REMEDIATION OF ANIONIC SURFACTANTS AND AMMONIUM BY BIOLOGICAL MATERIALS

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MASTER OF SCIENCE

By

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July 2012

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master of Science.

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ABSTRACT

REMEDIATION OF ANIONIC SURFACTANTS AND AMMONIUM BY BIOLOGICAL MATERIALS

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M.S in Materials Science and Nanotechnology

Supervisor: Dr. Turgay Tekinay

July, 2012

Surfactants are the main components in detergents and they are primarily discharged from household and industry. Ammonia (or ionized form ammonium) is a byproduct of animal and human metabolism and it is formed in and discharged from aquaculture. Contamination of soil and water sources by surfactants and ammonium is becoming a big problem because of their harmful effects. These substances are highly toxic to many organisms, leading to possible mass deaths in the freshwater ecosystem. As their presence causes a potential environmental risk, industrial and household wastewater systems should be adequately treated to reduce the concentration of ammonium and surfactants.

Chemical and biological methods are primarily used to treat wastewater systems. Biological treatment methods are more eco-friendly in comparison to chemical methods. Among biological treatment methods, the use of specific bacteria strains for removal of chemical contaminants is a widely applied process for treatment of industrial and municipal wastewater. However, those bacteria may not be capable of withstanding harsh environmental conditions or they may not specifically degrade the contaminant of interest, so isolation of bacterial strains more resistant to environmental extremes and more suitable for bioremoval is a possible strategy to improve current wastewater treatment strategies. By isolating bacteria well-adapted to the environmental and physical

conditions of the system to be cleaned, very high efficiencies can be obtained for wastewater cleaning. To this end, a two-step approach was used.

In the first part of this project, our aim was to find an integrated efficient biological based method to clean up industrial wastewater from anionic surfactants. Two main strategies were utilized to solve this problem: Finding and applying a more biodegradable and eco-friendly detergent alternative, and developing a biological treatment method specific for the anionic surfactants in the wastewater system of interest. It is expected that, by combining these two strategies, anionic surfactants in wastewater can be removed more efficiently.

In the second part of this project, a novel bacterial strain, which we termed STB1, was isolated from a commercial sea bass farm and found to display high heterotrophic ammonium removal characteristics. The species identity of STB1 was determined to be *Acinetobacter calcoaceticus*. We evaluated ammonium removal characteristics of STB1 at varying ammonium concentrations, and observed that STB1 can almost completely remove ammonium at low (50 mg/l) and medium (100 mg/l) concentrations within 72 h, while 45% ammonium removal was observed at a higher concentration (210 mg/l) during the same time period. Trace amounts of metabolized ammonium was converted to nitrite or nitrate and 22.16% of ammonium was introduced to cell biomass, while 4.34% of total nitrogen was initially incorporated into biomass and subsequently released to the supernatant fraction in the 100 mg/l sample. Most of the remaining conversion products are expected to be gaseous denitrification products. Toxicological studies with *Artemia salina* (brine shrimp) nauplii revealed that STB1 strain is non-toxic to *Artemia* larvae, which suggests that STB1 can be safely and efficiently utilized for water quality enrichment in aquatic ecosystems.

Keywords: bioremediation; environmental biotechnology; anionic surfactants; heterotrophic ammonium removal; *Artemia salina*

ÖZET

ANYONİK YÜZEY AKTİF MADDELERİN VE AMONYUMUN BİYOLOJİK MATERYALLERLE REMEDİASYONU

Ömer Faruk Sarıoğlu

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Tez yöneticisi: Dr. Turgay Tekinay

Temmuz, 2012

Yüzey aktif maddeler deterjanların esas bileşenleri olup evsel ve endüstriyel atık olarak deşarj edilmektedir. Amonyak (veya iyonize olmuş formu amonyum) ise insan ve hayvan metabolizması sonucu oluşan bir yan ürün olup, su ürünleri yetiştiriciliği ve kuluçkahane bulunan işletmelerde oluşmakta ve deşarj edilmektedir. Toprak ve su kaynaklarının yüzey aktif maddeler ve amonyum ile kirlenmesi bu maddelerin zararlı etkilerinden dolayı büyük problemlere yol açmaktadır. Bu maddeler birçok organizmanın sağlığı için tehdit oluşturmaktadır ve tatlı su ekosistemindeki organizmaların toplu ölümlerine yol açabilmektedir. Bu durum ciddi sorunlara sebebiyet verdiğinden dolayı atık su sistemlerindeki amonyum ve yüzey aktif maddelerin konsantrasyonunun azaltılması için etkili yollara ihtiyaç vardır.

Atık su sistemlerinin arıtılmasında kullanılan kimyasal ve biyolojik metotlar mevcuttur. Biyolojik metotlar kimyasal metotlara göre daha çevreci özelliklere sahiptir. Biyolojik arıtım metotları arasında özel bakteri suşlarının kimyasal atıkların yıkımı amacıyla kullanımı endüstriyel ve belediye atık sularının arıtımında yaygın olarak kullanılmaktadır. Ancak bazen bu bakteri karışımları zorlu çevresel koşullara karşı dayanıklı olamamakta ve istenilen atığa yönelik bir arıtım sağlayamamaktadır. Zorlu çevre koşullarına daha dayanıklı ve biyoyıkım amacıyla kullanılabilecek daha iyi ve daha güçlü bakterilerin bulunması ve izole edilmesi olası bir stratejidir. Uygun çevresel ve

fiziksel koşullar sağlandığında bu yöntem atık suların arıtımında başarı sağlayabilmektedir. Bu amaçla iki adımlı bir yaklaşım takip edilmiştir.

Projenin ilk kısmında, belirli bir endüstriyel atık suyunun anyonik yüzey aktif maddelerden arındırılması için etkili bir biyolojik yöntem geliştirilmesi hedeflenmiştir. Bu kısımda çözüme yönelik iki ana strateji geliştirilmiştir: daha biyobozunur ve daha çevreci bir deterjanın bulunması ve uygulanması, ve arıtılması istenilen endüstriyel atık suyun içindeki yüzey aktif maddeye özgü nitelikte bir biyolojik arıtma metodunun geliştirilmesi. Bu iki stratejinin birleştirilmesi ile atık sulardaki anyonik yüzey aktif maddelerin arıtımının daha etkili olması hedeflenmiştir.

Projenin ikinci kısmında, STB1 bakteri suşu, ticari bir deniz levreği çiftliğinden izole edilmiş ve amonyumu temizleme karakteristikleri yüksek olarak bulunmuştur. STB1 suşunun tür olarak tespiti *Acinetobacter calcoaceticus* olarak bulunmuştur. STB1 suşunun 72 saat içinde, aynı koşullarda, değişen konsantrasyonlardaki amonyumu parçalama karakteristikleri değerlendirilmiş, düşük (50 mg/l) ve ara konsantrasyonlardaki amonyumda (100 mg/l) neredeyse tamamen parçaladığı görülürken yüksek konsantrasyonda (210 mg/l) %45 oranında parçalama gözlemlenmiştir. 100 mg/l örneği için metabolize olmuş olan amonyumun düşük bir miktarı nitrit veya nitrata dönüşüp %22.16 oranında amonyum hücre biyokütlesine aktarılırken, %4.34 oranında toplam azot ilk etapta hücre biyokütlesine katılıp daha sonra süpernatanta aktarılmıştır. Geri kalan dönüşüm ürünlerinin büyük bir kısmının ise gaz halindeki denitrifikasyon ürünleri olduğu umulmaktadır. *Artemia salina* (su piresi) ile yapılan toksikoloji çalışmalarının sonucuna göre STB1 suşunun *Artemia* larvaları için toksik olmadığı bulunmuştur. Bu sonuç STB1 suşunun güvenli ve etkili bir şekilde sucul ekosistemlerin su kalitesi zenginleştirilmesi amacıyla kullanılabileceğini önermektedir.

Anahtar kelimeler: biyoremediasyon; çevresel biyoteknoloji; anyonik yüzey aktif maddeler; heterotropik amonyum yok edilimi; *Artemia salina*

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Canım babama,

LIST OF ABBREVIATIONS

ABS	branched-chain alkyl benzene sulfonate
DATS	dialkyltetralin sulphonates
FT-IR	fourier transform infrared spectroscopy
HPLC	high performance liquid chromatography
LC-MS	liquid chromatography mass spectroscopy
LABSA	linear alkyl benzene sulfonate
MBAS	methylene blue active substances assay
MS	mass spectrometry
OD	optical density
SDS	sodium dodecyl sulfate
SLES	sodium lauryl ether sulfate
SPAC	sulpho-phenyl carboxylic acids
TN	total nitrogen
UV	ultraviolet

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Parts of this study was published as “Heterotrophic ammonium removal by a novel hatchery isolate *Acinetobacter calcoaceticus* STB1” Omer Faruk Sarioglu, Rabia Suluyayla and Turgay Tekinay *International Biodeterioration and Biodegradation,* July 71, 2012 (Web)”, Reproduced (or 'Reproduced in part') with permission from Elsevier Ltd. Copyright 2012 Elsevier B.V. doi:10.1016/j.ibiod.2012.04.012.

Parts of this study was submitted as “Fourier Transform Infrared Spectroscopy as a Novel Approach for Analyzing the Biochemical Effects of Anionic Surfactants on a Surfactant Degrading *Arcobacter butzleri* Strain” to *Applied Spectroscopy* and got positive reviews. The revision has done and the paper is resubmitted to the journal (June).

CHAPTER I: BIOREMEDIATION OF ANIONIC SURFACTANTS

INTRODUCTION

1.1. Surfactants and their effects on the environment

Surface active agents (surfactants) are distinct chemicals which have both hydrophobic and hydrophilic groups and are primarily used for lowering the surface tension. Due to the high potential to use surfactants in different fields, they are widely used for industrial purposes and discharged to industrial wastewater. In general, surfactants come from cleaning detergents. However, detergents may be used for different purposes in industry. For instance, for glassware production, detergents are used as lubricants for shaping process. Since it brings an important problem, there should be a fine way to treat wastewater systems. In today's world, insufficient wastewater treatment leads to accumulation of high concentration of surface active agents (surfactants) in the recycling water, which causes a number of problems. The surface active agents accumulate on the surface of water and prevent the penetration of oxygen through water leading to death of the organisms in the water. Also excess foaming on the surface, which is detrimental to both the ecology and the tourism. In Fig. 1 and Fig. 2, examples for excess foaming of detergent surfactants on the surface of aquatic environments can be seen. These molecules could be also harmful for humans if they contaminate the water resources and people consume

contaminated water or agricultural products produced in these contaminated areas.

Until the end of 80's, detergent products mostly contained ABS (Alkyl Benzene Sulphonate) type surfactants. However, these types of surfactants are highly branched and not easily degraded in the environment. Since 1987, most countries have started to use LAS (Linear Alkyl Sulphonate) type surfactants, since these type of detergents are more easily degraded by various organisms in the environment, especially by bacteria [1, 2, 3]. After starting the use of LAS type surfactants, the concentrations of detergents in wastewaters have decreased significantly [6, 7]. Nevertheless, this application could not decrease the detergent concentration in the wastewater to values low enough so that there would be no risk to human and animal health; thus new techniques for the treatment of detergents are being developed [8].

Figure 1: Foaming in an industrial effluent. Adopted from: Sherwood Institute: Currently Browsing BLOG – Development [4].

Figure 2: Foaming in a domestic sewage. Adopted from: LIFE Magazine [5].

1.2. Current treatment methods and need for novel approaches

Chemical treatment methods are widely used to remove surfactants from wastewater systems and very efficient for cleaning up surfactants from wastewater systems. Chemical treatment methods are essentially oxidization reactions in which surfactants are destroyed by free radicals.

Fenton and photo-fenton reactions are common examples for chemical treatment methods to clean up the surfactants, but these reactions require high amount of free radicals and high acidic medium, which have severe detrimental effects on biological systems [9]. Moreover, if wastewater from Fenton reaction pool leaks to a river or any types of freshwater system, living organisms would be affected and might die due to these free radicals. In Fenton reactions unstable heavy metals are formed and accumulate in the pool, and the use of oxidants like H_2O_2 to initialize Fenton reactions may increase the COD (Chemical Oxygen Demand) values [9].

New approaches are required to resolve the problems associated with chemical treatment methods, which in turn bring in new costs. As such, it is also feasible to abandon chemical-based treatment methods and use special bacteria for biodegradation of surfactants, which is suggested as a more ecologically friendly way to clean up the surfactants. For this purpose, some biotechnology companies market specific bacteria mixes as commercial products [10]. Furthermore, some plant species can also be used for detergent absorbance and biodegradation [11]. This technique is called as phytoremediation and it is a very effective method in some cases. Relatively few plant species are currently used for this purpose, though the discovery of new and more effective phytoremediation agents may make this method more efficient for wastewater treatment.

Detergents may contain various chemicals in their formulas, for different purposes. However, toxicity displayed by detergents is due to the presence of surface active agents [12]. These surface active agents are divided into anionic, cationic and amphoteric surface active agents [13]. Since the most used surface active agents are anionic ones, biodegradation studies have focused on this type of substances. LAS (Linear Alkyl Benzene Sulphonate) type surfactants are in this class [14, 15]. Normally, the microorganisms in the environment do not encounter with these substances, and may therefore have difficulties to degrade these chemicals without the prior adaptations required for the task. However, after they encounter with these chemicals, they may evolve new strategies to deal with these substances and survive. Using different microorganisms together for detergent biodegradation is more reasonable, since they are more resistant to

environmental conditions in a consortium. Using bacteria for detergent biodegradation is both more effective and faster than the other methods. Furthermore, bacteria production is very economical in comparison to other methods.

The prime advantage of plants for bioremediation is the ability of plants to extend their roots into deeper parts of the soil and cleaning these hard-to-reach regions as well as the surface. In addition to this, the plant species that can survive nearby the factorial regions lend support to the idea that at least some plants are well-adapted to tolerate surfactants [16]. On the other hand, even though plants can be effective for the remediation of lower concentrations, unlike bacteria they cannot endure higher concentrations of surfactants and rapidly die in such environments, so the applicability of this method is very limited. As such instead of using phytoremediation as the primary remediation method, phytoremediation can be applied as a supportive method after completing other, more effective primary methods. Due to the aforementioned problems associated with phytoremediation, the most studied biological method is using bacteria for biodegradation. A large variety of bacterial species can be used for that purpose [17].

Since detergents contain diverse chemicals such as Sodium Lauryl Ether Sulfate (SLES), Sodium Dodecyl Sulfate (SDS), Triethanolamine (TEA); the enzymes involved in bacterial biodegradation also differ [18]. Moreover, the end products of biodegradation differ according to the biodegradation process applied by bacteria [19]. The main bacterial species for detergent biodegradation are

belonged to: *Vibrio, Klebsiella, Flavobacterium, Pseudomonas, Escherichia, Enterobacter, Proteus, Shigella* and *Citrobacter* genera [20]. However, many different bacterial species can also be utilized for detergent biodegradation.

1.3. Remediation of surfactants by bacteria

Classic wastewater treatment systems are not enough to clean up some of the chemicals. In particular classic wastewater treatments are insufficient in reducing the detergent concentration to minimal values. To complement or replace chemical methods, recent developments in biotechnology have led to the development of commercial consortia of bacteria produced to be used for the degradation of certain chemicals in water (Table 1).

- **Amnite F250 (BioPond):** contains bacteria that keep water clear and free of toxins.
- **Amnite L250 (BioSolv):** this bacterial formulation degrades deposited fat, oil and grease.
- **Amnite S250 (BioGest):** this bacterial formulation is efficient at degrading organic solids.

Table 1: Some commercial bacteria mixes from *Cleveland Biotech Limited* [21].

