



Thermophilic Bacteria Investigation in Petroleum Contaminated Soils by Different Isolation Method

Olca GULCICEK^{1,*} , Ali UNYAYAR¹ , Erman OZER¹ , Ozlem SANSARCI¹ 

¹ Department of Environmental Engineering, Faculty of Engineering, Mersin University, Mersin, TURKEY

Article Info

Received: 04/09/2018

Accepted: 19/11/2018

Keywords

Petroleum contaminated soil
Thermophilic bacteria
Isolation

Abstract

There are many and various microorganisms earth flora, ranging from macroscopic to microscopic forms. When soil ecosystem is contaminated by complex compounds and heavy metals, which are difficult to separate from each other, in petroleum and petroleum derived products, for that reason it would be unable to utilization of soil due to decomposition of its physical, chemical and biological characteristics. Biological decomposition of petroleum and petroleum derived products by natural microorganism populations, is the primary mechanism for eliminating the petroleum and other hydrocarbon pollution of environment. The purpose of this study is to research the best isolation method on the isolation of thermophilic bacteria from the soils which are contaminated by petroleum and petroleum derived products. Recent studies related to thermophilic bacteria have been intensified since these bacteria are more resistant to extreme environmental conditions than other bacteria species. With this purpose, 4 different isolation methods have been investigated in isolating the thermophilic bacteria from the petroleum contaminated soil which is contaminated by petroleum and petroleum products. At the end of the research, in order to identify bacteria colonies which are considered to be pure, DNA isolation, Conventional PCR (polymerase chain reaction) Process, Gel Electrophoresis processes are applied. Different incubation times, carbon resources and enrichment solutions are tried out to obtain a superior method and it is revealed that with the increase in the incubation period, more known/unknown species can be isolated without using additional carbon source from the soil.

1. INTRODUCTION

Microorganisms are spread over a wide ecological area. One of these areas is soil which contains the necessary water, oxygen, and basic nutrients for microorganism growth [1, 2].

Soil is a complicated and dynamical biological system. It is estimated that there are 10 billion bacteria and thousands of bacteria species in one gram of soil [3,4]. Physicochemical structure of soil is mostly determined by climatic changes, agronomic practices effect the distribution and diversity of bacteria [3,5,6,7]. This distribution and diversity presents a large resource for biotechnological practices. Bacteria, which are proliferate rapidly than plants and animals, in terms of biochemical effects, constitute a very important group in providing the balance of living beings.

Generally, microorganisms are the most important recycling species of our world. Environments that deteriorated with pollutants can be remediated by microorganism activities. They are able to decompose wide range of waste and even toxic materials into harmless or beneficial components. Organisms around the world are tended to be sensitive towards to the drastic changes around themselves. High temperature and pressure, drought, hyper salinity and harsh pH conditions destroys the cellular integrity rapidly by ruining the structure and functionality of biomolecules [8].

*Corresponding author, e-mail: olcayozer@mersin.edu.tr

Extreme environmental conditions can be considered as natural or simulated forces which make it difficult for most living systems to survive and develop. Various microorganisms are observed in environments which have extreme conditions in the research and it is revealed that, not only the microorganisms tolerate these conditions, but it is also necessary for them to be alive. Over the past decade, scientists have made significant alteration in their research for the limits of habitable environmental conditions [8,9].

Temperature is one of the most important variables around us. In order to classify organisms, we strictly depend upon their relationship with temperature. For that reason, it is regarded as the most fundamental condition for a microbial colony to thrive. Microorganisms are generally divided into 3 groups as psychrophiles, mesophiles and thermophiles for their physical abilities [10,11]. Thermophilic organisms are adapted to survive in high temperatures and divided into two categories which are; thermophiles (45-65 °C) and hyperthermophiles (85 °C) [12,13].

Temperature immensely effects the biodegradation of hydrocarbons by altering their chemical composition and physical structure of petroleum, the rate of metabolism by microorganisms and the structure of microbial communities. The viscosity of oil is increases at low temperatures. Evaporation rates of short chain alkanes are reduced and the water solubility of the hydrocarbons are increased accordingly. Biodegradation rates are usually decreased with respect to temperature decline [14]. Thermophilic bacteria, one of the extreme environment adapted life forms, living under very different conditions from regular living conditions of ourselves, has been one of the focus centers of intense interest in recent years [13,15]. Cellular elements (cell membrane, ribosomes etc.) and components (enzymes, proteins, nucleic acids etc.) of thermophilic organisms are resistant to high temperatures (65-85°C). They are also resistant to denaturant and proteases such as extreme acidic and alkaline conditions [10,16].

