
5. DMF containing 40 mg/mL X-gal was spread to petri plates prepared by LB-agar mediums for blue/white screening.
6. 10-50 µL from each transformation on prewarmed LB-agar mediums and incubate overnight at 37°C.

3.2.2.4. Blue/White screening and plasmid isolation

The cultures were screened by blue/white screening method. Colonies, with the gene sequence that we insert, take white color in the presence of X-gal. Colonies with blue color do not have the insert within. One of the LB-agar medium plate, where transformed cell mixture were incubated for 16 h, is shown in Figure 3.3.

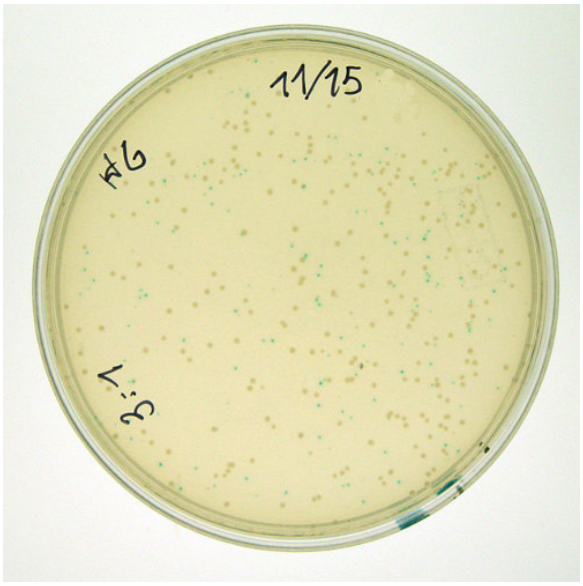


Figure 3.3. LB-Agar plate

After 16 hours incubation, white colonies were selected from the plate and transferred to 15 mL tubes containing 2 mL LB medium and 2 µL ampicilline. Colonies were incubated for 16 hours. For the isolation of plasmids Roche High Pure Plasmid Isolation Kit (Catalog #1 754 785) was used.

- **Plasmid isolation procedure**

1. Culture mediums were centrifuged in order to form cell pellets at 6000 g for 30 sec.
2. Supernatants were discarded.
3. 250 µL Suspension Buffer + RNase mixture was added to the tubes and resuspended.
4. 250 µL Lysis Buffer was added and mixed by inverting the tube 6 times.
5. Mixture was incubated for 5 min at room temperature.
6. 350 µL pre-chilled Binding Buffer was added to lysed solution, mixed gently and incubated on ice for 5 min.
7. Samples were centrifuged for 10 min at 13.000 g.
8. Entire supernatant of the centrifugation was transferred to High Pure Filter Tubes and centrifuged for 1 min at 13.000 g.
9. Flow through was discarded from collection tube.

10. 500 µL Wash Buffer I was added to filter tube and centrifuged for 1 min at 13.000 g. Flow through was discarded from collection tube.
11. 700 µL Wash Buffer II was added to filter tube and centrifuged for 1 min at 13.000 g.
12. Flow through was discarded from collection tube and centrifuged for additional 1 min at 13.000 g.
13. Filter tube placed into a 1.5 microfuge tube and 100 µL Elution Buffer ws added.
14. Tubes were centrifuged for 1 min at 13.000 at room temperature and purified plasmid DNAs were stored at -20ºC.

Steps of the procedure are briefly given in Figure 3.4.