Moreover, these bacteria mixes can be stored for long periods in industry without the need for repurchase, in case the same problem is encountered again or long-term remediation is required, [21] thus using bacterial consortia are

economical compared to chemical treatment methods. Most of detergent-degrading bacteria are Gram negative, and as such more resistant to change in environmental conditions, and more suitable for bioremediation processes compared to Gram positive bacteria. Gram negative bacteria have a lipopolysaccharide (LPS) layer in their outer membrane, which makes these bacteria more resistant than their Gram positive counterparts to toxic substances. As such, most Gram-negative bacilli are common amongst detergent-degrading bacteria. Some previously studied bacteria genera and species are: *Klebsiella liquefasciens*, *Enterobacter liquefasciens*, *Klebsiella aerogenes*, *Escherichia coli*, *Enterobacter agglomerans*, *Staphylococcus albus*, *Pseudomonas aeruginosa*, *Proteus* sp., *Klebsiella oxytoca*, *Brevibacterium, Vibrio, Klebsiella pneumonia, Flavobacterium, Shigella* and *Citrobacter*. There is no a distinct relationship with bacteria's spore or biofilm forming ability and biodegradation capability of surfactants. Only *Staphylococcus albus* and *Brevibacterium* are gram positive among these bacteria. Only *Staphylococcus albus* is not rod-shaped, it is coccus while the other bacteria species are all bacilli [22]. *Pseudomonas* is the most studied genus for surfactant biodegradation. As a result of these studies, enzymes that are responsible for detergent biodegradation, P1 (*Primary alkylsulphatase),* P2 *(Primary alkylsulphohydrolase*) and a number of other bacterial enzymes were previously discovered [23, 24]. P1 and P2 are involved in the primary biodegradation, they initialize the biodegradation process for ultimate degradation of surfactants.

It was found that detergent degradation is inversely correlated with the concentration of detergent in the medium [24]; when detergent concentration

increases, the toxicity of the surfactant makes it difficult for bacteria to survive and degrade detergents, even death of the bacteria may occur at high concentrations.. Thus, at limited concentration ranges, bacterial treatment can be very useful to reduce detergent concentration to minimal values. Furthermore, it may be better to use bacterial consortiums instead of single species when conducting biodegradation studies since each species may be involve in a different step of the biodegradation process. Finding optimum bacterial consortia for surfactant biodegradation is current hot topic, and there are many successful studies for finding such consortia for surfactant removal or applying them to contaminated sites [25, 26].

Scott and Jones [27] have studied the bacterial degradation of surfactants from a biochemical point of view, and have obtained that the degradation of SDS (Sodium Dodecyl Sulphate) is initiated by the release of inorganic sulphur by basic sulphatase. Then the released alcohol is oxidized to lauryl acid by alcohol dehydrogenase, which is coded by a specific region in the genome that also encode supplementary proteins essential for the degradation of 5-12 carbon linear alkanes. Finally, lauryl acid is degraded in the β–oxidation process. This pathway was discovered in *Pseudomonas* species, but other detergent-degrading bacterial strains can also initiate the process of SDS biodegradation in a similar manner or participate in later stages of SDS degradation if they do not have the full set of required enzymes [27]. Some studies about LAS biodegradation have shown that the primary biodegradation begins with oxidation of the external methyl group and is followed by shortening of the alkyl chain via oxidative cleavage of C_2 units. After primary biodegradation, formation of the sulpho-phenyl carboxylic

acids (SPACs) occur [28]. Secondary biodegradation (ultimate biodegradation or mineralization) involves opening of the aromatic ring and desulphonation of SPACs, so that the formation of CO_2, H_2O, inorganic salts and biomass occur and SDS is completely degraded. It was also shown that dialkyltetralin sulphonates (DATS) and iso-LAS (co-products of LAS) form carboxylated intermediates upon bacterial biodegradation process [26].

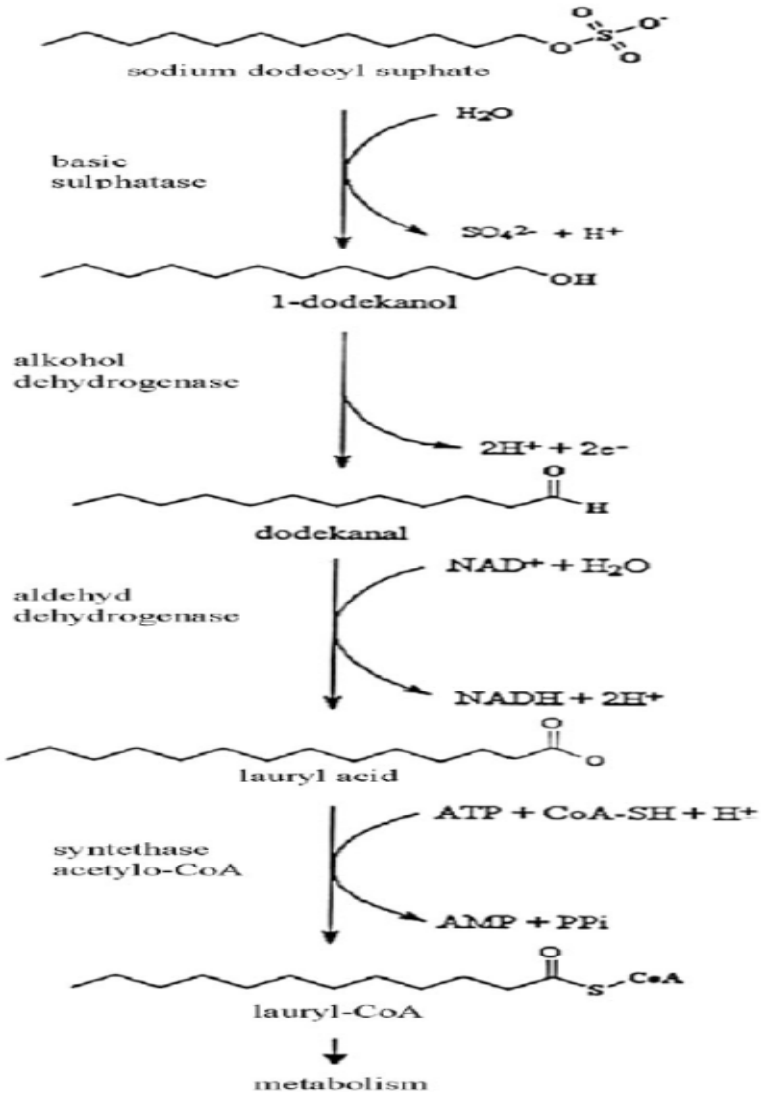


Figure 3: Scheme of SDS biodegradation. Adopted from M. Walczak et al. 2004, "Decomposition of Anionic Surface Active Substances by Bacteria from the Surface Microlayer of Lake Jeziorak Maly". [29].

1.4. Using of plants for bioremediation: phytoremediation

Plants can also be used for bioremediation processes. Some plants have the ability to degrade certain organic chemicals in the soil or water. Nevertheless, this is a very recent research area and only few plants are used for this process. Also, if we compare with microorganism based systems, it is not so effective, because plants can't tolerate high levels of surfactants. However, they can be used for supporting microorganism based systems. There are studies on different plants to adapt or engineer them for biodegradation. Some known plants which are used for this process are: Thlaspi plant, Tobacco, Wheat, Corn etc [30].

There is no study analyzing detergent degradation by plants. However it is possible for hydroponic plants, which grow in water, to reduce the detergent levels to some extent. The growth of some hydroponic plant species near factories and other industrial areas supports this idea. However, the question remains whether plants can completely degrade the detergents that it absorbs or whether the chemicals are mainly stored in plants vacuoles and are not metabolized in cells. For bioremediation of surfactants by bacteria, there are many studies that also demonstrate the enzymatic pathways by which surfactants are degraded. However, since phytoremediation is a very recent topic there is a dearth of studies for this topic so far, information on the actual mechanisms of phytoremediation is very limited. To elucidate what occurs to surfactants in plant cells, enzymatic studies should be conducted for plants that can be used for phytoremediation and the chemistry of this process must be understood.

1.5. Bioremediation of anionic surfactants: examples from literature

Surface active agents (surfactants) are the most widespread contaminant xenobiotics and continuously enter into the wastewater and aquatic environments [31-35]. The main principle for their ecological behavior is biodegradability of their chemical structures [36-39]. The biodegradation of surfactants can be performed by various microorganisms found in nature. The fundamental agents for surfactant biodegradation are bacteria [33, 35, 38-40]. Normally, microorganisms can degrade anionic surfactants in nature under standard conditions at a very low rate. [34, 35, 41-44]. Therefore, to improve the degradation of these contaminants, bio-augmentation techniques may be used and biotechnological approaches can be applied for efficient removal of surfactants from industrial wastewater [34, 45]. Membrane bioreactors have been used successfully to rapidly increase bacterial concentrations and enhance biodegradation rates of surfactants [46, 47].

Since removal of surfactants from wastewater systems is an essential issue, this topic has been extensively studied to find better solutions and more efficient approaches. As mentioned previously, chemical based methods can be used for degradation of surfactants, however, there is a tendency for using biological methods for the remediation of surfactants since the latter are less harmful [48] and very efficient at optimized conditions [27]. The hazardous effects of different types of surfactants are well-studied, and the effects of these chemicals on a number of organisms across different taxa have already been tested. For example, a study in the Netherlands [49] demonstrates the potential risk of a range of surfactants on aquatic environments. This study reveals

surfactants and soap have toxic properties on aquatic ecosystems above certain concentrations. Mieure et al. [50] has studied the risk estimation of LAS on terrestrial plants and animals. In this study, they found that orchids and hydroponically grown vegetables are the most vulnerable plant species to surfactants. As animal models, earthworms *Eisena foetida* and *Lumbricus terrestris* were used and harmful effects were observed at concentrations as low as 10 mg/l LAS. For toxicity risk assessment studies of aquatic environments, both surfactants themselves and their intermediate products must be considered and analytical tests should be regularly conducted for monitoring purposes [51]. It is more difficult to monitor LAS and their degradation products in marine environments, since the potential interferences may occur from other natural surfactants and other organic compounds [52]. For detection of surfactants in such environments, biosensor based systems have been devised as an alternative to classical analytical methods [53] however, improvements are required for practical application of this technology [27].

Anionic surfactants' biodegradation process is affected by several factors [27]. For instance, biodegradation of one of the most popular anionic surfactants, LAS, is affected by the concentration of dissolved oxygen [54], complexing with cationic surfactants [55, 56], formation of insoluble magnesium and calcium salts [57], the presence of other organic contaminants [58, 59] and pH changes during biodegradation process [60].

Although single bacterial species can efficiently degrade anionic surfactants under optimized conditions, the use of bacterial communities is

preferred for degradation of anionic surfactants [35] Therefore, bacterial consortia consisting of several bacterial strains are required for more efficient surfactant utilization under aerobic conditions [61, 62].

It was previously noted that some species of *Acinetobacter* are able to degrade different pollutants such as biphenyl or chlorobiphenyl, aniline, phenol, benzoate, crude oil and acetonitrile [63, 64]. A facultative anaerobic species; *A. calcoaceticus,* was able to degrade a greater proportion of alkanes compared to aromatic fractions in crude oil [65]. Another facultative strain of *Pantoea agglomerans* was involved in the biodegradation of kerosene, toluene and vaseline [66]. In a recent study, newly isolated strains of those two bacterial species; *Acinetobacter calcoaceticus* and *Pantoea agglomerans* were used in a consortium for removal of anionic surfactants SDS and LAS and this consortium was able to degrade both surfactants at extremely high concentrations (100% removal of SDS at 4000 ppm in 120 h, 60% removal of LAS at 300 ppm in 150 h) [25].

The strain *Serratia odorifera* 2 was previously described by Grimont et al. [67]. This strain is a member of the Gram-negative order Enterobacteriales and its cells are small and rod-shaped. These bacteria are widely found in water, soil, manure, bedding and feedstuff. The production of three special enzymes: DNase, lipase and gelatinase by this group of bacteria allows them to be distinguished from other genera which belong to the family Enterobacteriaceae [68]. A consortium of *Pantoea agglomerans* and *Serratia odorifera* 2 was previously tested for LAS biodegradation and found as adequately successful at

concentrations up to 200 ppm. This novel consortium has shown complete mineralization of LAS at 200 ppm in 72 h under optimized conditions [68].

In a different study, two different consortia: *Acinetobacter calcoaceticus-Klebsiella oxytoca* (A-K), and *Serratia odorifera-Acinetobacter calcoaceticus* (S-A) were tested for their biodegradation capability of SLES [26]. Both consortia have shown great efficiency, completely degrading 3000 ppm of SLES under optimized conditions. However, A-K bacterial consortium demonstrated a better efficiency (A-K completely degraded 3000 ppm of SLES in 96 h while the S-A consortium degraded the same concentration in120-144 h), higher growth rate and greater viability than the co-culture S-A.

MATERIALS AND METHODS

2.1. Materials and procurement of organisms

In early experiments of bacterial bioremediation of anionic surfactants, *Arcobacter butzleri, Bacillus subtilis, Proteus vulgaris, Klebsiella oxytoca, Pseudomonas aeruginosa* and a commercial bacteria mix (ESI EcoClear ^{TM} wastewater cleaning bacteria mix, which includes: *Bacillus subtilis, Bacillus amyloliquiefaciens, Nitrosomonas, Nitrobacter, Cellulomonas biazotea*), were used to test for biodegradation of SDS. ESI, EcoClear ^{TM} wastewater cleaning bacteria mix was purchased from ESI, Eco Scientific, Inc., Ohio, USA. *Klebsiella* and *Pseudomonas sp*. were obtained from Hacettepe University, Biology Department, Ankara, Turkey; *Enterobacter* and *Proteus sp*. were obtained from METU, Biology Department, Ankara, Turkey; and *Arcobacter butzleri* was obtained from Izmir Institute of Technology, Food Engineering Department, Izmir, Turkey.

Moreover, to find and isolate more specific and useful bacteria for bioremediation of anionic surfactants, different water samples were taken from the factory area nearby the wastewater effluent and named (according to the area they were taken) as: after biological treatment sample, detergent mix sample, river sample, the lower platform sample from machine, biological treatment sample, oily detergent sample after processing, detergent-water mix sample from machine, before biological treatment sample.

For our initial studies, SDS was utilized. After collection of bacterial isolates from different areas at the factory, we started to use SLES and LABSA

type surfactants for biodegradation studies in addition to SDS. SDS was purchased from Sigma-Aldrich (USA). SLES and LABSA were purchased from a local cleaning materials selling company (Tarmay Chemistry).

In all surfactant biodegradation experiments, LB (Lysogeny-broth) medium was used for growth of bacteria. In addition to this, minimal salt medium M9 which contains varying amounts of basal salts: 6.3 g/l Na_2HPO_4, 3.0 g/l KH_2PO_4, 0.5 g/l NaCl, and 1.0 g/l NH_4Cl was added to the medium. All reagents were purchased from Sigma-Aldrich (USA).

Duckweed samples were obtained from Ankara University, Faculty of Science, Department of Biology, Ankara, Turkey. The used bamboos in our studies were commercial ornamental bamboos, which were obtained from a local florist shop.

2.2. Analysis of anionic surfactants, MBAS Assay

MBAS (Methylene Blue Active Substances) assay in which, methylene blue binds with anionic surfactants in a liquid and gives an absorbance at 652 nm is accepted as the optimal method to measure surfactant concentration in water [69]. Besides MBAS assay, there are some other methods to measure surfactant concentration in water such as GC/MS (Gas Chromatography/Mass Spectroscopy) and HPLC (High Performance Liquid Chromatography) [70] however, these methods were not suitable for our studies, because they are labor intensive, more expensive and we had to measure a lot of samples. The basic difference between MBAS assay and the other measurement methods is; while we can observe just primary degradation by MBAS assay; GC/MS and HPLC

can also allow observing ultimate degradation of surfactants. Since our goal does not involve screening of ultimate degradation of surfactants, MBAS assay is sufficient for characterization of bacterial strains for their capability to degrade surfactants. In addition to this, primary degradation of surfactants is enough for surfactants to lose their surfactant properties. Construction of a calibration curve for the surfactant is required before applying this assay for experimental samples. We constructed calibration curves for different surfactants which were used in biodegradation studies, and the concentration changes of surfactants were calculated based on these data.

2.3. Finding alternative chemicals or detergents those are more biodegradable and more eco-friendly

Before starting this project, initial observations were done at the factory to have a better understanding of the problem and to have an opinion about how large scale bio-treatment is performed at the industrial area. Glassware industry uses detergents or similar lubricants for lubricating properties, to obtain smooth surfaces on glassware products. The basic problem of the factory was, difficulty of reducing the anionic surfactants level in wastewater to legal limits, which is 1 mg/l for European Union countries. Besides high concentration of anionic surfactants in wastewater, excess foaming leads serious problems such as collection of foams on the water surface and prevention of gas exchange for freshwater organisms. Due to all these problems, an R&D project was needed to solve this problem with minimal cost and maximum benefit.