Utilization of thermophiles as a biotechnological manner can be divided into two groups; a whole cell catalyst or the macromolecules or metabolites of the cell. Also, thermophiles are known as a potential in producing bioenergy, thermoenzymes and biosurfactants. The use of thermophiles in biotechnological applications has superior to other methods in many ways, such as possessing high metabolic activity which leads to the formation of advanced products, the inactivation of pathogenic/contaminants by means the removal of mesophile organisms from medium, the production of heat-resistant macromolecules and metabolites, the absence of cooling steps after heating steps in biotechnological processes, the increase of the solubility of medium, ionization and diffusion of chemicals, decreasing medium density, increasing in reaction rate with surface tension and viscosity, the expression of thermostable enzymes [17].

Petroleum and derivative products is the most important energy source of the world from the beginning of industrial revolution. It is clear that unless a novel energy source is discovered equivalent to petroleum, this importance seems to be continued until running out of sources [18]. To remove petroleum out of contaminated soils expensive and difficult processes have to be carried out. Petroleum contamination deteriorates the soil structure via scattering soil crumbs, thus spoils the air and water economy of the soil. It is widely known that in disintegrating the components in contaminated areas, microorganisms use their own metabolic enzymes [19]. In a study which the disintegration rate of hydrocarbons in soil by bacteria and fungi are compared, it was observed that 82% of the n-hexadecane use was made by bacteria and only 13% by fungi in a place full of sand [20].

Owing to this study, four different isolation methods are investigated to isolate “soil thermophilic bacteria”, which adapts themselves to extreme conditions by being exposed to petroleum products for many years which have detrimental effects on living beings, can be used in environmental biotechnology practices, from the soil contaminated by petroleum and petroleum products. The effects of employ different enrichment solutions (Ringer solution, mineral salt medium, NPK fertilizer solution) and different incubation times (1 hour, 1 day, 35 days, 180 days) are experimented on the isolation of thermophilic bacteria. During the incubation period at 65 °C, n-hexane/hexadecane were used as sole carbon source in three isolation methods and no additional carbon source was added to growth medium in one of the isolation methods.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Soil Sample

Soil sample has been taken from the ground surface (0-15 cm in depth) which is contaminated with petroleum and mineral oils (Adana, Turkey) where vehicle maintenance and repair have been made for many years. Soil samples that are brought to Mersin University Environmental Engineering Laboratory were sieved by means of a 2-mm sieve and preserved at 4 °C in the dark, after air-dried and ground.

2.1.2 Solutions

Ringer solution; NaCl 2,25 g/L; KCl 0.105 g/L; dehydrated CaCl₂ 0.06 g/L; NaHCO₃ 0.05 g/L is weighed and completed to 1 L with distilled water and the solution sterilized for 15 min. in 121 °C autoclave.

Mineral Salt Medium; K₂HPO₄ (0.9 g/L); KH₂PO₄ (0.5 g/L); NH₄Cl (0.5 g/L); CaCl₂·2H₂O (0.1 g/L); MgCl₂·6H₂O (0.2 g/L); FeCl₂·4H₂O (0.1 g/L); 1mL/L is completed to 1 L with distilled water by adding trace element stock solution and the solution is sterilized for 15 min. in 121 °C autoclave.

Trace element solution; ZnCl₂ (0.5 g/L); MnCl₂·4H₂O (0.3 g/L); H₃BO₃ (3g); CaCl₂·6H₂O (2 g/L); CuCl₂·2H₂O (0.1 g/L); NiSO₄·6H₂O (0.2 g/L); Na₂MoO₄·2H₂O (0.3 g/L) is completed to 1 L with distilled water and the solution is sterilized for 15 min. in 121 °C autoclave.

Nitrogenous solution content; (0.1 g) urea, (0.1 g) KH₂PO₄, (0.1 g) NH₄SO₄ is completed to 100 mL with distilled water and the solution is sterilized for 15 min. in 121 °C autoclave.

NPK fertilizer solution; Including 15% nitrogen (N), 15% phosphorus (P) and 15% potassium (K), 9% sulfur (S) and 1% zinc (Zn) in its compound, 100 g NPK fertilizer is weighed and completed to 1 L with distilled water and the solution is sterilized for 15 min. in 121 °C autoclave.

2.1.3. Used Growth Media

Nutrient Agar (NA); 20 g pulverized nutrient agar (Merck) is weighed and completed to 1 L and the solution is sterilized for 15 min. in 121 °C autoclave (pH: 7.10). Sterilized media than poured to sterile petri dishes in laminar flow cabinet).

N-hexane/hexadecane media (n,h/ h,d); K₂HPO₄ (0.9 g/L); KH₂PO₄ (0.5 g/L); NH₄Cl (0.5 g/L); CaCl₂·2H₂O (0.1 g/L); MgCl₂·6H₂O (0.2 g/L); FeCl₂·4H₂O (0.1 g/L); 20g agar-agar, 1mL/L trace element stock solution is completed to 1 L with distilled water, sterilized 121 °C for 15 minutes without adding carbon source. 2% (v/v) n-hexane (Merck) to the 500 mL media which was taken to sterile laminar flow cabinet, 2% (v/v) hexadecane (Sigma) to the 500 mL media was added and transferred to the petri dish.