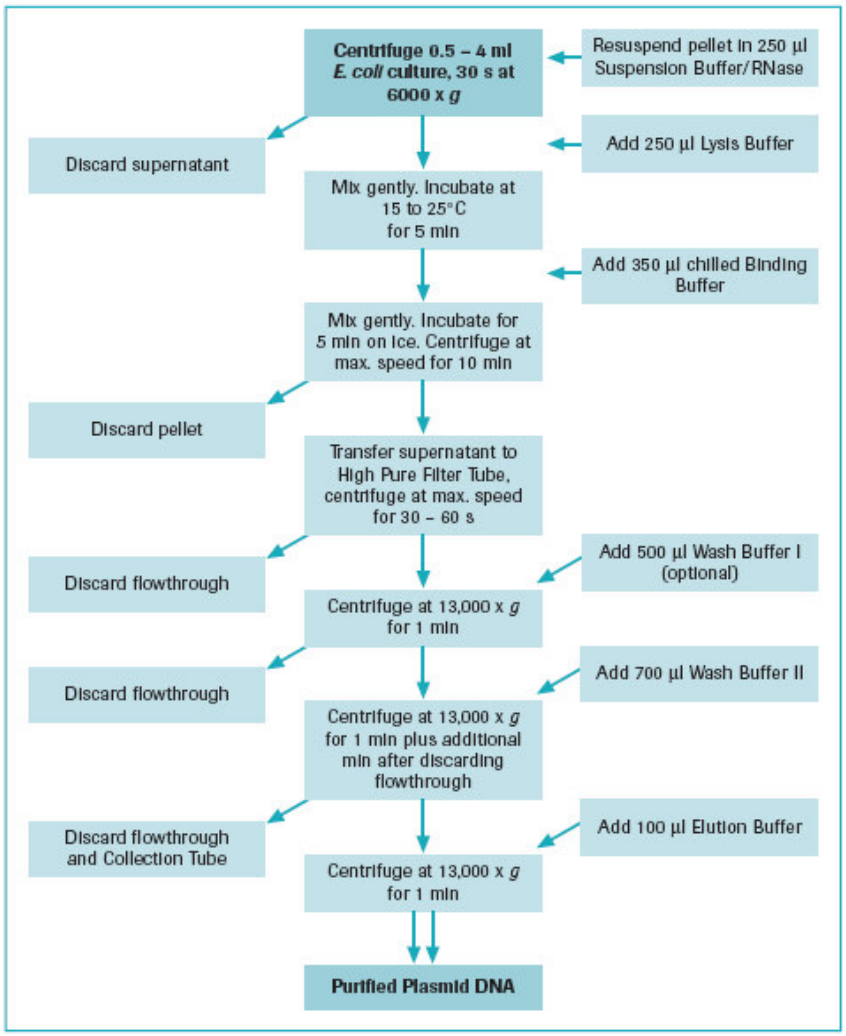


Figure 3.4. Plasmid isolation procedure

3.2.3. Sequencing

3.2.3.1. Sequence PCR

Sequence PCR reaction was set with the chemicals (provided by Big Dye Terminator v3.1) listed in Table 3.4. All the chemicals were mixed in separate tubes for each sample and placed into PCR device.

Table 3.4. Chemicals used for sequence PCR

Chemicals	Amount
ABI RR-100 dye	2 µL
ABI 5X PCR Buffer	2 µL
M13 Forward/Reverse Primer	3.2 pmole (≈ 1 µL)
Template	0,5 µL
Water	to 10 µL

Device was first heated up 95ºC and held for 5 min. Then temperature was set to 95ºC, 55ºC and 72ºC for 10 sec, 5 sec and 4 min, respectively. Last three step was repeated for 35 cycles and finally temperature was fixed at 4ºC until samples were taken. Reaction condition is briefly given in Table 3.5.

Table 3.5. Sequence PCR conditions

Temperature	Duration	
95°C	5 min	
95°C	10 sec	30 cycle
55°C	5 sec	
72°C	4 min	
4°C	∞	

After sequence PCR, PCR products were purified by sodium acetate precipitation.

- **Procedure for purifying sequence PCR products**

1. 1:1 premix solution of 3M sodium acetate and 125 mM EDTA was prepared and 4 µl of the premix solution was added to each of the sequencing reactions.
2. 50 µl of 100% ethanol was added to each sequencing reaction and vortexed briefly so that the content is at the bottom of the tube.
3. Mixtures were incubated at room temperature for 15 min.