To solve the factory's wastewater problem, we divided our project into two main topics; the first one is finding equivalent chemicals or detergents for glassware production which have low toxicological properties and high biodegradability, and the second one is activating the bio-treatment facility of the factory by using specific living organisms to the existing detergent. For conventional glassware production process in Turkey and other countries, a chemical is needed for its lubricating properties. Due to their low cost and high efficiency, diluted detergent mixes are used frequently for glassware production process. Nevertheless, the main chemicals in detergents, anionic surfactants are difficult to be cleaned up from wastewater and cause significant problems. Our first goal was finding an easily biodegradable chemical lubricant. There are many petroleum based commercial lubricants, but their biodegradability is low. Moreover, besides common lubricants, more biodegradable biolubricants are produced for their self removal abilities; unfortunately, such biodegradable biolubricants are extremely expensive in comparison to detergents. We prepared a long list (>100 chemicals) for metal working fluids and lubricants and we started to order them [71]. Our samples were chosen for their low cost and increased biodegradability. The samples were tested in our laboratory for some of their physical properties. The parameters which are considered in physical tests are:

1. pH of the samples (The target pH range was between 6 – 8)

2. Foaming (Our aim was to find low foaming samples)

3. Solubility of machine oil by the detergent (Our aim was to obtain samples that did not solve the machine oil)

4. Quality of the sample for glassware production process

The range of pH was described by the factory according to the specification list for detergents. Low foaming of the samples is an expected property for all samples since this issue was one of our goals. Low solvent property of samples for machine oil is important since previous detergents solve machine oil and make a pasty mix which plugs the pipes and prevents the transfer of after-process water. The actual test for seeing the production quality of samples for glassware production process can be done at the factory -*in situ*- however, before *in situ* trials, we did preliminary quality testing in laboratory environment by using an apparatus which mimics the rotational motion of the glassware production machines. This helped us eliminate some of the samples. The main problem in the factory about quality of the detergents is scratches on the surface of glassware products, which renders the glass defective. The defective glasses are disposed, increasing production costs. If detergent used is very low or ineffective, scratches occur; and if the detergent levels are too high, then white spots form on the glass.

2.3.1. pH analysis

The pH of all of the chemicals and the detergents were tested. We considered the pH of samples in the range of pH 6-8 and most of the samples were appropriate for pH in accordance to pH analysis results.

2.3.2. Foaming test

Diluted samples were prepared at equal concentrations (in Fig. 4a, 1/5 ratio) and 2 ml from each sample was taken and transferred to a 15 ml Falcon tube. Then 15 ml Falcon tubes were shaken vigorously for 30 second by Vortex and samples were classified according to the foaming differences between them.

2.3.3. Solvent of machine oil test

The main purpose for applying this test is do not encounter with the problem of presence of a pasty mix due to the solution of machine oil by the chemical or detergent sample and preventing the possibility of plugging the pipes with this pasty mix. In Fig. 4b, first sample is more appropriate since it solves the machine oil less than the second sample.

(a)

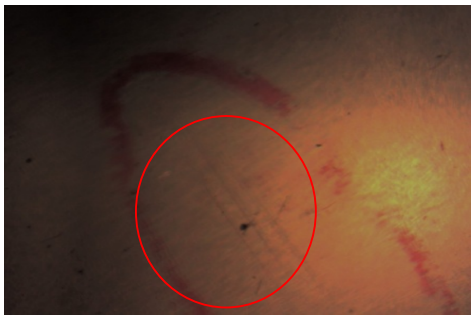
(b)

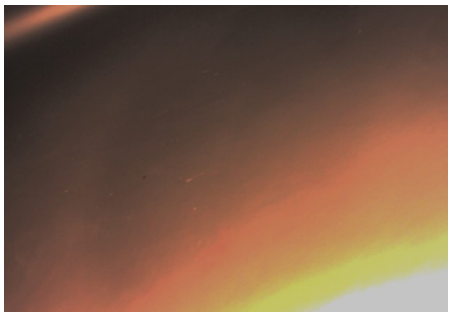
Figure 4: Foaming (a) and solvent of machine oil (b) differences between two different diluted detergent samples at equal dilution ratios (1/5 dilution ratio for both samples).

2.3.4. Mould scratching test

For glassware production process, a metallic mould which is coated with a special cork powder is utilized for shaping glassware products. Diluted detergent mixes which lower the surface tension are used to reduce the risk of mould scratching on glassware products. We tested the likelihood that chemicals or detergents cause mould scratching on glass cups. The system works with the aid of a rotational motor (Fig. 5a). By the mould scratching test apparatus, which mimics the production, we were able classify the samples in accordance to their qualities, and eliminate some of them. Before starting the test, the surface of the mould is wetted by a diluted chemical or detergent sample and then the glass cup is fixed on the mould scratching test apparatus to allow rotational motion of the glass cup on the mould. The system was opened for one minute and after this time, the system was stopped, glass cup is taken and observed under the microscope for the presence of mould scratches on the glass surface.

(a)





(b) **(c)**

Figure 5: Mould scratching test apparatus (a). At the end of the test, microscope images of the detergent sample that leading to mould scratches on glass surface (b), and the detergent sample that does not lead to mould scratches on glass surface (c).

2.4. Factory trials for alternative detergent samples

After eliminating many of the chemicals in the laboratory tests (mould scratching test, foaming test, solubility of machine oil by the detergent test), 16 different alternative detergent samples were tested in a factory in pilot trials. We compared the production quality of alternative detergent samples with previously tried high quality detergents. While some of alternative detergent samples could fulfill our criteria, some of them could not. The main expected criteria from alternative detergent samples are shown below:

- Higher Production Quality; low (acceptable) mould scratching and lower white stain viability on glass surfaces
- Lower mould change frequency
- Lower foaming levels
- Lower solubility of machine oil by the detergent
- Higher biodegradability

2.5. Biodegradation of SDS by *Arcobacter butzleri*

2.5.1. Culture media and procurement of bacteria

LB (Luria-Bertani) broth was utilized as the base growth medium in this study [72, 73].This medium was supplemented with M9 minimal salts, including 6.3 g/l Na_2HPO_4, 3.0 g/l KH_2PO_4, 0.5 g/l NaCl and 1.0 g/l NH_4Cl [74]. All reagents were obtained from Sigma-Aldrich (USA). The *Arcobacter butzleri* strain used in this study was isolated and characterized as previously described [75]. Briefly, the strains were isolated from chicken carcasses by using Arcobacter enrichment broth (AEB) to specifically isolate *Arcobacter* species, and the *A. butzleri* isolate was identified at the species level by a multiplex-PCR assay. No specific designation was given to the isolated strain. This strain was grown in LB-broth medium and upon visible growth; new inocula were prepared for surfactant biodegradation studies.

2.5.2. Shaking-culture experiments for SDS biodegradation

Bacterial inocula were grown at SDS concentrations of up to 100 mg/l to observe surfactant degradation capability of *A. butzleri* at varying initial surfactant concentrations. LB-broth samples containing 0, 10, 40 and 100 mg/l SDS were prepared for biodegradation studies. Bacterial growth ratios were determined by OD_{600} measurements. Samples were incubated at 30^{o}C and 125 rpm. Remaining SDS concentrations were determined at days 0, 1, 2, 3, and 6 by MBAS (Methylene Blue Active Substances) assay [69]. All tests were done in triplicate.

2.5.3. Fourier Transform Infrared spectroscopy analysis (FT-IR)

Arcobacter butzleri samples were inoculated in 50 ml M9 salts supplemented LB medium containing 0, 40, 100 mg/l and 3 g/l SDS. Samples were taken at days 0, 1 and 3 (1 ml for each aliquot) and centrifuged at 14000 rpm for 5 min, the supernatants were removed and the remaining pellets were washed with physiological saline (0.90% w/v of NaCl) twice and stirred with distilled water. 50 µl of this final solution was dried on a 96-well plate at 45^{o}C for 1 h. When the samples in each loaded well were dried, the 96-well plate was utilized in FT-IR transmittance analysis by using Nicolet 6700 FT-IR Spectrometer (Thermo-Scientific, US). OMNIC^{TM} software was used for measurements and basic modifications such as baseline and background corrections. Background corrections for H_2O and CO_2 were carried out for each analysis. Experiments were repeated for four times and duplicate samples were utilized in each experiment.

2.5.4. Scanning Electron Microscopy (SEM)

The *A. butzleri* isolate was inoculated in 50 ml M9 salts supplemented LB medium with and without 3 g/l SDS and incubated for 48 h at 125 rpm and 30°C. 0.2 ml of evenly distributed bacteria-containing medium was taken for each sample. The bacteria-containing medium was poured on a filter membrane and dried at 45^{o}C for 1 h. After drying, filter membranes were fixed for SEM analysis as described by Greif and colleagues [76]. Images were taken using a Quanta 200 FEG scanning electron microscope (FEI Instruments, USA).

2.6. Preliminary characterization of bacterial and plant samples for surfactant biodegradation studies

2.6.1. Isolation of Surfactant Degrading Bacteria

Various bacterial species were tried in early surfactant biodegradation studies such as; *Bacillus sp.*, *Proteus sp.*, *Enterobacter sp.*, *Pseudomonas sp.* and ESI, EcoClear ^{TM} wastewater cleaning bacteria mix. Those isolates were obtained from different sources and stored at -80^{o}C for further studies. Water samples nearby the wastewater effluent were taken from the factory to isolate surfactant degrading bacteria. Equal amounts of those samples (1 ml) were inoculated to 100 ml M9 salts supplemented LB medium. Then we spread those samples to LB-agar plates to figure out if there is more than one colony in each sample. Just for River sample, we observed two different colonies. One is larger and darker and one is smaller and lighter on LB-agar plates. We named those isolates as River 1 isolate and River 2 isolate. We then inoculated all isolates to M9 salts supplemented LB medium for further use in surfactant resistance experiments. The incubation of samples was done at 30^{o}C and 100 rpm initially, and at 30^{o}C and 125 rpm later.

Bacterial samples were grown in SDS, SLES or LABSA containing LB media. Initially, low concentrations of SDS containing bacterial growth media were used, and then SDS concentrations were increased gradually until observable negative effects could be seen. For initial surfactant biodegradation studies, upper limit was 10 mg/l, although bacterial samples can grow at higher concentrations of SDS as well. For further biodegradation studies, bacterial

inocula were grown at surfactant concentrations of up to 300 mg/l (a defined concentration based on literature survey and discharge requirements) to observe surfactant biodegradation capability of different isolates at higher initial surfactant concentrations. Bacterial growth ratios were determined by OD_{600} measurements. Remaining surfactant concentrations were determined sequentially by MBAS assay.

2.6.2 Phytoremediation studies

Duckweed samples were grown in SDS containing water, and upper limit for SDS concentration was around 10 mg/l. The remaining SDS concentrations in the water were calculated by converting the absorbance data of the MBAS assay to SDS concentrations in mg/l unit by using the calibration curve of SDS.

Similar to duckweeds, bamboos were also grown in SDS containing water and remaining SDS concentrations in the water were calculated by converting the absorbance data of MBAS assay to SDS concentrations in mg/l unit by using the calibration curve of SDS. There are several different experiments for bamboos and the upper limit for SDS concentration is 100 mg/l. To observe the toxicological effects of SDS on bamboos, bamboos were grown at higher concentrations of SDS (1000 mg/l).

RESULTS AND DISCUSSION

3.1. Finding appropriate detergent alternatives for glassware production

16 different alternative detergent samples were tested in a factory in pilot trials. We labeled these detergents with letter initials. Our expected criteria from alternative detergent samples are shown below:

- Higher Production Quality; low (acceptable) mould scratching and lower white stain viability on glass surfaces
- Lower mould change frequency
- Lower foaming levels
- Lower solubility of machine oil by the detergent
- Higher biodegradability

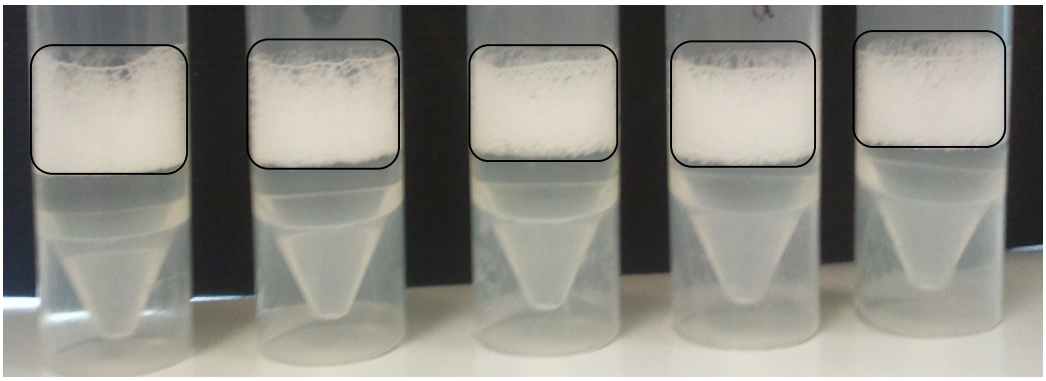
3.1.1. First trial

- Tried detergents: A detergent, B detergent, and C detergent.
- Hayat detergent is a previously used detergent in the factory.
- PK detergent is an alternative detergent which was used in another factory at that time.
- A, and C detergents are our own detergent mixes, B detergent is from Universal Chemistry.
- C detergent was not tested in our laboratory for physical tests. It is a control detergent mix that contains very low amounts of surfactants.

3.1.1.1. Physical test results

3.1.1.1.1. Foaming test

According to Fig. 6, at 1/4 dilution ratios, foaming of 4 different detergent samples are very close. There are slight differences for foaming levels among 4 different samples. Hayat, PK and B detergent samples seem very close at 1/4 dilution ratio for foaming levels, however A detergent 1/4 and B detergent 1/5 samples display nearly same foaming levels and their foaming level is slightly lower than the other samples.

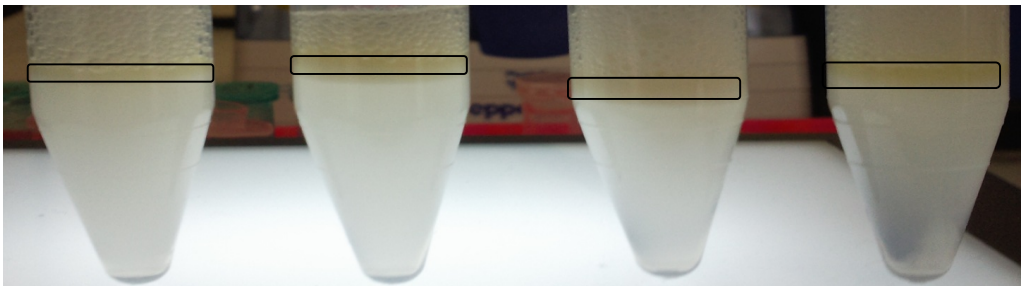


Hayat det. 1/4 PK det. 1/4 A det. 1/4 B det. 1/4 B det. 1/5

Figure 6: Comparison of foaming levels for 5 different samples at equal dilution ratios (1/4).

3.1.1.1.2. Solvent of machine oil test

According to Fig. 7, B detergent solves machine oil less in comparison to the other samples. Nevertheless, there are very slight differences for the other samples solvent of machine oil capability.



Hayat det. PK det. A det. B det.

Figure 7: Comparison of solvent of machine oil differences for 5 different samples at 1/4 dilution ratios.

3.1.1.2. First trial results

3 different detergent samples were tested in this trial. The length of this trial was 1 day. The basic parameters that were considered in this trial were: *foaming, solvent of machine oil*, and *production quality*. The dark black stains at the floor reflect unsolved machine oil droplets.

Figure 8: After processing appearance of A detergent.

Figure 9: After processing appearance of B detergent.

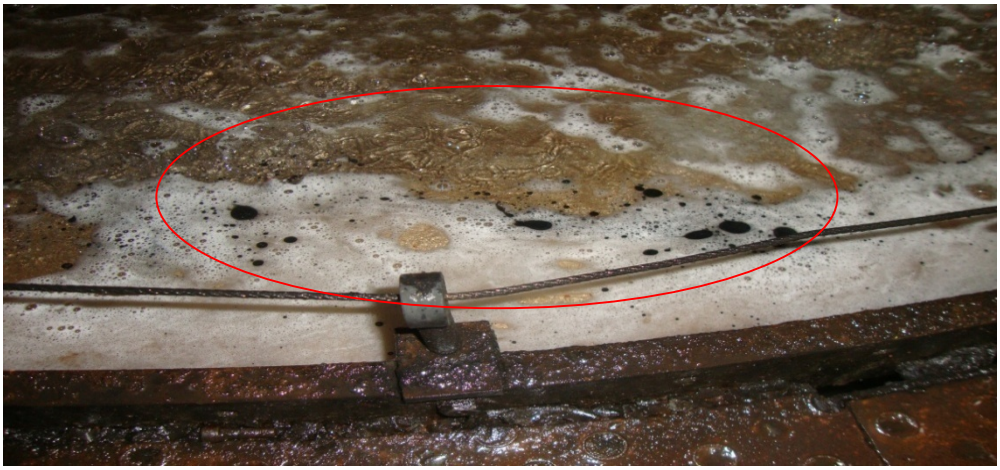


Figure 10: After processing appearance of C detergent.