NPK agar media; 100g NPK fertilizer, agar-agar (Merck) is weighed and completed to 1 L with distilled water. The prepared solution was sterilized 121 °C for 15 minutes in autoclave. Media that is taken to sterile laminar flow cabinet and poured to the sterile petri dishes.

2.2. Method

Isolation methods used on soil which is given for the study and applied practices to the colonies which are considered to be pure are explained in Figure 1.

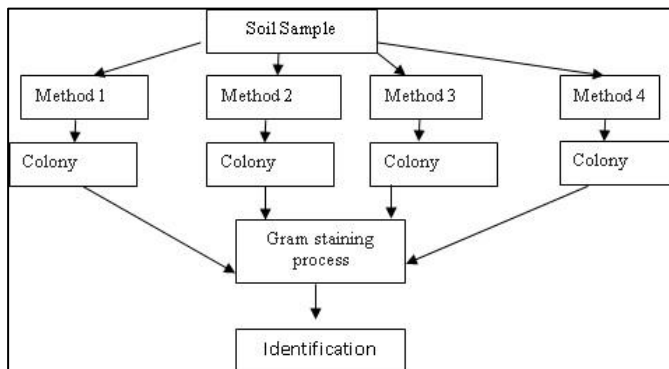


Figure 1. Process summary chart on isolation methods

Method 1

- 1 gram of soil was weighed and transferred to heat resistant 10 mL tubes. 9 mL Ringer solution was added.
- The solutions formed from the soil and the solution were mixed at 2500 rpm for about 1 minute until a homogeneous solution was formed. The tubes were kept at room temperature for 1 hour until clear water was formed.
- 200 μ L surface fluid was added to nutrient agar (NA) medium with sterilized pipette in sterilized environment.
- Bacteria cultures in petri dishes was allowed to grow at 65 °C for 2 days.

Method 2

- 1 gram of soil was weighed and transferred to heat resistant 10 mL tubes. 9 mL mineral salt solution was added. The solution was stirred at 2500 rpm for 1 minute. Experiment was designed to set up as 4 parallel (T1-T2-T3-T4) and 10 tubes were used in each set. Nitrogenous solution was added to the first five tubes (Figure 2).
- The tubes were incubated at 65 °C for 24 hours.
- 200 μ L surface fluid was added to nutrient agar (NA) medium with sterile micropipette in sterile environment and were allowed to incubate at 65 °C for 2 days.
- The colonies observed at the end of the incubation were passaged 4 times to the NA medium for purification of the colonies.
- At the end of passage fourth, different types of colonies were inoculated on n-h / h.d medium and the growing colonies were passaged 4 times for purification.

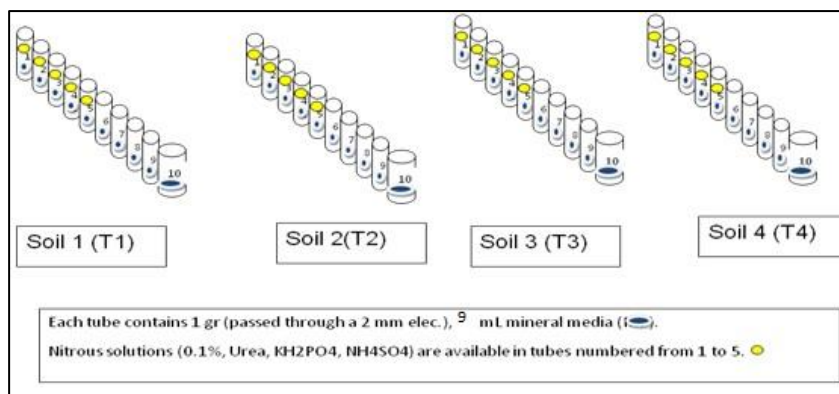


Figure 2. Method 2 Testing set

Method 3

- 100 gr of soil sample, 400 mL NPK fertilizer solution used in agriculture (10%) were mixed in rectangular PVC box and left at 65 °C incubator (Figure 3).
- After it was placed in the incubator with no additional carbon source in the box, the liquid was taken at the end of 2 days, 2 months and 6 months and the NPK agar plantation was made first. In order to purify the colonies, it was passaged into hexadecane (H.d) and n-hexane (n-H) media after passaged 4 times in NPK agar.
- The fourth passaging was made in order to purify the colonies which show reproduction in H.d and n-H media.