4. Mixtures were centrifuged for 15 min at 13.000 rpm at room temperature.
5. Supernatants were aspirated off using a pipette.
6. 70µl of 70% ethanol was added to each tube and centrifuged for 10 min at 13.000 rpm.
7. Supernatants were aspirated off using a pipette.
8. A second was step was performed by adding 70µL of 70% ethanol and centrifuging for another 10 minutes at 13000rpm.
9. Supernatants were aspirated off using a pipette. Samples were incubated at 95ºC for the removal of entire ethanol.
10. DNA pellets were dissolved in 20 µL formamide and become prepared for sequence analysis. Samples were stored at 4ºC packed in foil paper until analysis.
11. Samples were kept at 95ºC for 3 min and 20ºC for 5 min, respectively before loading to sequence analyzer.

Sequence analysis was performed by automized device, ABI 3100 Avant (PE, Applied Biosystem, CA).

3.2.3.2. Phylogenetic analysis

After the sequence analysis, all of the results were compared in NBCI Database using BLAST tool. Results with a similarity of 97-100% were accepted to be true for species and results with a similarity of 90-96% were accepted to be true for genus.

The results were also transferred to MEGA (Molecular Evolutionary Genetics Analysis, version 4.0) program for building a sequence library. Phylogenetic trees were built by neighbor-joining method using MEGA program.

4. RESULTS AND DISCUSSION

Two washing machines were collected from consumer's because unpleasant odor and visual pollution complaints. Source of the complaints turn out to be microbial formation, called biofilm, not only causing bad odors and visual pollution but also seriously threatening human health.

In this study, we have tried to find the eukaryotic sources of the biofilm formation in three parts of two different washing machines, which were primarily used in different conditions. Some parts of the washing machines were found to be extremely contaminated by biofilm formation. Study was performed by the samples collected from these parts; detergent drawer case, tub seal and discharge hose. While biofilm on detergent drawer case causes bad odors; on tub seal, biofilm formation leads to significant both unpleasant odor and visual pollution.

Samples were collected from the parts stated above and the eukaryotic species were identified by 18s ribosomal DNA cloning methods.

Results are given in comparison with same parts of different machines.

4.1. Detergent Drawer

Microorganisms, identified in detergent drawer of each washing machine; sequence similarity, frequency in the analysis, pathogenity and health risks are given in the Table 4.1.

Some of the species identified in detergent drawer belong to *Fusarium* genus, a very large genus of filamentous fungi. Having approximately 20 species, *Fusarium* is one of the most common fungi observed in soil and plants. Some of the species are opportunistic pathogens to human and may cause several infections such as eye and nail infections. Species of *Fusarium* genus may also contaminate blood circulation and cause high fever [29].

Table 4.1. Microorganisms identified in detergent drawer [29-32].

	Microorganism	Sequence Similarity	Frequency*	Kingdom	Pathogenity / Health Risks	Other
MACHINE A	*Colpoda* sp. PRA-118	97%	25%	Protozoa		
	Castrella truncata	92%	25%	Metazoa		
	Exophiala oligosperma strain CBS 725.88	98%	16%	Fungi	*Olecranon bursitis* (elbow inflammation)	Optimum growth at 25-30°C
	Uncultured eukaryote clone RT5in1	96%	16%			
	Fusarium oxysporum	98%	8%	Fungi	Plant pathogen Opportunistic human pathogen Nail and eye infection Blood circulation	Appr. 20 species Very common in soil and plants
	Penicillium griseofulvum strain 3.5190	98%	8%	Fungi	Pathogen	

	Microorganism	Sequence Similarity	Frequency*	Kingdom	Pathogenity / Health Risks	Other
MACHINE B	*Fusarium oxysporum*	98%	67%	Fungi	Plant pathogen Opportunistic human pathogen	Appr. 20 species Very common in soil and plants
	Fusarium solani strain 421502	97%				
	Tritirachium sp. IAM 14522	96%	8%	Fungi	Ear & corneal infections	
	Hartmannella vermiformis	97%	8%	Protozoa	Not pathogen	Promotion for *L. Pneumophila* to form biofilm