3.1.1.3. Discussion of the first trial

B detergent sample is an eco-friendly and highly biodegradable detergent, and the surface active agents in this detergent are fully biodegradable according to statement of the producing company. A, and C detergent samples are also biodegradable detergent samples and the actual surfactant in these detergent samples, LABSA, is a highly biodegradable surfactant type. Although

the actual surfactant in A and C detergent samples are same, the percentages of this surfactant in these samples are different and so they are called differently as A, and C. Since C detergent sample is much more diluted with water, T.A.S (total active substances) ratio is lower for this sample (3.8 %). Although A detergent sample contains more surfactant in its formulation, the expected production quality for this sample could not be reached, probably because of the T.A.S ratio is not enough (< 20%).

Comparison of 4 different detergent samples					
	Biodegradability	Foaming	Solvent of machine oil	Production quality	T.A.S percentage
PK detergent sample	Unknown	High	High	Good	23%
Hayat detergent sample	Good	High	High	Good	24%
A detergent sample	Good	Low	High	Not good	14%
B detergent sample	High	Low	Medium	Good	13,80%

Table 2: Comparison of 4 different detergent samples for different properties.

3.1.2. Second trial

- Tried detergents: E detergent, F detergent, G detergent, and H detergent.
- E, and F detergents are our own detergent mixes; G, and H detergents are from Universal Chemistry.
- D detergent was not tested in the factory; it was eliminated due to its high foaming property.

3.1.2.1. Physical test results

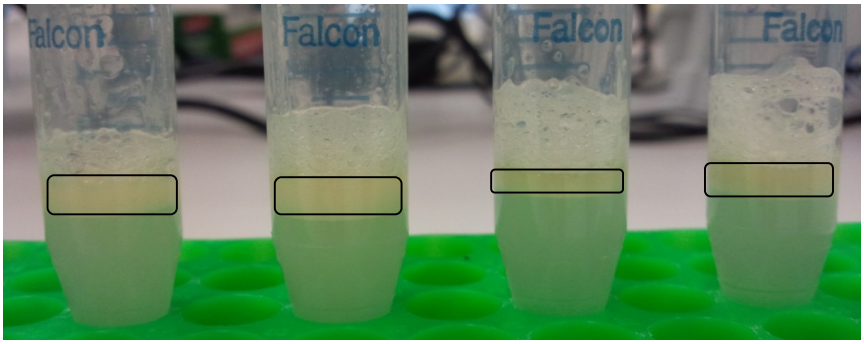
3.1.2.1.1. Foaming test

According to Fig. 11, foaming levels of samples at 1/4 dilution rates were different from each other. G and H detergent samples are very close for their foaming levels, and their foaming levels are higher than other samples. F detergent sample has lower foaming level than G and H detergent samples, and E detergent sample has the lowest foaming level among 4 samples.

E det. 1/4 F det. 1/4 G det. 1/4 H det. 1/4

Figure 11: Comparison of foaming levels for 4 different samples.

3.1.2.1.2. Solvent of machine oil test



E det. 1/4 F det. 1/4 G det. 1/4 H det. 1/4

Figure 12: Comparison of solvent of machine oil differences for 4 different samples at 1/4 dilution ratios.

According to these results, G detergent solves machine oil more comparing to the other samples. Nevertheless, there are very slight differences for the other samples' solvent of machine oil capability.

3.1.2.2. Second trial results

4 different detergent samples (E, F, G and H) were tested in this trial. The length of this trial was 1 day. The basic parameters that were considered in this trial were: *foaming, solvent of machine oil*, and *production quality*. The dark black stains at the floor reflect unsolved machine oil droplets.

Figure 13: After processing appearance of E detergent.

Figure 14: E detergent, solvent of machine oil test.

Figure 15: After processing appearance of F detergent.

Figure 16: After processing appearance of G detergent.

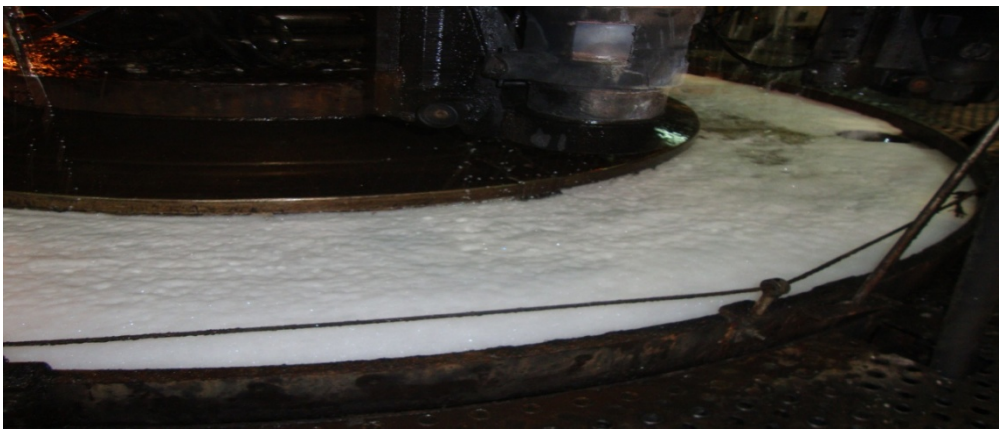


Figure 17: After processing appearance of H detergent.

3.1.2.3. Discussion of the second trial

G and H detergent samples are eco-friendly and highly biodegradable detergents, and the surface active agents in this detergent are fully biodegradable according to statement of the producing company. E, and F detergent samples are also biodegradable detergent samples and the actual surfactant in these detergent samples, LABSA, is a highly biodegradable surfactant type. Although the actual surfactant in E and F detergent samples are same, the percentages of this surfactant in these samples are different, and so they are called differently as E and F detergents. In this trial, only E and F detergents are good for production quality.

Comparison of 4 different detergent samples					
	Biodegradability	Foaming	Solvent of Machine oil	Production quality	T.A.S percentage
E detergent sample	Good	Normal	Medium	Good	27,32%
F detergent sample	Good	High	Medium	Good	34,14%
G detergent sample	High	High	High	Not good	14,70%
H detergent sample	High	High	Medium	Not good	17,37%

Table 3: Comparison of 4 different detergent samples for different properties.

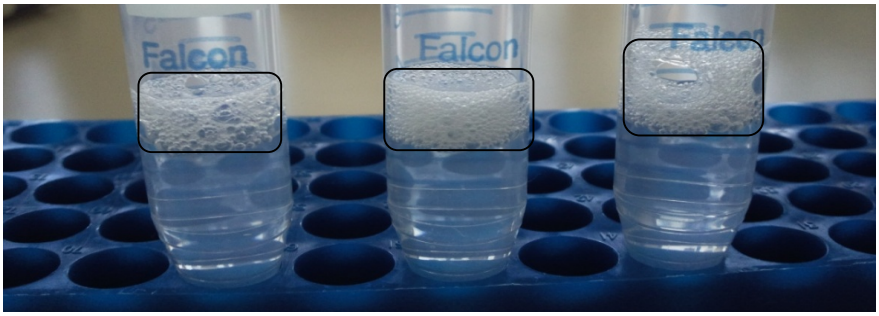
3.1.3. Third trial

- Tried detergents: I detergent, and J detergent samples.
- Both detergents are our own detergent mixes.
- No Universal Chemistry detergent samples were tested in this trial.

3.1.3.1. Physical test results

3.1.3.1.1. Foaming test

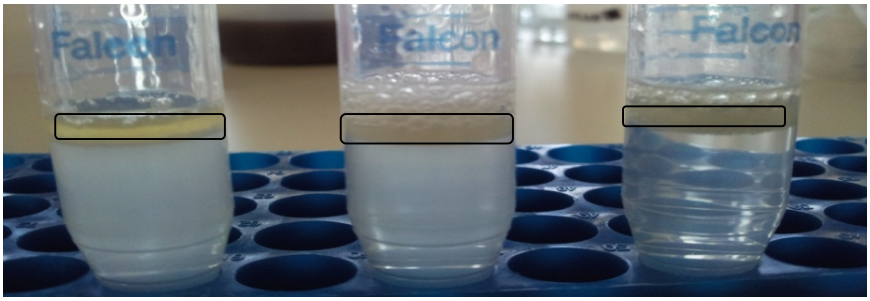
According to Fig. 18, at 1/5 dilution ratios, foaming of 3 different detergent samples are very close. There are slight differences for foaming levels among 3 different samples. I and PK detergents display lower foaming, and J detergent sample displays higher foaming in comparison to these samples.



PK det. 1/5 I det. 1/5 J det. 1/5

Figure 18: Comparison of foaming levels for 3 different samples.

3.1.3.1.2. Solvent of machine oil test



PK det. 1/5 I det. 1/5 J det. 1/5

Figure 19: Solvent of machine oil differences for 3 different samples.

According to these results, solvent of machine oil for 3 different detergent samples is different. I detergent solves machine oil higher than other samples. PK detergent solves machine oil less than I detergent however, J detergent has the lowest solvent of machine oil, and the clearer appearance at the bottom of the tube supports the low solvent of machine oil property of this sample.

3.1.3.2. Third trial results

2 different detergent samples were tested in this trial. The length of this trial was 1 day. The basic parameters that were considered in this trial were: *foaming, solvent of machine oil*, and *production quality*. The dark black stains at the floor reflect unsolved machine oil droplets.

Figure 20: After processing appearance of I detergent.

Figure 21: I detergent, solvent of machine oil test.

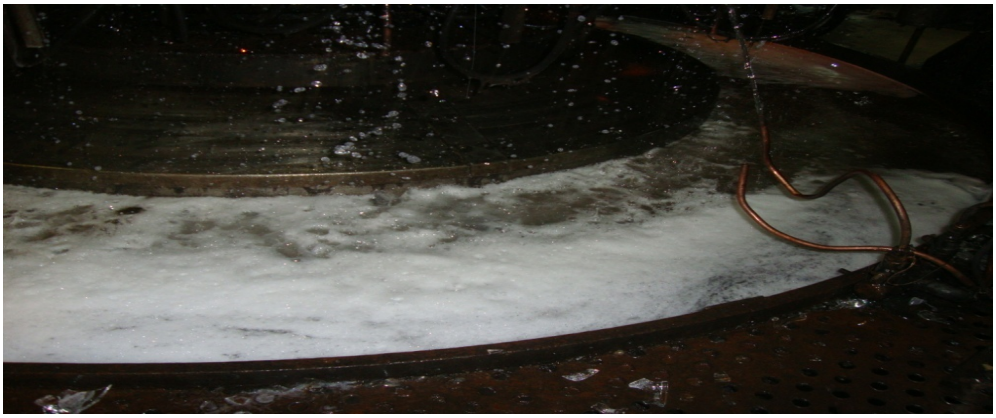


Figure 22: After processing appearance of J detergent.

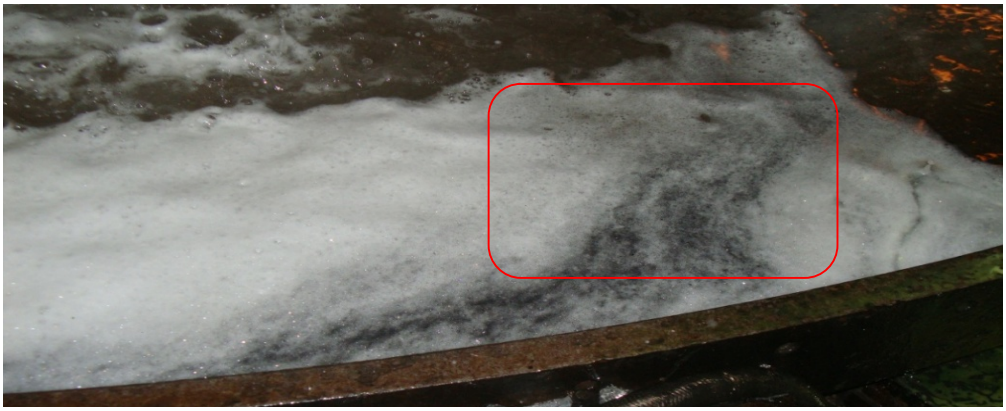


Figure 23: J detergent, solvent of machine oil test.

3.1.3.3. Discussion of the third trial

I detergent sample is an efficiently biodegradable detergent sample and the actual surfactant in these detergent sample, LABSA, is a highly biodegradable surfactant type. J detergent sample is also an efficiently biodegradable detergent; the actual surfactant in this detergent mix, SLES is another highly biodegradable surfactant type. Since LABSA has a benzene ring in its formulation, biodegradation of LABSA is supposed to be more difficult in comparison to SLES in the nature. Nevertheless, the production quality is good for both samples.

Comparison of 3 different detergent samples					
	Biodegradability	Foaming	Solvent of Machine oil	Production quality	T.A.S percentage
PK detergent sample	Unknown	Normal	Low	Good	23,00%
I detergent sample	Good	Normal	Low	Good	23,40%
J detergent sample	High	Higher	Lower	Good	21,40%

Table 4: Comparison of 3 different detergent samples for different properties.

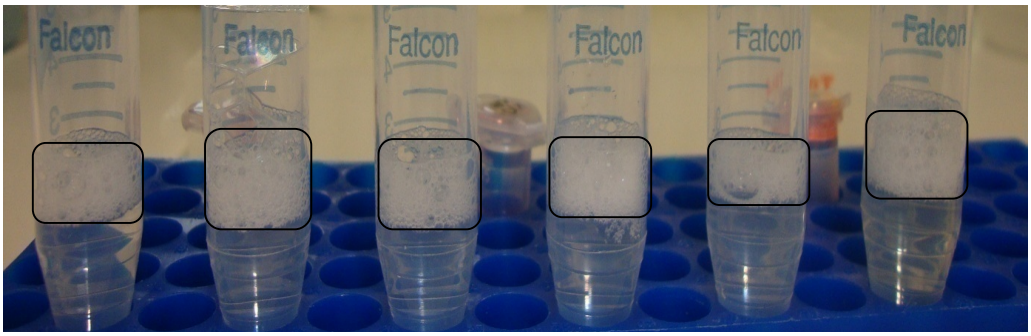
3.1.4. Fourth trial

- Tried detergents: K detergent, L detergent, M detergent and O detergent samples.
- K, L, and M detergent samples are from Universal Chemistry, O detergent sample is our own detergent mix.

- K, L, and M detergent samples are new offered alternatives by Universal Chemistry for using in glassware production process (L detergent sample was previously tried in another Turkish glassware production company and found as successful).
- PE detergent is an alternative detergent which was used in another factory at that time (like PK detergent).

3.1.4.1. Physical test results

3.1.4.1.1. Foaming test



K det. 1/5 L det. 1/5 M det. 1/5 N det. 1/5 O det. 1/5 PE det. 1/5

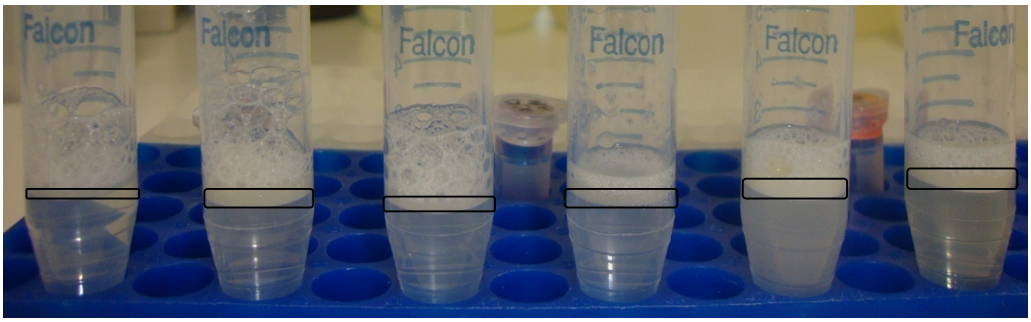
Figure 24: Comparison of foaming levels for 6 different samples.