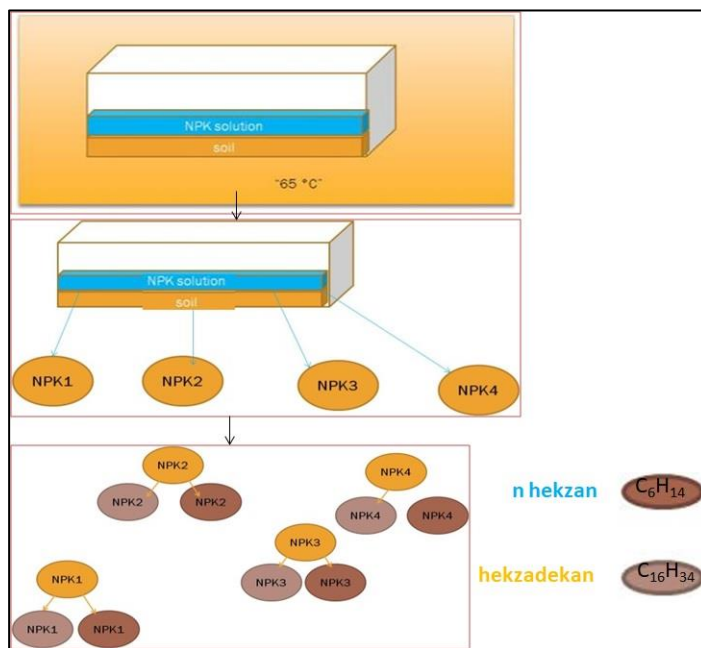


Figure 3. Method 3 Test flow chart

Method 4

- 90 mL of mineral solution, 5 gr of soil and 5 ml of n-hexane/hexadecane was added as a carbon source to the 250 mL erlenmeyer.
- At the end of the processes erlenmeyer were left to incubate for 7 days at 150 rpm at 65 °C temperature.
- At the end of the 7 days, 5% (v/v) of fresh MM (90 mL) which contains carbon source was injected after taken from 10 mL of incubated fluid in sterile laminar flow cabinet.
- The refresher process was repeated 5 times, with a total incubation time of 35 days.
- At the end of the refresher process, parallel to the carbon sources in the liquids, the same carbon source was added to the medium (liquid to solid) and left to incubate for 2 days at 65 °C.

Processes Applied on Colonies

It encapsulates the applied processes after the purification of observed colonies in their media, as a result of all isolation methods that are tried in the study

Gram staining process

All of the methods investigated in thermophilic bacteria isolation from the soil contaminated by petroleum, classification was made with Gram staining process which Hucker modified, as a result of 18-24 hours of incubation in Nutrient Agar media [21].

Bacteria identification study

Towards the end of methods colonies considered as pure was taken to liquid cultures and DNA isolation, Conventional PCR (Polymerase Chain Reaction) Process, Gel Electrophoresis processes were made for bacteria identification in a commercial laboratory (Triogen Biotechnology Lab., Ankara).

DNA isolation

Bacteria Genomic DNA Purification Kit (GMbiolab Co, Taiwan) is used in order to isolate DNA from the bacteria in liquid culture. A portion of the bacteria in the liquid medium was removed and centrifuged at 14000g for 2 minutes, then incubated with lysozyme and the isolation was continued according to the chitin procedure.

Conventional PCR process

PCR process is the proliferation of nucleic acids under appropriate conditions. In vitro (in test-tube) is a method which is defined as the enzymatic synthesis of copies of a specific DNA fragment by directing it by primers. The method is based on the enzymatic synthesis of these two primer-restricted genes using a pair of synthetic oligonucleotide primers complementary to base sequences in this region, specific for the two ends of the region to be proliferated. The polymerase chain reaction is a distinctive and reliable method for proliferation of target nucleic acid chains of disease effects such as bacteria, viruses, fungi, parasites and protozoa in the laboratory using primers (specific complementary oligonucleotides) with heat-resistant enzymes. The genetic material which is studied, can be proliferated even in very few or irrelevant DNA molecules, can be made into a homogeneous DNA material and therefore can be identified easily [22].

PCR process was proceed in Bio Rad T100 Thermal Cycler device. Primers are suitable sequences for bacteria to proliferate their ribosomal RNA parts. It gives a product approximately 1400bp in length. The ingredient of the reaction mixture used in the process is 10X Buffer (3 ul), 2.5 mM dNTP (2 ul), 10 uM Forward Primer (0.6 ul), 10 uM Reverse Primer (0.6 ul), Template DNA (3 ul), GeneTaq DNA Polymerase (0.2 ul), dH₂O (20.6 ul).

Gel Electrophoresis

Gel electrophoresis is a molecular examination method widely used in the determination of molecular weight, quantity and subtypes of purified nucleic acids and proteins. Agarose-gel electrophoresis and polyacrylamide gel electrophoresis are the most used gel electrophoresis methods. After PCR cycles completed, products were made with SYBR Green nucleic acid dye (Invitrogen™) in 2% agarose gel.