*Frequency is identified by the percentage of species observed in experiments

Two other species of fungi identified in one of the machines; *Exophiala oligosperma* and *Penicillium griseofulvum*, are also pathogen microorganisms. *Exophiala oligosperma* species may cause *Olecranon bursitis* (simply defined as elbow inflammation) and its optimum growth temperature is defined as 25-30ºC [30]. This temperature interval is also parallel to cold-wash program used in washing machine.

Two other species, except *Fusarium* genus; were identified in detergent drawer of machine B. One of them is *Tritirachium*, a species of fungi, which may cause ear and corneal infections [31]. The other species identified is a protozoa, *Hartmannella vermiformis*, not a pathogen on its own but known to promote biofilm formation in the presence of *Legionella pneumophila* [32].

4.2. Tub Seal

Microorganisms, identified in tub seals of each washing machine; sequence similarity, frequency in the analysis, pathogenity and health risks are given in the Table 4.2.

In the tub seal of machine A, different species of *Rhodotorula* genus were identified. The genus is known to be widely observed fungus in daily products besides of air, soil, ocean and lake-like aquatic mediums and may establish host interactions with plants, humans and other mammalians. A small number of *Rhodotorula* species are pathogen to humans. But pathogen species may cause deadly infections to AIDS and leukemia patients with weakened immune system [33]. 38% of the species found in the machines couldn't be identified in database because of non-significant similarity.

Metazoa species were identified in tub seal of machine B; *Microdalyellia rossi* and *Castrella truncata.* There is no available information about these species.

Table 4.2. Microorganisms identified in tub seal [33].

	Microorganism	Sequence Similarity	Frequency	Kingdom	Pathogenity / Health Risks	Other
MACHINE A	*Rhodotorula slooffiae*	96%	38%	Fungi	Opportunistic pathogen Infection risk for AIDS and leukemia patients	Present in air, soil, lake and ocean water, daily products Plant, human and other mammallians as host
	Rhodotorula minuta	95%	15%			
	Rhodotorula cassiicola	93%	8%			
	No significant similarity found		38%			

	Microorganism	Sequence Similarity	Frequency	Kingdom	Pathogenity / Health Risks	Other
MACHINE B	*Microdalyellia rossi*	92%	75%	Metazoa		
	Castrella truncata	92%	25%	Metazoa		

*Frequency is identified by the percentage of species observed in experiments

4.3. Discharge Hose

Microorganisms, identified in discharge hose of each washing machine; sequence similarity, frequency in the analysis, pathogenity and health risks are given in the Table 4.3.

Bodo cautus, a species of protozoa, is identified in machine A. The species is typically found in the intertidal zone at the water's edge at a mean distance from sea level of 21 meters and it is known to be found in specimens of human feces (especially in tropical regions) and have flagellates which is especially necessary for microorganism to attach on a surface for biofilm formation [34]. Another protozoa species present in discharge hose is *Colpoda*, which is pathogen to human and may cause intestinal infections. The species is also known to be present in humid soil. Two fungi species were also identified and none of them are human pathogen.

Fusarium oxysporum were also identified in machine B's discharge hose as well as in detergent drawer of both machines. Another microorganism, found in machine B's discharge hose, *Acanthamoeba*; a protozoa species, is known to cause eye infection and is known to be present in soil and water.

Table 4.3. Microorganisms identified in discharge hose [34].