According to these results, at equal concentrations, foaming of 6 detergent samples is different. O detergent sample displays the lowest foaming; K and N detergent samples follow this sample with similar foaming levels. M detergent and PE detergent samples display similar foaming and their foaming levels are higher than K, N, and O detergents. L detergent sample displays the highest foaming level among 6 samples. Therefore, O detergent is the favorable

detergent sample according to this test. Previously utilized L detergent displays high foaming and it does not match with our expected criteria.

3.1.4.1.2. Solvent of machine oil test

According to Fig. 25, solvent of machine oil for 4 detergent samples is very close. L, N, O, and PE detergent samples solve machine oil similarly. M detergent sample solves machine oil more than these samples. K detergent sample solves machine oil more than all samples.



K det. 1/5 L det. 1/5 M det. 1/5 N det. 1/5 O det. 1/5 PE det. 1/5

Figure 25: Comparison of solvent of machine oil differences for 6 different samples at equal dilution ratios (1/5).

3.1.4.2. Fourth trial results

5 different detergent samples were tested in this trial. The length of this trial was 2 days. The basic parameters that were considered in this trial were: *foaming, solvent of machine oil*, and *production quality*. The dark black stains at the floor reflect unsolved machine oil droplets.

Figure 26: After processing appearance of PE detergent.

Figure 27: After processing appearance of K detergent.

Figure 28: After processing appearance of L detergent.

Figure 29: After processing appearance of M detergent.

Figure 30: M detergent, solvent of machine oil test.

Figure 31: After processing appearance of N detergent.

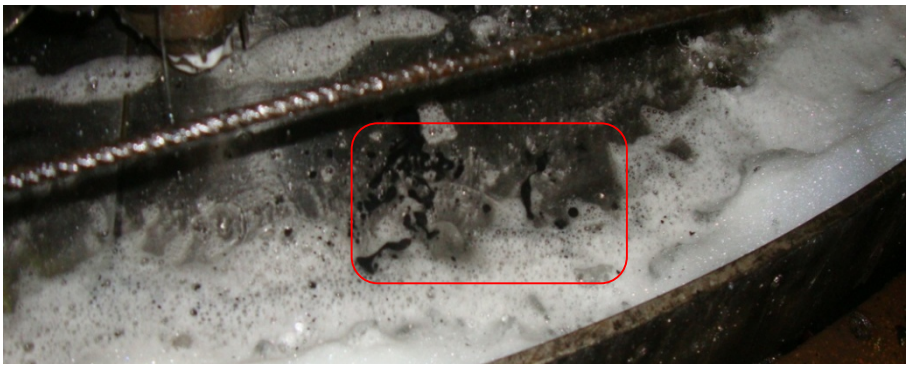


Figure 32: N detergent, solvent of machine oil test.

Figure 33: After processing appearance of O detergent.

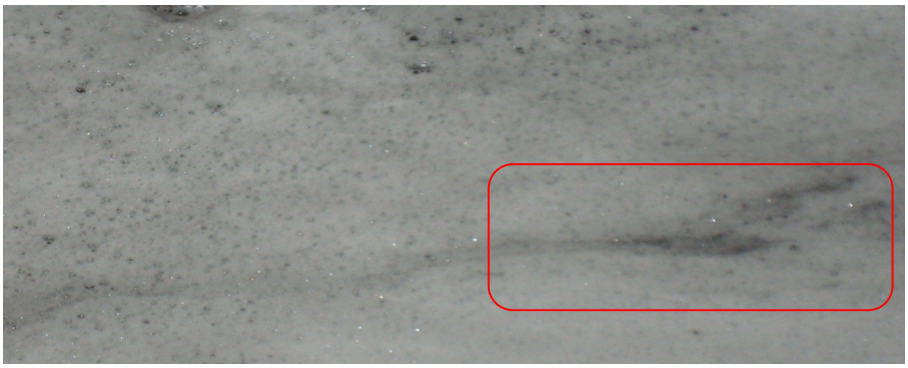


Figure 34: O detergent, solvent of machine oil test.

3.1.4.3. Discussion of the fourth trial

K, L, M detergent samples are eco-friendly and highly biodegradable detergents, and the surface active agents in this detergent are completely biodegradable according to the statement of the producing company. O detergent sample is an efficiently biodegradable detergent sample and the actual surfactant in this detergent sample, LABSA, is a highly biodegradable surfactant type. N detergent sample is also an efficiently biodegradable detergent; the actual surfactant in this detergent mix, SLES is another highly biodegradable surfactant type. Since LABSA has a benzene ring in its formulation, biodegradation of LABSA is supposed to be more difficult in comparison to SLES in the nature. Nevertheless, the production quality is good for both samples. K, L, and M samples are also good for production quality.

Comparison of 6 different detergents					
	Biodegradability	Foaming	Solvent of machine oil	Production quality	T.A.S percentage
PE detergent sample	Unknown	High	Low	Good	22%
K detergent sample	Unknown	High	Normal	Good	19,60%
L detergent sample	Unknown	High	Low	Good	15,40%
M detergent sample	Unknown	High	Low	Good	15,00%
N detergent sample	High	Lower	Low	Good	21%
O detergent sample	Good	Low	Low	Good	26,60%

Table 5: Comparison of 6 different detergent samples for different properties.

3.1.5. Fifth Trial

- Tried detergents: P and R detergent samples.
- After this trial, P and R detergent samples were tested in the factory for the last time and then accepted as the appropriate detergent for production quality.

3.1.5.1. Physical test results

3.1.5.1.1. Foaming test

According to Fig. 35, the detergent samples are different for foaming levels at equal concentrations. P detergent displays the lowest foaming at 1/4 dilution ratio. R detergent follows P detergent; it displays lower foaming than Hayat and PE detergent. Hayat and PE detergents display higher foaming, and their foaming levels are very similar. As a result, P and R detergent samples are more appropriate than other samples for their foaming properties. P and R detergent samples are modified forms of previously tried N and O detergent samples. The foaming levels of P and R detergent samples are significantly decreased by additional use of an efficient anti-foam, SILFOAM® SE 39.

P det. 1/4 R det. 1/4 PE det. 1/4 Hayat det. 1/4

Figure 35: Comparison of foaming levels for 4 different samples.

3.1.5.1.2. Solvent of machine oil test

According to Fig. 36, solvent of machine oil for 4 detergent samples is different. Hayat and PE detergent samples solve machine oil similarly and more than other alternatives, P and R detergents. P detergent sample solves machine oil less than Hayat and PE detergent samples. R detergent sample solves machine oil less than all samples. R detergent is found as more suitable for solvent of machine oil property according to this test however, P detergent is also eligible since its solvent of machine oil property is very close to R detergent sample.

P det. 1/4 R det. 1/4 PE det. 1/4 Hayat det. 1/4

Figure 36: Comparison of solvent of machine oil differences for 4 different samples at equal dilution ratios (1/4).

3.1.5.2. Fifth trial results

2 different detergent samples were tested in this trial. The length of this trial was 1 day. The basic parameters that were considered in this trial were: *foaming, solvent of machine oil*, and *production quality*. The dark black stains at the floor reflect unsolved machine oil droplets.

Figure 37: After processing appearance of PE detergent.

Figure 38: After processing appearance of P detergent.

Figure 39: After processing appearance of R detergent.

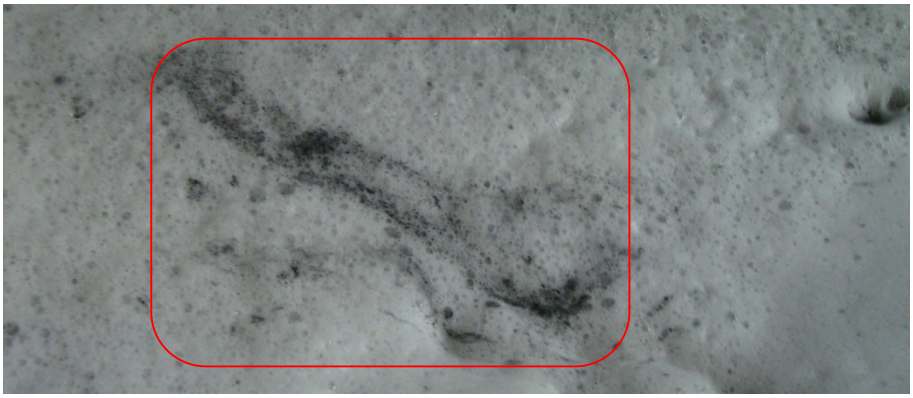


Figure 40: R detergent, solvent of machine oil test.

3.1.5.3. Discussion of the fifth trial

R detergent sample is an efficiently biodegradable detergent sample and the actual surfactant in this detergent sample, LABSA, is a highly biodegradable surfactant type. P detergent sample is also an efficiently biodegradable detergent; the actual surfactant in this detergent mix, SLES is another highly biodegradable surfactant type. Since LABSA has a benzene ring in its formulation, biodegradation of LABSA is supposed to be more difficult in

comparison to SLES in the nature. P detergent sample is the developed version of N detergent, and R detergent sample is the developed version of O detergent. The foaming levels of detergents are adjusted as lower by addition of SILFOAM® SE 39 antifoam at a standard concentration for both detergent samples. Total active substances ratio in the detergent mixes are also adjusted as optimum values and the production quality was found as good for both samples.

Comparison of 4 different detergents					
	Biodegradability	Foaming	Solvent of machine oil	Production quality	T.A.S percentage
PE detergent sample	Unknown	High	Normal	Good	22%
Hayat detergent sample	Unknown	High	Normal	Good	24%
P detergent sample	High	Lower	Low	Good	24,80%
R detergent sample	Good	Low	Low	Good	20,10%

Table 6: Comparison of 4 different detergent samples for different properties.

3.1.6. Overall results of the factory trials

At the end of the factory trials with alternative detergent samples, we have achieved:

- *A very successful and biodegradable detergent which was developed by us and the recipe belongs to us. (P detergent)*
- *High production quality for glassware production,*
- *No white stain problem anymore,*
- *Mould change frequency is in expectation,*
- *Lower foaming levels,*
- *Lower solvent of machine oil,*

- *T.A.S percentage, pH, and salt concentration of the detergent are in expectation.*

Comparison of the all tried detergent samples in the PE factory					
	Biodegradability	**Foaming**	**Solvent of machine oil**	**Production quality**	**T.A.S percentage**
A detergent sample	Good	Low	High	Not good	14%
B detergent sample	High	Low	Medium	Good	13,80%
C detergent sample	Good	Low	Medium	Not good	3,80%
E detergent sample	Good	Normal	Medium	Good	27,32%
F detergent sample	Good	High	Medium	Good	34,14%
G detergent sample	High	High	High	Not good	14,70%
H detergent sample	High	High	Medium	Not good	17,37%
I detergent sample	Good	Normal	Low	Good	23,40%
J detergent sample	High	High	Low	Good	21,40%
K detergent sample	Unknown	High	Normal	Good	19,60%
L detergent sample	Unknown	High	Low	Good	15,40%
M detergent sample	Unknown	High	Low	Good	15%
N detergent sample	High	Low	Low	Good	21%
O detergent sample	Good	Low	Low	Good	26,60%
P detergent sample	High	Low	Low	Good	24,80%
R detergent sample	Good	Low	Low	Good	20,10%

Table 7: Comparison of 16 different detergent samples for different properties.

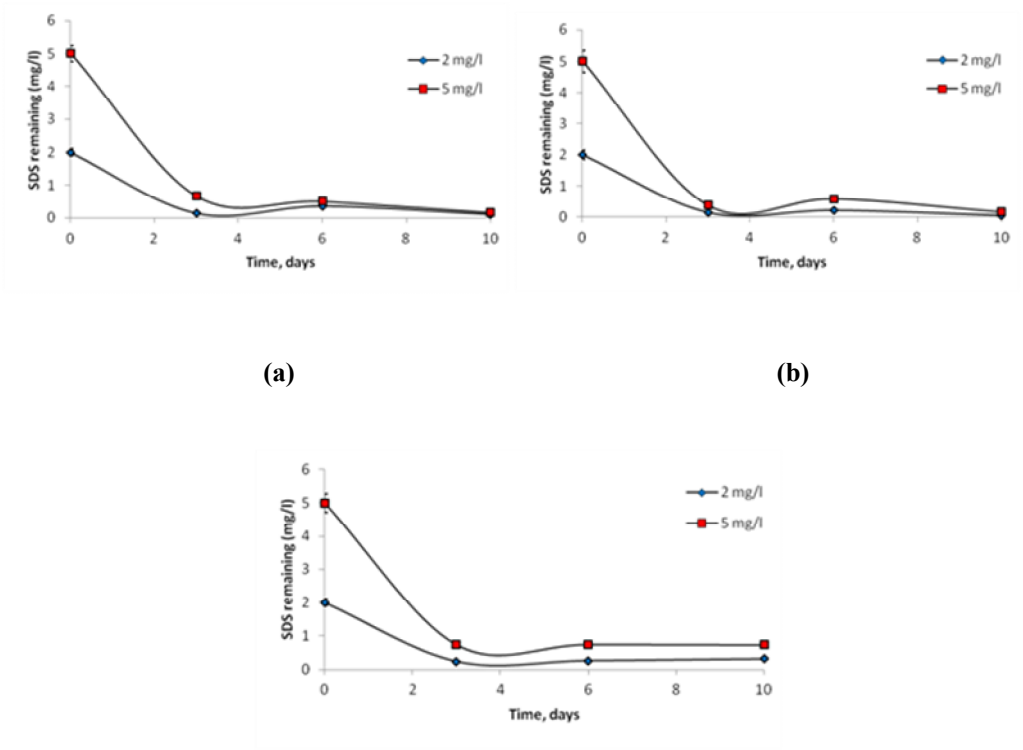
P detergent was chosen as the optimal detergent for its high biodegradability, low foaming, low solvent of machine oil, and higher production quality properties. Since the main surfactant in the P detergent sample is SLES, we have attempted to isolate specific bacterial strains capable of efficiently degrading SLES.

3.2. Identification of bacterial isolates that degrade surfactants

3.2.1. Initial characterization of surfactant degrading bacteria

Initially, low concentrations of SDS containing bacterial media were used, and then SDS concentrations were increased gradually until negative effects could be seen. Among different bacterial samples, *Arcobacter butzleri* was chosen as one of the most important ones, since the success of *A. butzleri* was high, and this species is novel for surfactant bioremediation. Besides *A. butzleri*, some other bacterial species were found as successful however, most of them was already mentioned in the literature such as *Bacillus sp.*, *Proteus sp.*, *Enterobacter sp.*, and *Pseudomonas sp*. ESI, EcoClear ^{TM} wastewater cleaning bacteria mix was found as successful as well, although this bacteria mix is not specific for surfactant biodegradation. It was observed that, surfactant biodegradation is higher and faster initially, later it significantly decreases. Optimum time for nearly complete surfactant bio-removal was observed between 6-10 days, and biostimulation, stimulation of bacterial growth by sugars, mineral salts or nitrogen sources, is related to the removal efficiency. As a result of these studies, *Arcobacter butzleri*, *Bacillus subtilis* ATCC 6633, and EcoClear ^{TM} wastewater cleaning bacteria mix were selected as successful. In those studies, it was observed that, as expected bacterial samples are more resistant to toxicological effects of SDS than plant samples. Even if bacteria will start to die at very high concentrations of SDS, it started to adapt to this environment and maintain their life stable for a while until nutritional values in the medium becomes too low. For preliminary bacterial biodegradation studies,

upper limit was 5 mg/l however, bacterial samples grown well at higher concentrations of SDS as well (Fig 41a, Fig.41b, Fig. 41c).



(a) **(b)**

(c)

Figure 41: Biodegradation of SDS by *Arcobacter butzleri* (a) *Bacillus subtilis* (b) and ESI, EcoClear ™ wastewater cleaning bacteria mix (c) in 10 days at different concentrations of SDS. (Error bars represent means ± SEM, n=2).

3.2.2. Biodegradation of SDS by *Arcobacter butzleri*

3.2.2.1. Biodegradation Capability of *A. butzleri* at different SDS concentrations

The genus *Arcobacter* is a potential candidate for use in bioremediation efforts as the bioremediation capability of *Arcobacter* is similar to those of *Pseudomonas* and *Klebsiella* which are widely used in bioremediation studies [77]. Otth et al. [78] have reported *Arcobacter* strains displaying resistance to a number of heavy metals and therefore are promising candidates that may be used for bioremediation, alone or in a consortium with other bacteria. We have tested surfactant biodegradation capability of an *Arcobacter butzleri* strain at varying SDS concentrations. The growths of each sample were very similar at experimented concentrations in 10 days (Fig. 42A). The *A. butzleri* isolate showed efficient biodegradation for each tested concentration of SDS (Fig. 42B). Biodegradation of SDS varied between 80% (10 mg/l sample) and 99% (100 mg/l sample).