Molecular phylogenetic analysis

DNA sequence results which belong to all the samples obtained as a result of bacteria identification process were submitted in NCBI (The National Center for Biotechnology Information) GenBank ® and compared with other gene sequences. BLAST® analysis of obtained DNA sequences results were edited with BioEdit Sequence Alignment Editor accordingly [23].

3. RESULTS

3.1. Method 1 Isolation Results

Colony formation was not observed at the end of the first and at the end of the second day as a result of inoculation on NA media made according to Method 1. Leaving the soil/Ringer solution mixture at room temperature for 1 hour was not enough for the thermophilic bacteria to pass through the liquid.

The soil / Ringer solution mixture prepared by the same method was left for 48 hours at 65 °C, and growth was observed in the petri dishes 24 hours after inoculation on the NA medium.

3.2. Method 2 Isolation Results

Petri dishes that were prepared according to Method 2 were left to incubate for 48 hours at 65 °C. At the end of the 48 hours, almost every petri dish proliferated with similar colony characteristics. Selected colonies were passaged 4 times to NA medium to purify the colonies. At the end of the fourth passaging, selected colonies were passaged to medium which contain n-hexane and hexadecane as a carbon source.

Although in many biological processes, the C/N ratio is a critical factor, in previous studies have reported that nitrogen does not seem to play significant role in biodegradation of petroleum crude components [24, 25]. No significant difference in colony formation and growth rate was observed in sowing tubes made from nitrogen added solution in this study. In other words, in the second method was reported that 0,1 % nitrogen supplementation did not play an important role in microorganism growth.

According to Method 2 two different colony types were observed and these are; round, flat-edged, raised, moist and homogeneous colonies, wet, sticky, coarse and camber mucoid colonies (Figure 4).

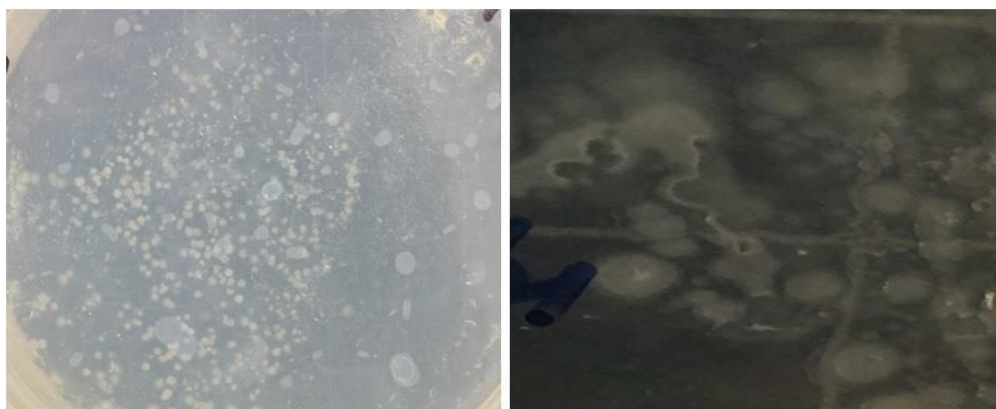


Figure 4. Colonies observed as a result of Method 2

3.3. Method 3 Isolation Results

According to method 3, the liquid taken from NPK solution / soil mixture that does not contain carbon source, was similar to colony types observed in Method 2 as a result of planting to NPK agar at the end of the second day.

Leahy and Colwell (1990), have reported petroleum hydrocarbons do not contain significant concentrations of other nutrients substances necessary for vital activities [26]. These lack of nutrients can be adjusted by adding urea, phosphate, N-P-K fertilizers, ammonium and phosphate salts in the growth media [27,28]. In this study, the addition of NPK fertilizer has been shown to positively affect the growth of microorganisms.

At the end of second month, as result of the planting to NPK agar, they were determined as smooth-edged, sticky, and small in size, and common, whitish, sticky colonies (Figure 5).



Figure 5. The colony observed as a result of Method 3

At the end of the 2. month, more common types of colonies were found in the n-h / h.d medium (Figure 6).



Figure 6. Colonies observed in n-h/h.d medium at the end of the second month

At the end of the sixth month, developed colonies as a result of sowing on NPK agar and n-h / h.d agar medium were determined as common, whitish and sticky colonies. (Figure 7).