	Microorganism	Sequence Similarity	Frequency	Kingdom	Pathogenity / Health Risks	Other
MACHINE A	*Bodo caudatus*	92%	27%	Protozoa		At least 13 species Present in feaces, app 20 m above sea level… Having flagellas
	Uncultured eukaryote clone RT5in1	93%	27%			
	Pseudodidymium cryptomastigophorum	90%	18%	Protozoa		
	Troposporella fumosa strain MUCL 15695	93%	9%	Fungi	Plant pathogen	
	Colpoda sp. PRA-118	90%	9%	Protozoa	Intestinal infections	Present in humid soil
	Acremonium-like hyphomycete KR21-2	97%	9%	Fungi		Present in aquatic mediums and soil Mn cycle

	Microorganism	Sequence Similarity	Frequency	Kingdom	Pathogenity / Health Risks	Other
MACHINE B	*Fusarium oxysporum*	92%	64%	Fungi	Plant pathogen Opportunistic human pathogen Nail and eye infection Blood circulation	Appr. 20 species Very common in soil and plants
	Acanthamoeba sp. KA/MSG26	92%	36%	Protozoa	Eye infection	Present in soil and water

*Frequency is identified by the percentage of species observed in experiments

4.4. Phylogenetic Analysis

Eukaryotic sources of biofilm formation in washing machines are found to belong to protozoa, metazoa and fungi species. 50% of the species identified are fungi while 38% and 12% are protozoa and metazoa, respectively.

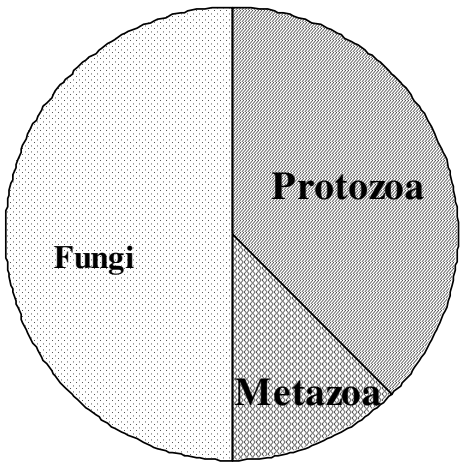


Figure 4.1. Distribution of microorganisms identified in washing machines

In order to present the taxonomic relationship of microorganisms identified in washing machines; phylogenetic tree was built using MEGA program and Neighbor-joining method. The tree is given in Figure 4.2. Empty shapes stand for the samples from machine A while filled shapes stand for the samples from machine B. It is well observed that biodiversity of washing machine B is less than washing machine A.

In general, the highest biodiversity is observed in detergent drawers and lowest biodiversity is observed in tub seals for both machines.