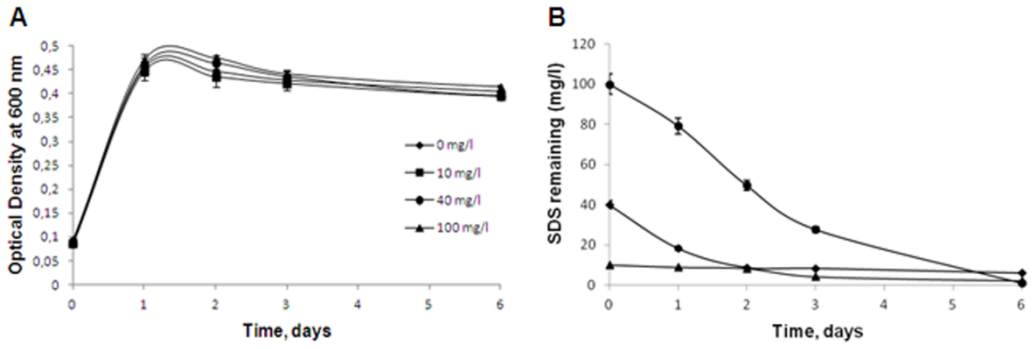


Figure 42: Growth curve (A) and biodegradation of SDS (B) by *Arcobacter butzleri* in 6 days at different concentrations of SDS (10, 40, and 100 mg/l). Error bars represent means ± S.E.M, n=3.

This result indicates that at certain concentration ranges, SDS does not reduce the biodegradation capacity; on the contrary, it may support this process because of the enhanced metabolic activity. The growth curves of bacteria for each experimental concentration are very similar, which suggests the presence of SDS in the growth medium at the concentrations between 10-100 mg/l does not have a significant effect on bacterial growth and the increase in degradation rate may be caused by changes in the expression of detergent metabolizing genes instead.

3.2.2.2. FT-IR spectroscopy analysis results

In this study, most of the specific regions and chemical groups of FT-IR spectroscopy are determined based on Movasaghi and colleagues' report for biological tissues [79]. FT-IR spectroscopy analysis of bacteria grown at experimental concentrations displayed significant peak differences in spectra compared to the 0 mg/L control sample (Fig. 43A, Fig. 43C and Fig. 43D). We observed that amide I (1544 cm^{-1}) and amide II (1655 cm^{-1}) peaks greatly decreased in intensity for the 3 g/l sample after 72 h. A similar result was also observed for the 100 mg/l sample after 3 days but not for day 0 and day 1. However, for the 0 mg/l, and 40 mg/l samples, there is no such peak difference among the spectra with respect to different days. While distinct peaks are expected to be observed for S-O stretching vibrations of SDS for experimental samples in the region of 1250-1200 cm^{-1} [80], no such peaks were observed for those samples.

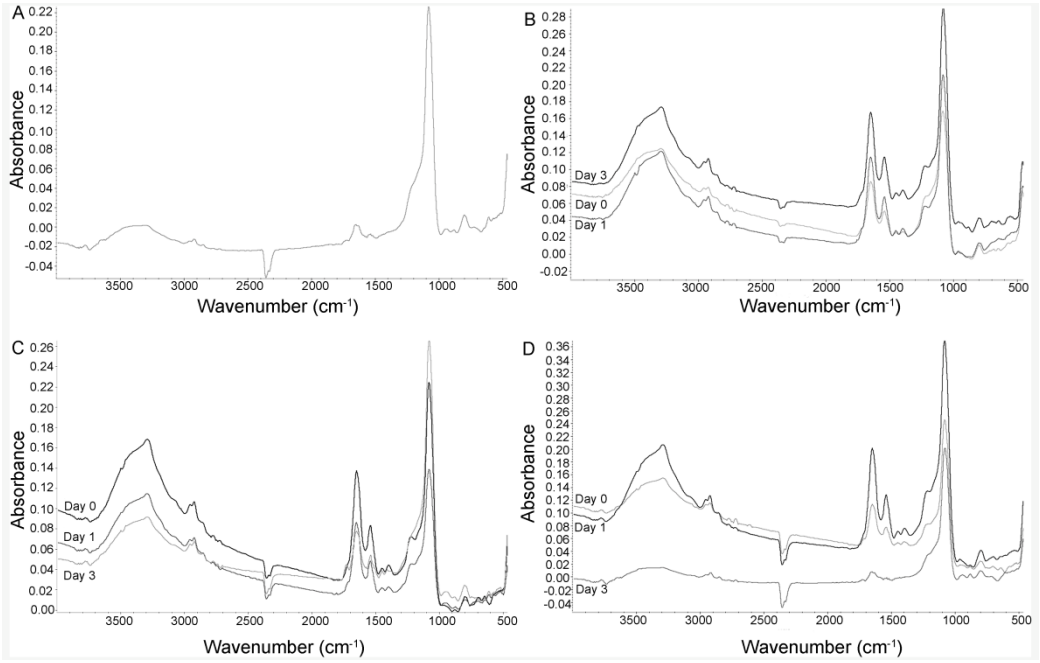


Figure 43: FT-IR spectra of *Arcobacter butzleri* grown at 3 g/l SDS after 72 h incubation period (A); 0 mg/l SDS (B), 40 mg/l SDS (C), and 100 mg/l SDS (D) at day 0, 1, and 3.

FT-IR analysis was utilized to screen the effects of SDS biodegradation on bacteria as a novel approach. Photometric tests like MBAS assay or chromatographic analyses like HPLC can be performed for screening surfactant biodegradation however, to screen biochemical interactions and alterations that occur as a result of those interactions, spectroscopic applications may be performed like Circular Dichroism, Raman and FT-IR spectroscopy. Since FT-IR spectroscopy allows a rapid analysis of chemical interactions that take place in bacterial cells, and it is simpler to perform, this technique was utilized for further analyses. We aimed to observe differences in specific chemical bonds and groups by FT-IR spectroscopy as a result of biodegradation process,

especially at higher concentrations of SDS. We have performed FT-IR spectroscopy to also screen specific peaks for the metabolites of SDS (e.g. dodekanal and lauryl acid) and the effects of them together with SDS itself on the biochemistry of a SDS metabolizing bacterial isolate. Although we could not observed specific peaks for neither SDS nor its metabolites due to the overlapping effect, we observed several significant peak differences in spectra of the experimental samples. For instance, at higher SDS concentrations (100 mg/l and 3 g/l), amide I and amide II peaks, which reflect the proteins of the bacterial cell, are greatly reduced in intensity (Fig. 43A, Fig. 43D). This change in the amide regions can be explained by alterations in the secondary structures of proteins [81]. The anionic headgroup of SDS and positively charged proteins interact electrostatically. Moreover, the tail of SDS and proteins that have hydrophobic characteristics may participate in hydrophobic interactions. Therefore, it is likely that SDS and its metabolites interact with proteins via hydrophobic and electrostatic interactions and alter their secondary structures at sufficiently high concentrations, which might lead to the observed changes in the amide I and amide II regions [82]. In a recent study by Rocha et al., a similar behavior was observed for amyloid-β peptide. When surface pressure in the environment decreases to a certain level due to the presence of surfactants, amide I and amide II peaks of amyloid-β were disappeared according to IRRAS (Infrared Reflection Absorption Spectroscopy) results [83]. Finally, the expected peaks of SDS in the region of 1250-1200cm^{-1} (S-O stretching vibrations) were not seen in the experimental samples. This result is probably due to the overlapping effect; CH_2 stretching of the bacterial carbohydrates leads to this

spectral overlap and makes it impossible to detect S-O stretching vibrations, which are highly specific to SDS [79, 80].

3.2.2.3. Effects of high concentrations of SDS on bacterial cell morphology

3 g/l SDS appears to induce stress conditions for *A. butzleri,* since the growth of bacteria was negatively affected (data not shown). This concentration was used to observe the effects of high concentrations of SDS on bacterial cell morphology. SEM images of this isolate revealed that, in contrast with the smoother cell walls of unstressed control samples, there are small burrs on bacterial cell wall in the 3 g/l sample (Fig. 44A and Fig. 44B). It is likely that the presence of high concentration of SDS in the medium may be the reason of this difference.

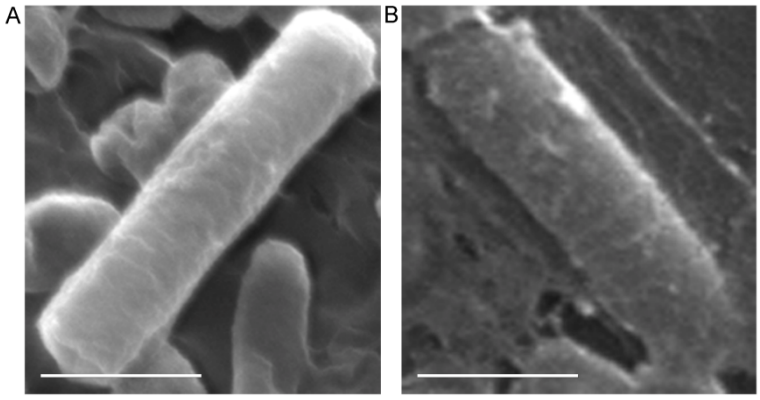


Figure 44: Scanning electron microscope (SEM) images of single *Arcobacter butzleri* cells. (A) corresponds to non stressed bacterium, and (B) corresponds to SDS stressed bacterium which was grown in 3 g/l SDS containing medium. Bars stand for 1µm.

3.2.2.4. The potential use of *A. butzleri* isolate for bioremoval of anionic surfactants

Arcobacter is widely found in aquatic environments such as river water, drinking water reservoir and canal water [84] and it would be used in sewage and wastewater treatment plants where living organisms are not found or found in a very low number, therefore, it is concluded that *A. butzleri* isolate can be applied for bioremediation of anionic surfactants either alone or in a bacterial consortium similar to the one presented by Abboud and colleagues [25].

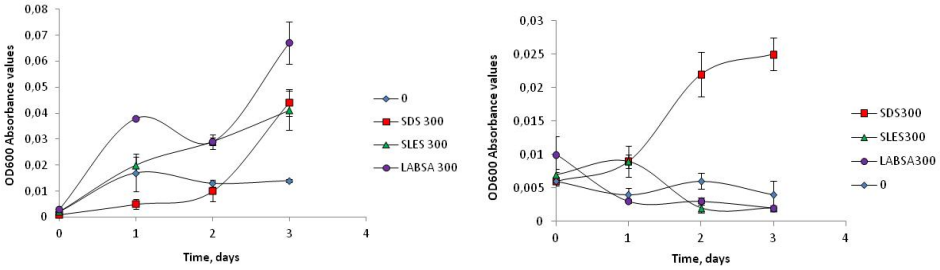
3.2.3. Isolation of bacteria from the factory area and construction of the AS bacterial consortium (STB3-STB4)

We also isolated bacteria from factory region and tried their surfactant resistance and biodegradation potential under different conditions. Those isolates were inoculated to media containing detergent surfactants, and their capability to utilize surfactants as a carbon and energy source was tested. The promising isolates according to the preliminary studies were tried further at higher surfactant concentrations. As mentioned above, it was observed that bacterial bioremediation of anionic surfactants was better than phytoremediation for factory isolates, since bacteria are more resistant to environmental changes than plants, and complete biodegradation of anionic surfactants by bacterial isolates is sooner. Even if duckweeds and bamboo were successful for bioremediation of SDS at certain concentrations, the sustainability and price of these plants limit their use in large scale environments. Moreover, such plants are more vulnerable to lower temperatures and higher surfactant concentrations,

and they are slower to remediate surfactants than bacteria. Therefore, we decided to focus on bacterial treatment, and condensed our studies for isolation and construction of an efficient bacterial consortium. Previously, a commercial bacterial consortium was tried for bioremediation of detergent surfactants in the factory but it did not work due to lack of optimization. However, in our project, since more biodegradable detergent surfactants that can be easily biodegraded by bacteria are offered and used, and a specific bacterial consortium for degradation of the offered detergent was developed, the prospects are expected to be higher than the previous case. Various bacterial isolates were tried for biodegradation of SDS, LABSA and SLES surfactants, either alone or in consortia. After deciding to use SLES as the surfactant of choice in our offered detergent (due to lower price, availability, and high biodegradability), we condensed our studies to generate a successful bacterial consortium to biodegrade high concentrations of SLES.

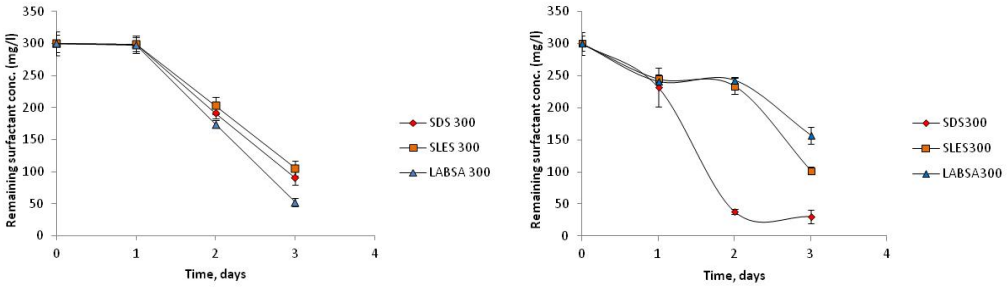
STB3 and STB4 isolates were tested for their surfactant biodegradation capacity after optimization of physical conditions. Growth curves of bacteria are very different at different environments which reveal their response to different surfactant types (Fig 45a, Fig. 45b). While STB3 displayed optimal growth in 300 mg/l LABSA containing medium, STB4 best grew in 300 mg/l SDS containing medium. Furthermore, STB3 showed maximum degradation capacity at 300 mg/l LABSA sample in 72 h, and STB4 showed maximum degradation capacity at 300 mg/l SDS sample in 72 h, (Fig 46a, Fig. 46b). However, both isolates exhibited similar degradation for SLES in 3 days. Based on these results, we constructed a potential bacterial consortium with STB3 and STB4

and tested their biodegradation efficiency for three different surfactants at concentrations of 300 mg/l. The growth curves of this consortium inoculated with three different surfactants was given in Fig. 47a. We observed that, this consortium was successful for degrading high concentrations of SDS, SLES and LABSA in 6-9 days; although biodegradation of LABSA could not pass 55% degradation threshold (Fig. 47b). Specifically, this consortium displayed >90% degradation for 300 mg/l SLES within 9 days, which is a highly promising result for remediating factory wastewater which contains SLES based detergent wastes. This study was repeated several times and in each study, at least three different replicates were utilized. After deciding to use this consortium in the factory for biological treatment, we bring 2 liter of bacterial consortium to the factory and activated the biological treatment plant after reaching a total volume of 100 liter bacteria in a week by inoculating our 2 liter bacterial consortium to a continuously aerated 100 liter growing tank which contains bacteria's essential and supplementary foods. Each week, half of the bacterial consortium was added to the factory's biological treatment pool and the remaining volume of the growing tank was completed to 100 liter by pure water and bacterial nutrients to provide further growth of bacteria. Bacterial growth was followed regularly by OD_{600} measurements; which was performed in the factory's chemical analysis laboratory.



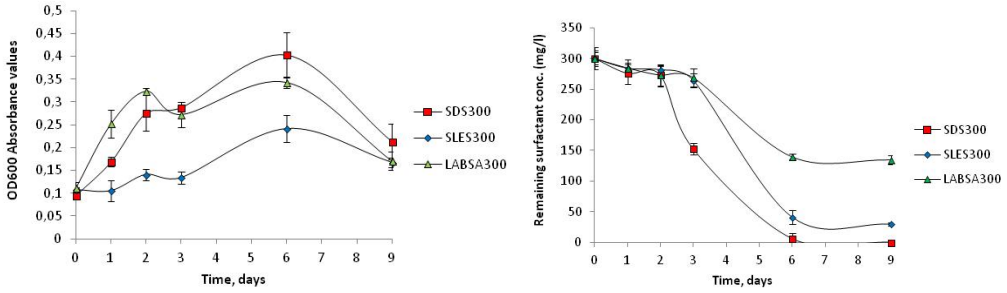
(a) **(b)**

Figure 45: Growth curves of STB3 (a) and STB4 (b) inoculated with three different surfactants at 300 mg/l in 3 days (Error bars represent means ± SEM, n=3).