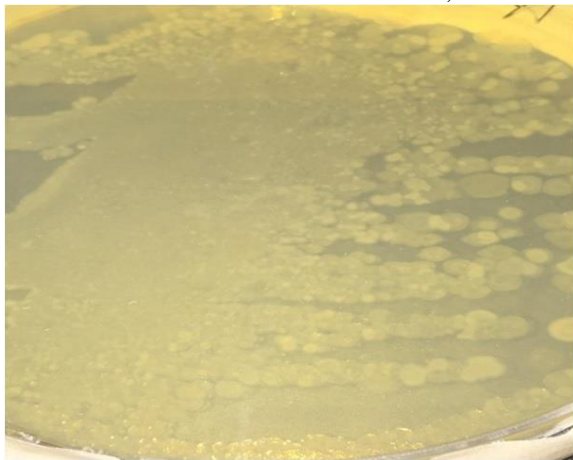


Figure 7. Colonies observed in NPK agar and n-h/h.d agar medium at the end of the sixth month

3.4. Method 4 Isolation Results

Plantation results that were made from the liquids taken at the end of the thirty fifth day which is the last step of method 4 instauration experiments, did not show significant differences according to carbon sources. Colonies formed as a result of hexadecane agar plantation taken from the liquid which has hexadecane as a carbon source, were determined to be whitish, with irregular edges and small in size. Colonies formed as a result of n-hexane agar plantation taken from the liquid which has n-hexane as a carbon source were determined to have irregular edges and small in size.

3.5. Gram Staining and Labeling Results Applied to Colonies

Gram staining results

As a result of Gram staining applied on the observed colonies in every method, it is determined that the colonies were in Gram (+), *Bacillus* form (Figure 8).

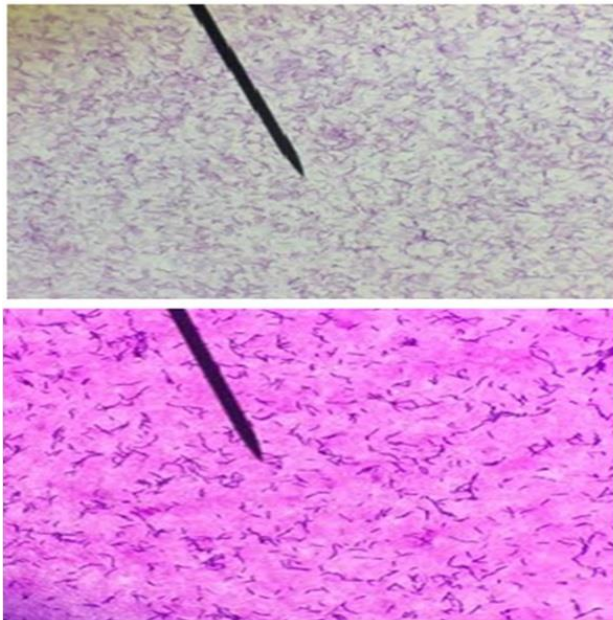


Figure 8. Gram staining results applied on observed colonies

Colony identification results

Colonies obtained as a result of all the methods were identified in a private laboratory after colonies produced in liquid culture. DNA sequence results, were scanned in the most popular and most frequently genetic sequence database NCBI (The National Center for Biotechnology Information) GenBank, Basic Local Alignment Search Tool (BLAST). In the evaluation of the scan results, the results with the lowest E-value, maximum similarity and the highest coverage were given priority.

Pre-exposure of a microbial community to hydrocarbons is important in clarifying how hydrocarbons can be broken down quickly. This incident which provides an increase in the potential of the community to oxidize hydrocarbons is known as adaptation. Being exposed to petroleum and petroleum products for long years, the soil sample we have used in this study is important in the diversity of the species which were isolated.

In the isolation studies which were conducted on the soils that have exposed to hydrocarbon pollution for a long time, new species' existence was determined the environments [24]. The effect of hydrocarbon pollution on the genetic composition of microbial community is thought to be related to environmental or local conditions.

Method 1, since there were no colonies as a result of the plantation that were made at the end of the first day, it is determined that the soil is not appropriate for thermophilic bacteria isolation. 1 hour of experiment time was not enough for method 1 and this was put forth by repeating the experiment as 48 hours and proliferate observation.

Method 2, (Soil + mineral salt solution, 24 hours of incubation) According to the results of identifying of colonies which develop in n-hexane / hexadecane medium, *Geobacillus stearothermophilus* (NR_115284), *Geobacillus thermoleovorans* (NR_036985) species were determined.

Method 3, (NPK solution + soil mixture) as a result of the passage to the NPK medium first and then to the n-hexane / hexadecane-containing medium at the end of second day, second month and sixth months;

Second day; *Geobacillus stearothermophilus* (NR_115284)

Second month; *Parageobacillus thermoglucosidasius* (NR_043022), *Geobacillus stearothermophilus*, *Geobacillus lituanicus* (NR_025657), *Geobacillus thermoleovorans* (NR_036985), *Geobacillus kaustophilus* (NR_115285), *Geobacillus subterraneus* (NR_025109), 3 unknown different species.

Sixth month; 1 unknown species were determined.

Fourth Method, (soil + mineral salt solution, 35 days) at the end of the 35th day, the colonies which were grown at the end of sowing were identified as *Parageobacillus thermoglucosidasius* (NR_043022), *Geobacillus kaustophilus* (NR_115285) and *Geobacillus jurassicus* (NR_116988).