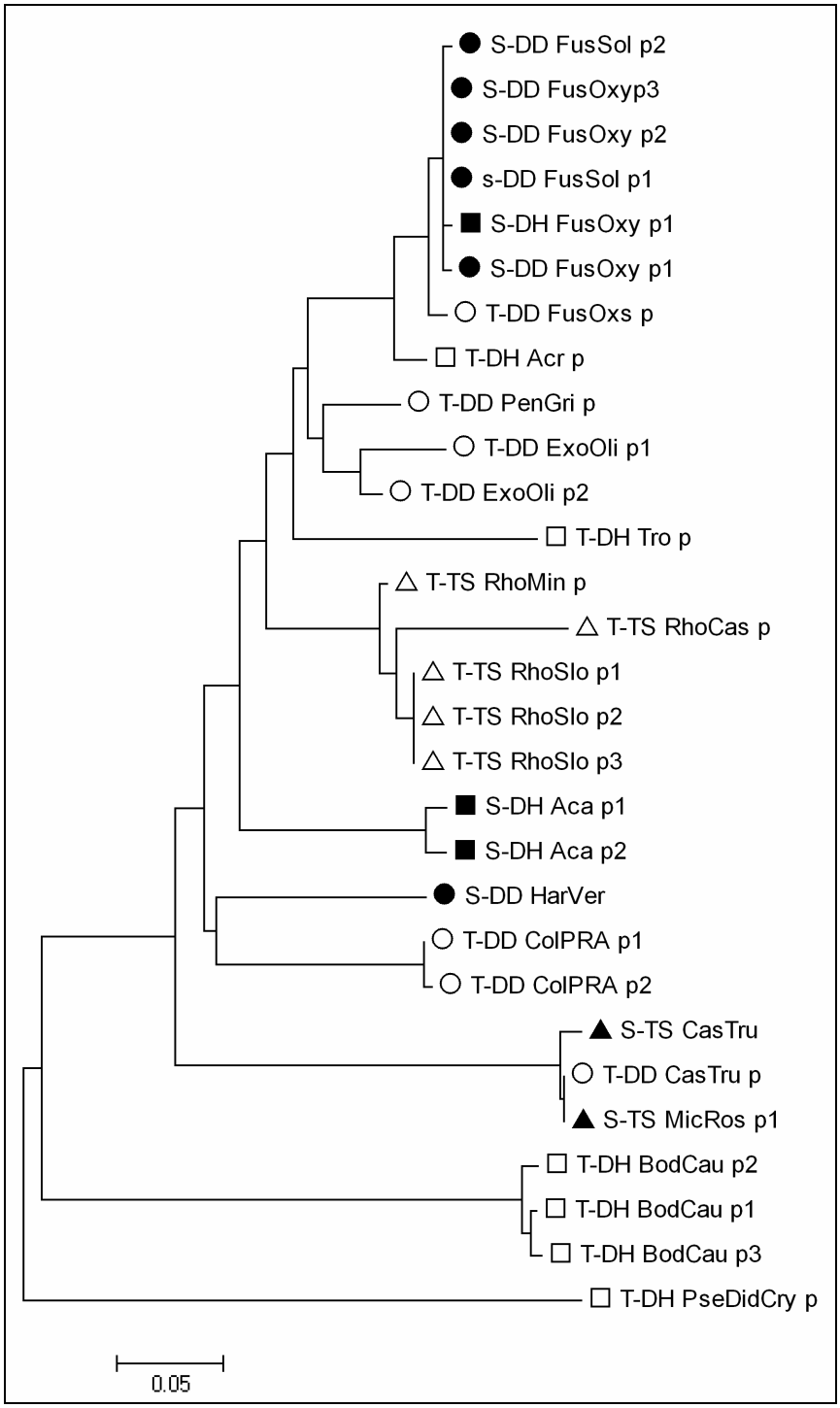


Figure 4.2. Phylogenetic tree; DD-Detergent Drawer, DH-Discharge Hose, TS Tub Seal, T-Machine A, S-Machine B

5. CONCLUSION

Biofilm formation is very common in humid surfaces, especially in aquatic mediums. It may cause several problems for the surface properties and system performance but most of all biofilm community may cause several infections and threaten human health.

Researches on biofilm formation have been developing very recently and almost all of the studies were made for medical devices, biomaterials and water distribution systems. Sources of biofilm on house-hold surfaces and especially white-goods have not been studied in detail yet.

In this study, we used samples from two washing machines which were initially operated in low temperatures and in geographic regions, and no significant similarity was found in biofilm communities of those machines.

Since different consumers in different geographies had used the machines, surfaces of the machines were exposed to a totally different contamination. Life styles of consumers and climatic properties of the geographic position may affect the contaminants coming from textiles (soil, stain, dirt), water distribution system, human skin etc.

For a more detailed comparison study, samples from closer regions with similar properties may be collected.