(a) **(b)**

Figure 46: Biodegradation of three different surfactants by STB3 (a) and STB4 (b) in 3 days (Error bars represent means ± SEM, n=3).



(a) **(b)**

Figure 47: Growth curves of the AS bacterial consortium inoculated with three different surfactants of the concentration of 300 mg/l in 9 days (Error bars represent means ± SEM, n=3) (a) and biodegradation of three different surfactants by AS bacterial consortium in 9 days (Error bars represent means ± SEM, n=3) (b).

3.3. 16S rRNA phylogenetic analysis of STB3 & STB4

The bacterial isolates collected from the factory region were tested for their surfactant resistance at extremely high concentrations of LABSA (2 g/l) and incubated for 48 h. In 48 h, River 2 and Detergent-Water mix isolates exhibited better growth in comparison to other isolates, and designated STB3 and STB4 respectively. STB3 and STB4 isolates 16S rRNA gene sequencing analysis. According to 16S rRNA gene sequencing analysis, STB3 was found as 97% similar to *Achromobacter xylosoxidans* and STB4 was found as 92% similar to *Serratia proteamaculans*. The phylogenetic tree of STB3 and STB4 is shown in Fig. 48. Since the initials of the species name of STB3 and STB4 are

A and S respectively, the consortium of STB3-STB4 was named as the AS bacterial consortium.

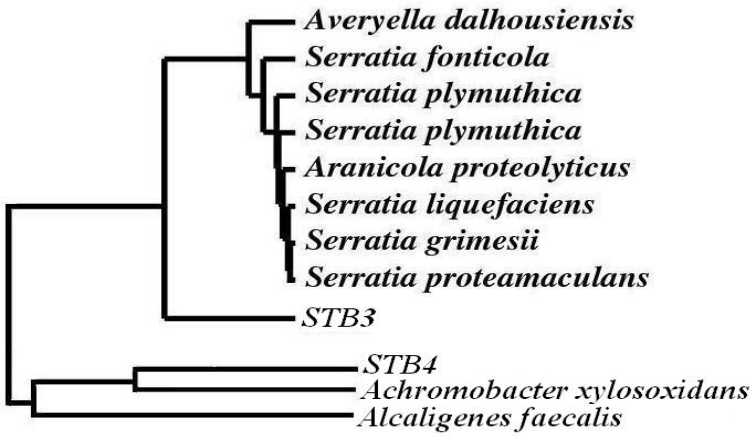


Figure 48: Phylogenetic tree of the STB3 and STB4 strains according to 16S rRNA gene sequencing analysis.

3.4. Assessment of SDS removal capability by different plants

For bamboo and duckweed, several experiments have been made and the best results are mentioned here. Both of them exhibited promising results at lower concentrations of SDS however, they started to die at higher concentrations and henceforth experiments were not proceeded. The experiments with duckweed were terminated earlier since this plant seemed more vulnerable to toxicological effects of SDS than bamboo. At the end of these studies, duckweed samples exhibited promising results at 1-10 mg/l initial SDS concentrations in 10 days (Fig. 49), and bamboo samples exhibited promising results at 2-100 mg/l initial SDS concentrations in 18 days (Fig. 50). However, we suspect that these bamboo plants do not metabolize detergent, they

just transfer them to their vacuoles and store them. After some time, detergent may be released back to the media.

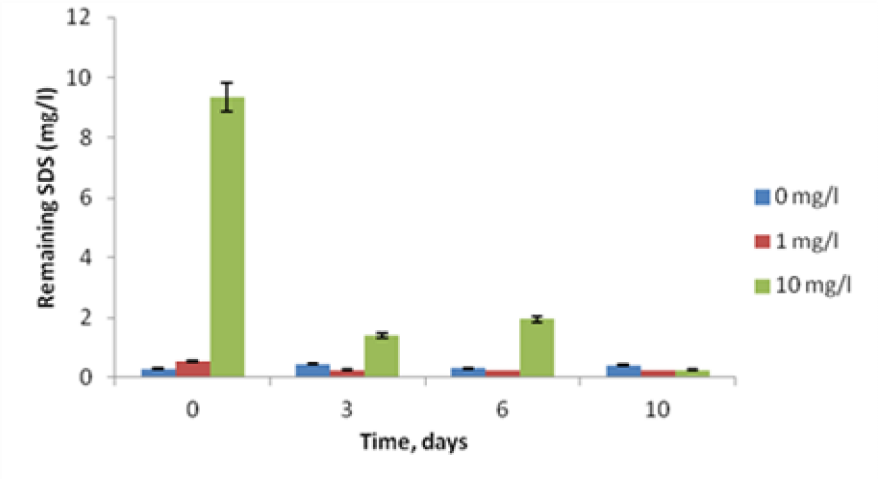


Figure 49: Removal of SDS by duckweed samples in 10 days at concentrations of; 0 mg/l (control), 1 mg/l, and 10 mg/l SDS. (Error bars represent means ± SEM, n=2).

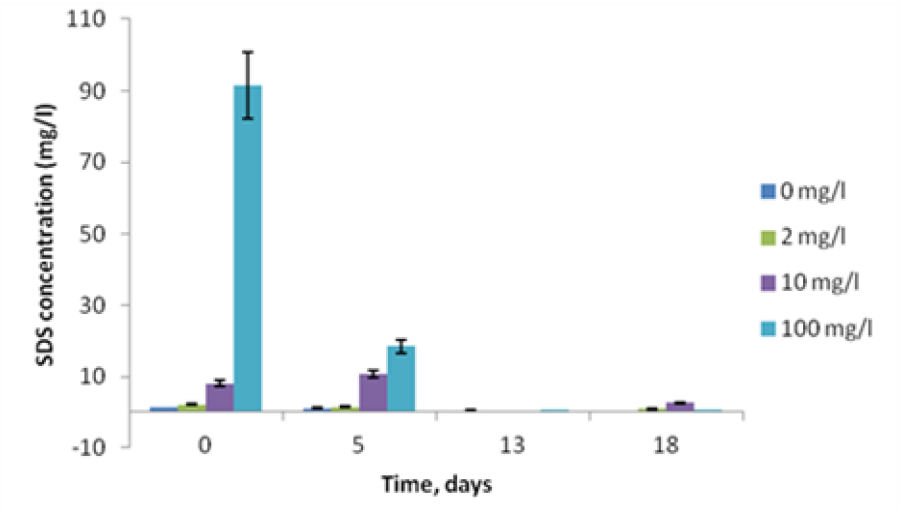


Figure 50: Removal of SDS by bamboo samples in 18 days at concentrations of; 0 mg/l (control), 2 mg/l, 10 mg/l, and 100 mg/l SDS. (Error bars represent means ± SEM, n=2).

CHAPTER II: BIOREMOVAL OF AMMONIUM

INTRODUCTION

1.1. Hazardous nitrogenic compounds and their effects on the environment

One of the main problems in water quality management is the inadequate remediation of high ammonium and nitrogen concentrations of the aquatic systems, which is primarily caused by industrial, agricultural, urban or domestic wastewater efflux into natural freshwater or marine environments [85]. Ammonia is one of the most toxic nitrogenous compounds to aquatic life and exists in water in two forms: as non-dissociated ammonia (NH_3) or mostly as ammonium ion (NH_4^+); the latter of which found to be severely toxic to aquatic organisms [86, 87]. The acute toxicity of ammonia is affected by several environmental factors such as water temperature, pH and salinity, as well as the amount of dissolved oxygen in water. Ammonia concentrations between 2–10 mg/l is usually lethal to most aquatic life, and the acceptable level for ammonia in drinking water is designated as 1.5 mg/l by the U. S. Environmental Protection Agency (US EPA) [86, 88].

Other forms of nitrogen, such as nitrite and nitrate, can also be highly toxic to aquatic life, with nitrite in particular being highly toxic to most aquatic organisms. However, nitrite is not stable in aquatic environments and is rapidly oxidized to nitrate, resulting in low nitrite concentrations in water [89]. Nonetheless, both compounds have low acceptable concentration thresholds: according to the US EPA the sum of the nitrite and nitrate concentration in

drinking water should be lower than 10 mg/l, and the separate limits of nitrite and nitrate are designated as 1 mg/l and 10 mg/l, respectively [90].

1.2. Biological removal of ammonium

Efficient removal of ammonia and other nitrogenous compounds from fresh and saltwater environments is highly desirable [85, 91]. Many methods are currently utilized to reduce the amount of ammonia present in water, and biological nitrogen removal is a common approach for ammonium remediation of both natural aquatic systems and industrial wastewater as it is significantly cheaper, more effective and devoid of undesirable side-products compared to the alternative physical and chemical remediation processes such as ion exchange and adsorption [91, 92, 93, 94, 95].

The biological remediation process of ammonium primarily involves its conversion into nitrogen, which is carried out mostly by autotrophic nitrifiers and some by heterotrophic denitrifiers [96, 97]. Autotrophic nitrifiers oxidize ammonium and convert it to nitrification products, such as nitrite or nitrate, while heterotrophic denitrifiers utilize those nitrification products and convert them to the primary denitrification product, nitrogen gas (N_2). Some heterotrophic denitrifiers have the ability to utilize ammonium as well, and those can simultaneously carry out both nitrification and denitrification. While heterotrophic microorganisms are tolerant to, or even dependent on, high concentrations of ammonium and organic matter, autotrophic microorganisms are generally incapable of surviving in such environments [98]. Recent studies have highlighted the fact that nitrogenous wastewater sources may be relatively high in

organic carbon and nitrogen. Therefore, ammonium removal by heterotrophic denitrifiers seems to be particularly advantageous, especially since autotrophic nitrifiers are usually sensitive to high amounts of ammonium and carbon and have difficulty growing in these conditions [99]. A wide variety of bacteria have been characterized with regards to their potential use in bioremediation of ammonium or other nitrogenous waste products. Those bacteria mostly act by converting the residual ammonia into nitrogen gas under heterotrophic conditions, and include strains belonging to a wide variety of species, such as *Alcaligenes faecalis*, *Acinetobacter calcoaceticus*, *Thiosphaera pantotropha, Microvirgula aerodenitrificans*, *Pseudomonas stutzeri*, *Pseudomonas putida, Bacillus sp., Comamonas sp.* and *Diaphorobacter sp.* [85, 98, 99]. Furthermore, some bacterial species have oligotrophic characteristics which can survive in low nutrient conditions with minimal growth and low rates of metabolism, though some of those bacteria also tend to be heterotrophic under nutrient-rich conditions [100].

1.3. Artemia as a model organism in toxicological studies

Artemia (pelagic crustaceans commonly known as brine shrimp) is widely used as live feed in aquatic ecosystems, and the nauplius stage in particular is commonly utilized as live feed in marine hatcheries [101]. *Artemia* can survive exposure to wide ranges of salinity, temperature and adverse environmental conditions; which combined with a short life cycle and the relative ease of rearing, make *Artemia* a very suitable model organism in ecotoxicity tests [102].

MATERIALS AND METHODS

2.1. Materials

All reagents were purchased from Sigma-Aldrich (USA). The test kits for ammonium, nitrite and nitrate were purchased from Merck Chemicals, Darmstadt, Germany (Merck Ammonium Cell Test 14559, Merck Nitrate Cell Test 14563 and Merck Nitrite Cell Test 14547; Merck, USA). *Artemia salina* cysts were purchased from a Taiwanese manufacturer (Artemia International Co., Ltd.).

2.2. Isolation and collection of bacterial isolates and the growth conditions

For isolating a possible nitrifier/denitrifier bacterial strain, brackish water samples were collected from a commercial sea bass farm in Mugla, Turkey and enriched by a medium containing 1.3 g of $(NH_4)_2SO_4$, 0.5 g of K_2PO_4, 3.8 mg of FeNaEDTA, 0.1 mg of $NaMoO_4.2H_2O$, 0.1 mg of $ZnSO_4.7H_2O$, 0.2 mg of $MnCl_2$, 0.05 g of $MgSO_4$, 4 mg of$CaCl_2$, 0.002 mg of $CoCl_2.6H_2O$ and 0.02 mg of $CuSO_4.5H_2O$ in 1 liter of distilled water [103]. The pH of the medium was adjusted to 7.8–8 via K_2CO_3 and supplied with either no additional salts (low salt medium, LS), 300 mM NaCl (high salt medium, HS) or 600 mM NaCl (sea salts medium, SS). Brackish water samples were inoculated in all three media (LS, HS, SS) and incubated at 125 rpm, 30°C for 33 days. 50% of media for each culture was replaced every five days and pH was maintained in the 7.8-8 range via K_2CO_3. Samples were streaked on LS, HS and SS agar plates every 5 days and incubated at 30°C for 5 days. All tests were done in triplicate. The term "survival" was used to indicate minimal growth of different isolates. While

lower survival rates were observed in the LS and HS media, a higher survival rate was observed in the SS medium sample at the end of this time period, and the potential ammonium-oxidizing isolate obtained from this plate was designated STB1.

2.3. 16S rRNA gene sequencing analysis

The species identity of STB1 was determined via 16S rRNA gene sequencing. DNA isolation was carried out via DNeasy Blood & Tissue Kit (QIAGEN, Germany). For the PCR amplification and further sequencing, a modified protocol in which 1.25 U Platinum Taq polymerase, 0.2 mM dNTP, 0.4 pmol T3 (ATTAACCCTCACTAAAGGGA) and T7 (TAATACGACTCACTATAGGG) primers encompassing the entire 16S gene, 1.5 mM MgCl and 1x Taq buffer were used in 50 µl volumes [104]. The PCR steps were carried out as follows; initial denaturation step of 96 °C for 5 min and 30 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s and a final elongation step for 72°C for 5 min. Sequencing was done via 3130xl Genetic Analyzer, with the help of BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). For analysis, ABI 3130 XL Genetic Analyzer was utilized. The 16S rRNA sequence of the isolate was analyzed by NCBI's Bacterial Blast and TreeDyn software was utilized to construct and visualize a phylogenetic tree.

2.4. Ammonium bioremoval experiments

A modified basal salts medium was utilized to grow STB1 in shaking cultures. The ingredients of the basal salts medium are as follows: 6.3 g/l Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/l NaCl, 2 g/l glucose (anhydrous), and 30% of trace elements solution that consists of: 6.1 g/l $MgSO_4$, 0.5 g/l $MnCl_2$, 3 g/l H_3BO_3, 0.5 g/l $FeSO_4.7H_2O$, 0.5 g/l $CaCl_2$, 0.5 g/l $CuCl_2$, 0.5 g/l $ZnCl_2$. Varying amounts of ammonium (in the form of NH_4Cl) were added to the basal medium for different samples. No additional carbon and nitrogen sources were utilized. STB1 cell cultures were inoculated in this medium and incubated for 72 h at 125 rpm and 30°C. Ammonium removal efficiencies at different concentrations were determined by adjusting initial ammonium concentration to 50, 100 and 210 mg/l, representing low, medium and high concentrations respectively. Periodically, samples were collected to analyze changes in growth, ammonium, nitrite and nitrate. Bacterial growth rates were determined by OD_{600} measurements and ammonium, nitrite and nitrate concentrations in the samples were determined by the spectrophotometric test kits of Merck (Merck Ammonium Cell Test 14559, Merck Nitrate Cell Test 14563 and Merck Nitrite Cell Test 14547). Before the tests, samples were centrifuged for 5 min at 4000 rpm and 4°C, and supernatants were extracted and transferred to test tubes. Pellets were discarded and transferred to 1.5 ml centrifugation tubes. Pellet samples were stored at 4°C for further use. Supernatants were used in analytical measurements of ammonium, nitrite and nitrate. All tests were done in triplicate. Experiments were repeated at least three times.

2.5. Determination of total nitrogen (TN) in the cell biomass

Pellets of bacteria grown in 100 mg/l ammonium containing medium were utilized for detection of total nitrogen amount introduced into the cell biomass and to the supernatant fraction from bacterial cells during the ammonium removal process. Samples were analyzed using an elemental analyzer for their TN content (Flash 2000 Organic Elemental Analyzer, Thermo Scientific USA) after dehydration and granulation of the cell pellets [105]. The conversion of total nitrogen amount in the unit of mg/l was based on the results of the total dry weight of bacteria in 1 liter of growing medium. All tests were done in triplicate.