Petroleum and petroleum products can be used as a carbon source via the biodegradation of microorganisms [29,30]. Various studies have been carried out on the effects of microbial surfactants on the biodegradation of these substances [31-33].

Biosurfactants are surfactants produced by microorganisms which have structurally different groups [25],[34-37]. Biosurfactants promote hydrocarbon pollutants breakup, bioavailability thus support hydrocarbon biodegradation. Microbial surfactants were added to the microorganisms in water insoluble substrates and experiments were conducted to investigate the mechanism of action of these substances. By adding microbial surfactants to the environment of hydrophobic substrates and microorganisms, between substrate and microorganisms has been investigated the mechanism of action. According to the results, these substances have been shown to increase the surface area of hydrophobic substrates and to facilitate the attachment of microorganisms to surfaces [38].

Biosurfactane production of the species determined in this study was investigated previously studies [32],[39-48]. *Bacillus* sp. are the best known bacteria capable of utilizing hydrocarbons as carbon and energy sources for producing biosurfactants [31,49].

In the previous studies were reported that nitrogen plays an important role in biosurfactants production [34-36],[38],[45],[50-54].

The third method was isolated more microorganism species than other methods. The NPK solution used in this method is thought to create a competitive environment between the species that are resistant to petroleum pollution for the growth and survival of microorganisms. We were thought that the NPK solution used in the third method increases the production of biosurfactant and that the microorganisms more used the petroleum and petroleum products in the soil.

4. DISCUSSION

Microorganisms are highly sensitive to growth environment and the growth of microorganisms are determined not only by the composition of their surroundings but also by sudden changes in the living environment [27],[32, 33].

Microorganisms play crucial role in maintaining ecosystem. Only adaptable microorganisms can live in polluted areas. Microorganisms isolated from contaminated areas, it is expected to utilizing/degrading these pollutants [48],[55-59]. Because of that Microorganisms can be used to mitigate adverse effects of pollutants [60,61].

It is well known that increasing the incubation time results in increased microorganism quantity [62-66]. When 4 methods that are tried on the isolation of thermophilic bacteria from the soil contaminated by petroleum is examined, it has been shown that contact time is important in the passing of bacteria from soil to liquid environment.

Different species were determined in the results of second method (1 day of incubation) and fourth method (35 days of incubation) where mineral salt solution was used commonly.

In the isolation of thermophilic bacteria adapted to the environment and can disintegrate petroleum and its products, more productive results were obtained by means of species diversity in third and fourth methods which have long incubation durations.

Especially in the study conducted with third method, as the result of plantation of soil-liquid solution held for 6 months without using additional carbon source, 4 unknown species and more thermophilic bacteria species were isolated than all the other methods. "Third Method" was selected as the most appropriate method in the isolation of species that can adapt themselves to the environment without using any additional carbon source, only using the petroleum as a carbon source which is already existing in the soil. Based on the results of this study, the effect of the biosurfactant type and amount on the isolation of thermophilic bacteria in the presence of NPK solution should be investigated.

In the areas contaminated with oil and petroleum products without using an additional carbon source, bioremediation efficiency should be investigated with only NPK fertilization.

Planting the bacteria isolated from the environments that are contaminated by petroleum and petroleum products to the environments contaminated by hydrocarbon, would provide great contributions in reforming the ecosystem's equilibrium.

This study will shed light on the isolation of bacteria from the environment, which will be selected for use in the biotechnological remediation of substances that are toxic in nature and difficult to break down.

There are many studies about biodiversity in our country and in the world. Every new detected organism specie is a potential source of genes for products that can be used for humanitarian purposes. It is thought that our work will contribute to the methodology and approach to determining the biodiversity our country has.

Unknown species that we have discovered in our studies and DNA sequence that codes 16S rRNA these species have will be recorded to the GenBank and therefore our country will contribute to the determination of thermophilic bacteria diversity and biodiversity studies.

Acknowledgement

The authors thank Mersin University, Scientific Research Project Funding (MEU BAP) for their financial support [Project number: 2017-2-AP5-2281].