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APPENDIX

Appendix A

Map of pCR® 2.1-TOPO® Vector

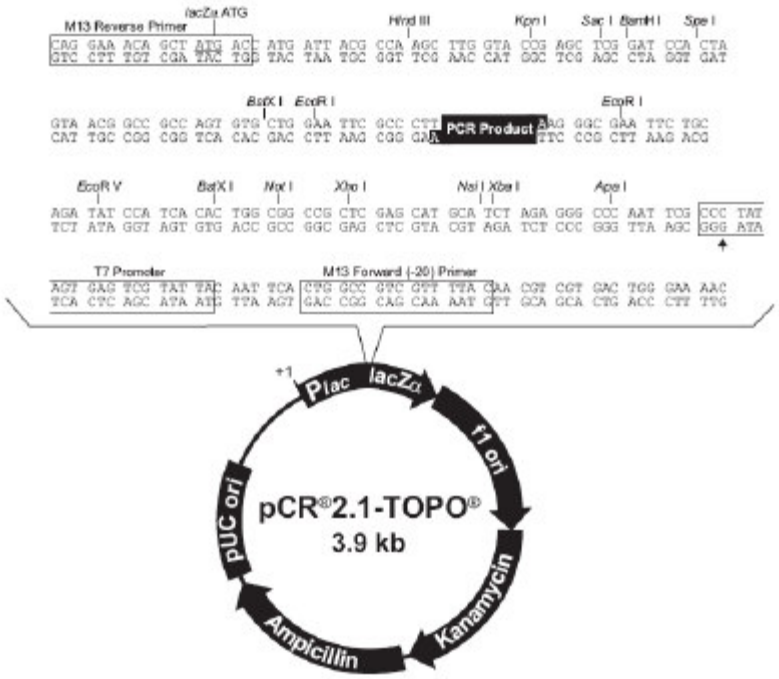


Figure A1. Map of cloning vector

Position (bp)	Element
1-547	LacZα fragment
205-221	M13 reverse priming site
234-357	Multiple cloning site
364-383	T7 promoter/priming site
391-406	M13 forward priming site
548-985	f1 origin
1319-2113	Kanamycin resistance ORF
2131-2991	Ampicillin resistance ORF

Appendix B

Lab Equipments

Autoclaves	**:** 2540 ML benchtop autoclave, Systec GmbH Labor-Systemtechnik.
	: NuveOT 4060 vertical steam sterilizer, Nuve.
Centrifuges	**:** Avanti J-30I, Beckman Coulter.
	: Microfuge 18, Beckman Couler.
Centrifuge rotors	**:** JA30.50Ti, Beckman Coulter.
	: F241.5P, Beckman Coulter.
Deep freezes and refrigerators	**:** Heto Polar Bear 4410 ultra freezer, JOUAN Nordic A/S, catalog# 003431.
	: 2021 D deep freezer, Arcelik.
	: 1061 M refrigerator, Arcelik.
Electrophoresis equipments	**:** E-C Mini Cell Primo EC320, E-C Apparatus.
	: Mini-V 8.10 Vertical Gel Electrophoresis System, Life Technologies GibcoBrl (now Invitrogen), Catalog# 21078.
Gel documentation system	**:** UVIpro GAS7000, UVItec Limited.
Ice Machine	**:** AF 10, Scotsman.
Incubators	**:** EN400, Nuve.
Orbital shake	**:** Certomat S II, product# 886 252 4, B. Braun Biotech International GmbH.
Magnetic stirrer	**:** AGE 10.0164, VELP Scientifica srl.
	: ARE 10.0162, VELP Scientifica srl.
Pipettes	**:** Pipetteman P10, P100, P1000, Eppendor

pH meter	**:** MP 220, Mettler Toledo International Inc.
	: Inolab pH level 1, order# 1A10-1113, Wissenschaftlich-Technische Werkstätten GmbH & Co KG.
Power supply	**:** EC 250-90, E-C Apparatus.
Pure water systems	**:** USF Elga UHQ-PS-MK3, Elga Labwater.
Spectrophotometer	**:** DU530 Life Science UV/ Vis, Beckman.
	: UV-1601, Shimadzu Corporation.
Sterilizer	**:** FN 500, Nuve.
Transilluminator	**:** UV Transilluminator 2000, Catalog# 170-8110EDU, Bio- Rad.
Vortexing machine	**:** Reax Top, product# 541-10000, Heidolph2.2.

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