2.6. Scanning Electron Microscopy (SEM)

STB1 was inoculated in 5 ml SS medium containing 1 cm^2 bioball pieces and incubated for 7 days at 125 rpm and 30°C. The bacterial plaques were then fixed as described by Greif and colleagues [76]. Initially, the bacterial plaques from the overnight cultures were washed twice with PBS buffer. Fixation of the cells was provided by incubating in 2.5% glutaraldehyde in PBS for overnight at room temperature. Then the plaques were rewashed twice by PBS. Dehydration of the samples was done by slowly exchanging them in a 30% to 95% series of ethanol. The plaques were coated with 10 nm gold-palladium before imaging the samples. A Quanta 200 FEG scanning electron microscope (FEI Instruments, USA) was used for taking the SEM images.

2.7. Toxicity studies on *Artemia salina* nauplii

Artemia salina cysts were added to 1.5 liter of autoclaved sea salt medium (35 g/l NaCl) to facilitate hatching. After hatching of *Artemia salina* cysts and initial development of the nauplius larvae for 2 days, equal amounts of *Artemia* nauplii were transferred to two different environments each with a total volume of 200 ml, one containing *Acinetobacter calcoaceticus* strain of STB1 (2.8×10^5 cfu/ml), and one containing no added bacteria. The aeration was provided by aquarium compressors. Survival of *Artemia* nauplii larvae was monitored every day. Samples were taken at days 0, 3, 5 and 7 following hatching for survival percentage and individual length measurements of the nauplii. All tests were done in five replicates. Experiments were repeated at least three times.

2.8. Statistical analysis

Student's t-test was applied for statistical analyses. Analyses were done by using the software Minitab Version 13.2 (Minitab Inc., USA) at a 0.05 level of probability.

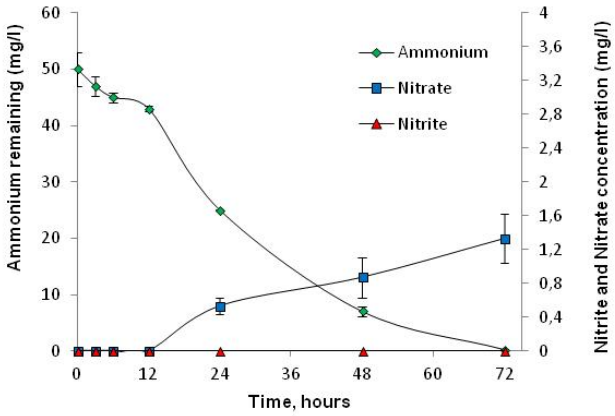
RESULTS AND DISCUSSION

3.1. Bacterial bioremoval of ammonium

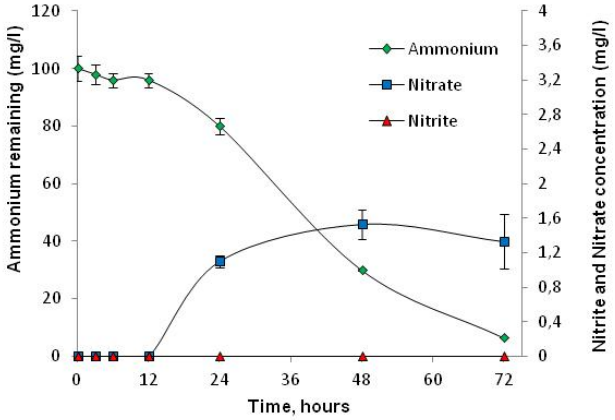
Initially, STB1 grown at lower concentrations of ammonium (≤ 10 mg l^{-1}, data not shown) in both heterotrophic and autotrophic conditions to determine which condition is better for ammonium removal by this isolate. While STB1 isolate displayed promising results for heterotrophic ammonium removal (complete removal of ammonium occurred in 72 h), minimal change was observed for ammonium concentrations in autotrophic conditions. This isolate was therefore chosen for heterotrophic ammonium removal experiments at higher concentrations of ammonium and we sought to identify its phylogeny.

STB1 isolate exhibited efficient heterotrophic ammonium removal at each concentration tested, and it was observed that the ammonium concentration is inversely proportional to the ammonium removal capability in 72 h (Fig. 51). The isolate displayed 100% ammonium removal for the 50 mg/l sample, 93% ammonium removal for 100 mg/l sample and 45% ammonium removal for 210 mg/l sample. Growth curves of each sample were very similar (Fig. 52). Nitrite concentrations were below detectable limits and nitrite production by STB1 was therefore assumed to be minimal. Nitrate concentrations were very close in each sample and between 1-2 mg/l at the end of 72 h growth period (Fig. 51).

A



B



C

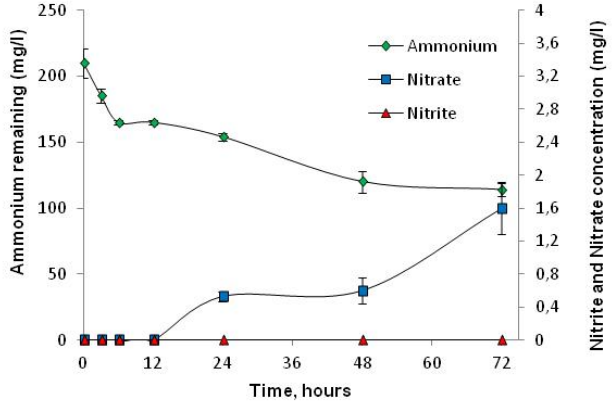


Figure 51: The decrease in ammonium, and increase in nitrate and nitrite concentrations by the STB1 isolate, with the initial ammonium levels of (**A**) 50 mg/l, (**B**) 100 mg/l (**C**) 210 mg/l in 72 h. Error bars represent means ± S.E.M of three replicates.

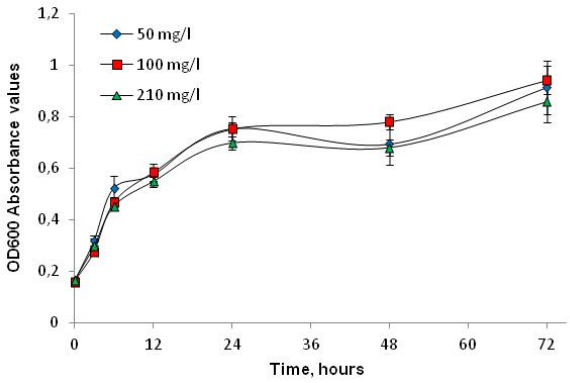


Figure 52: The growth curve of *A. calcoaceticus* STB1 in three different ammonium concentrations; 50 mg/l, 100 mg/l and 210 mg/l in 72 h. Error bars represent means ± S.E.M of three replicates.

3.2. Identification of STB1 by 16S rRNA gene sequencing analysis

16S rRNA gene sequencing analysis was performed to identify the phylogeny of the hatchery isolate, STB1 strain. A sequence of 1085 bp 16S rRNA gene was obtained from PCR and an accession number of JQ653966 was received from GenBank. STB1 was determined to be a member of the species *Acinetobacter calcoaceticus*, with 98% identity according to NCBI's Bacterial Blast algorithm. The phylogenetic tree of STB1 is shown in Fig. 53.

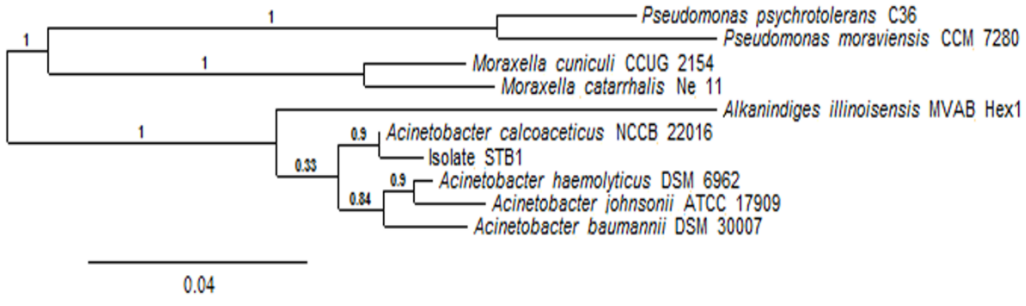


Figure 53: Phylogenetic tree of the STB1 strain according to 16S rRNA gene sequencing analysis.

3.3. TN incorporated into cell biomass

Total nitrogen (TN) analysis was done to the 100 mg/l sample to determine the percentage of ammonia metabolized (incorporated into cell biomass) during heterotrophic ammonium removal. TN analysis revealed that 22.16% of total nitrogen was incorporated into cell biomass, while 4.34% of total nitrogen was initially incorporated into cell biomass and subsequently released to the supernatant fraction during cell growth in 72 h (Table 8). Most of the remaining conversion products were expected to be gaseous denitrification products, a prediction supported by the high denitrification capability of *A. calcoaceticus* in a previous study [97].

Table 8

Conversion of nitrogen by removal of ammonium by *Acinetobacter calcoaceticus* STB1 in 72 h (in terms of mg/l)

	NH_4^+-N	NO_2^--N	NO_3^--N	Intracellular N	TN loss of cell biomass
Initial	99.87 ± 7.62	_	_	8 ± 0.11	_
Final	6.54 ± 0.05	_	1.33 ± 0.55	30.16 ± 1.18	4.34 ± 0.6

_, Below detectable limits.

Means and standard deviations were placed together in the form of mean of three different replicates ± SD.

TN loss of cell biomass = Maximum level of TN in cell biomass – latest level of TN in cell biomass

3.4. SEM results

SEM images of STB1 were taken to examine the biofilm forming capability of STB1 after fixing bacterial cells attached to bioballs at the end of a 7 day growth period. Fig. 58 reveals the distinct round shape of *Acinetobacter calcoaceticus* cells, which are physically close to each other, but there was no sign for biofilm formation or cellular fusion.

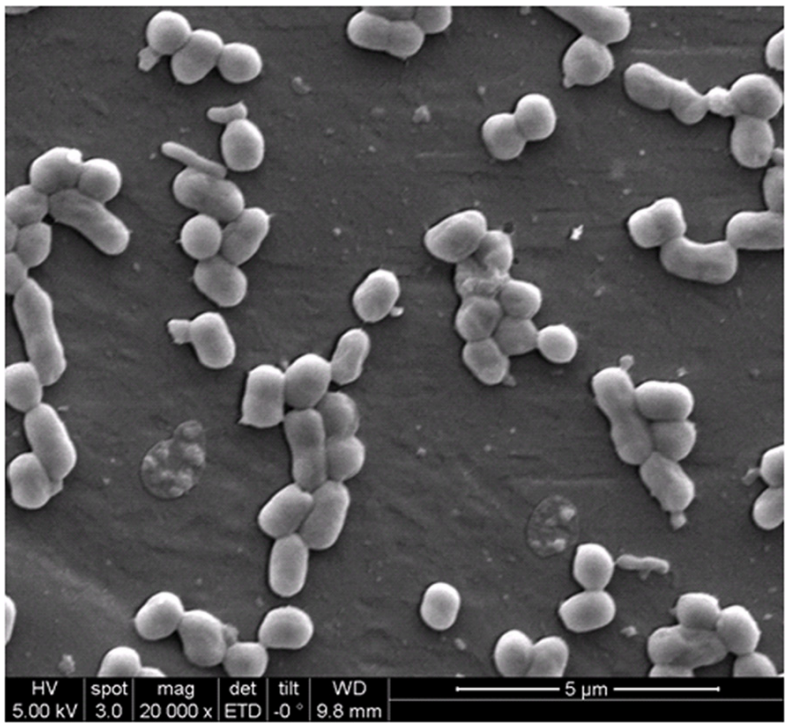


Figure 54: Scanning Electron Microscope (SEM) image of *A. calcoaceticus* STB1.

3.5. Toxicological studies with *Artemia salina* nauplii

Toxicological studies were performed to see whether STB1 strain can be safely utilized in aquatic ecosystems. As STB1 was isolated from the brackish water of a sea bass hatchery, it was assumed to be non-toxic. Toxicological properties of STB1 strain were tested on an aquatic organism, *Artemia salina*. According to the eco-toxicology studies, similar survival rates and total lengths were observed for both control and STB1 added samples during 7 days of growth period, and no significant difference was observed between those samples. Therefore, STB1 strain is found to be non-toxic for the nauplius development in *Artemia salina* (Table 9). The survival of larvae was sharply decreased for both samples after 5 days however; such decrease is normal, as

shown previously [106], which is possibly due to the excess contamination of both media by wastes and remains of *Artemia* larvae. This promising result supports our suggestion of this strain to be utilized safely in aquatic ecosystems as a heterotrophic ammonium remover in addition to the industrial applications. We believe this strain has a high potential to be used in recirculating aquaculture farms and other industrial settings for ammonia removal. Since it is isolated from a hatchery and nitrite or nitrate accumulation is minimal, we believe STB1 could thrive in these conditions and would not have any toxic or growth retarding affects on aquatic organisms.

Table 9

Average survival and individual length of *Artemia salina* nauplii [a]

Time	Treatment	Survival (%)	Individual Length (µm)
Day 0	Control	100 ± 0	428.3 ± 38.68
	STB1	–	–
Day 3	Control	68.7 ± 5.9	937 ± 191.4
	STB1	67.8 ± 5.56	978.3 ± 150.6
Day 5	Control	6.2 ± 1.03	938.3 ± 195.06
	STB1	6 ± 1.32	988.8 ± 159.2
Day 7	Control	4.34 ± 0.76	990.23 ± 139.8
	STB1	4.16 ± 0.49	994.52 ± 172.48

[a] STB1 represents treatment with *Acinetobacter calcoaceticus* STB1 strain and Control represents treatment without any bacterial strain. Means and standard deviations were placed together in the form of mean of five different replicates ± SD. Statistical analyses revealed that there is no statistically significant difference between same day samples for survival rates ($P > 0.05$).

CONCLUSION AND FUTURE PERSPECTIVES

In the first part, we have shown that AS bacterial consortium (A: *Achromobacter xylosoxidans* and S: *Serratia proteamaculans*) is capable of successfully degrading high concentrations (up to 300 mg/l) of anionic surfactants; SDS (complete bioremoval), SLES (~90% bioremoval) and LABSA (~55% bioremoval) in 9 days. The essential point in this project is high biodegradation capability of AS bacterial strain for SLES, since SLES is utilized as the main anionic surfactant in our offered detergent. The factory trials revealed that, our offered detergent has similar success rate of glassware production compared to previous detergent, with low solvent capacity of machine oil and low foaming properties. It is expected that, by applying a newly generated AS bacterial consortium, our offered more biodegradable detergent could be degraded more easily and more rapidly. This project brings to the literature a nice cooperation between university and industry, and a novel efficient bacterial consortium for bioremediation of anionic surfactants.

In the second part, we have described a novel strain of *Acinetobacter calcoaceticus* STB1 that is capable of removing ammonium in heterotrophic conditions. STB1 could effectively remove ammonium at concentrations up to 100 mg/l and was capable of remediating a significant portion of ammonium (~45%) at 210 mg/l concentration in 72 h. Moreover, the conversion of ammonium to nitrite or nitrate is limited and final levels are below the legal limits for drinking water [90]. The TN analysis results revealed that while around 22% of ammonium was introduced to cell biomass and around 4% of

total nitrogen was initially incorporated into cell biomass and subsequently released to the supernatant fraction, the remaining conversion products are unidentified and it is likely that N_2 gas has the highest ratio. Studies on *Artemia salina* nauplii show that, STB1 strain is non-toxic to this organism. It does not significantly affect the survival and growth of *Artemia* nauplii. Based on the findings mentioned above, STB1 is a suitable alternative for current chemical and physical treatment methods. Our findings strongly suggest the application of *Acinetobacter calcoaceticus* STB1 strain for efficient removal of ammonium from aquatic environments such as recirculating aquaculture systems, in addition to industrial or municipal wastewater treatment plants.

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LIST OF PUBLICATION

1. **Sarioglu, O.F., Suluyayla, R., and Tekinay, T.** Heterotrophic ammonium removal by a novel hatchery isolate Acinetobacter calcoaceticus STB1, 2012. International Biodeterioration and Biodegradation **71:**67-71.
2. **Sarioglu, O.F., Tamer, Y.T., Atabay, H.A., Molva, C., and Tekinay, T.** Fourier Transform Infrared Spectroscopy as a novel approach for analyzing the biochemical effects of anionic surfactants on a surfactant degrading *Arcobacter butzleri* strain. (**submitted for publication**).