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

REFERENCES

- [1] Başkaya, Y., Kocabaş, A., “Topraktan izole edilen mikroorganizmaların anti-mikrobiyal madde üretim potansiyellerinin belirlenmesi”, *KSU J. Nat. Sci.*,19(4): 393-398, (2016).
- [2] Singh, J. S. , Pandey, V.C ., Singh D. P., “Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development”, *Agriculture, Ecosystems & Environment*, 140, 3(4): 339-353, (2011).
- [3] Torsvik, V., Ovreas, L., “Microbial diversity and function in soil: from genes to ecosystems”, *Curr Opin Microbiol*, 5: 240-245, (2002).
- [4] Karapire M., Özgönen H., “Doğada Yararlı Mikroorganizmalar Arasındaki Etkileşimler ve Tarımsal Üretimde Önemi”. *Türk Bilimsel Derlemeler Dergisi*, 6 (2): 149-157, (2013).
- [5] Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., “Microbial Diversity and Soil Functions, *Eur J Soil Sci*, 54: 655–670, (2003).
- [6] Roesch, L.F.W., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K., Kent, A.D., Daroub, S.H., Camargo, F.A., Farmerie, W.G., Triplett, E.W., “Pyrosequencing enumerates and contrasts soil microbial diversity”, *The ISME Journal*, 1: 283–290, (2007).
- [7] Zhang Q., Wu J., Yang F., Lei Y., Zhang Q., Cheng X.,” Alterations in soil microbial community composition and biomass following agricultural land use change”. *Scientific Reports*, 6: 36587 , DOI: 10.1038/srep36587, (2016).
- [8] Rampelotto P. H., “Resistance of microorganisms to extreme environmental conditions and its contribution to astrobiology”, *Sustainability*, 2: 1602-1623, (2010).
- [9] Pikuta, E.V., Hoover, R.B., “Microbial extremophiles at the limits of life”, *Crit. Rev. Microbiol.*, 33: 183-209, (2007).
- [10] Kristjansson, M.M., Asgeirsson, B., Properties of extremophilic enzymes and their importance in food science and technology. *handbook of food enzymology* (ed. J.R. Whitaker), NY, USA, p.77-99, (2002).
- [11] Seckbach J.,” *Enigmatic Microorganisms and Life in Extreme Environments*”, Springer Science & Business Media, (2013).
- [12] Demirjian, D.C., Moris-Vara, F., Sassidy, C.S., “Enzymes from extremophiles”, *Curr Opin Chem Biol*, 5: 144–51, (2001).
- [13] Güven R. G., “Termofilik bakteriler ve biyoteknolojik açıdan önemli bazı enzimleri”, *Elektronik Mikrobiyoloji Dergisi TR* , 9 (1): 1-10, (2011).
- [14] Marshall K.C., *Advances in microbial ecology vol 12.*, Springer Science & Business Media, (2012).
- [15] Akkaya S. E., Kıvanç M., “Termofil bakteriler; sıcak su kaynaklarında yaşayan gram negatif basillerin izolasyon ve identifikasyon yöntemleri”, *Elektronik Mikrobiyoloji Dergisi Tr*, 7(1): 01-23, (2009).

- [16] Haki, G.D., Rakshit, S.K., “Developments in industrially important thermostable enzymes: a review”, *Bioresour Technol*, 89: 17–34, (2003).
- [17] Bekler F.M., “Türkiye’deki dibekli sıcak su kaynağından termofilik *geobacillus* sp.DB2 suşunun fizyo-biyokimyasal karakterizasyonu ve 16S rRNA sekanslaması”, *Iğdır Üni. Fen Bilimleri Enst. Der. / Iğdır Univ. J. Inst. Sci. & Tech.*, 7(1): 63-71, (2017).
- [18] Yılmaz M., “The energy potential of Turkey and its importance of renewable energy sources in terms of electricity production”, *Ankara Üniversitesi Çevre Bilimleri Dergisi*, 4(2): 33-54, (2012).
- [19] Dindar E., Şağban F. O. T., Başkaya H. S., “Kirlenmiş topraklarda arıtma çamuru uygulamasının enzim aktivitelerine etkisi”, *Uludağ Üniversitesi Mühendislik Fakültesi Dergisi*, 22(1): 81-94, (2017).
- [20] Bollag J.M. Stotzky G., *Soil Biochemistry volume 6*, Marcell Dekker Inc, (2017).
- [21] Society Of American Bacteriologist *Manual of Microbiological Methods*. McGraw Hill Book Company. Inc: London, (1957).
- [22] Çetinkaya E. Aykan K., “Mikrobiyolojide kullanılan bazı moleküler teknikler”, *Karaelmas Fen ve Mühendislik Dergisi /Karaelmas Science and Engineering Journal*, 2 (1): 53-62, (2012).
- [23] Hall, T.A., “BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT”, *Nucl. Acids. Symp. Ser*, 41: 95-98, (1999).
- [24] Silva G.P., Mack M., Contiero J.G. “A promising and abundant carbon source for industrial microbiology”, *Biotechnol. Adv*, 27: 30–39, (2009).
- [25] Varjani, S.J., Thaker, M.B., Upasani, V.N., “Optimization of growth conditions of native hydrocarbon utilizing bacterial consortium “HUBC” obtained from petroleum pollutant contaminated sites”, *Indian J. Appl. Res*, 4(10): 474–476, (2014).
- [26] Leahy J. G., Colwell R. R. “Microbial degradation of hydrocarbons in the environment”, *Microbiol. Rev*, 54: 305–315, (1990).
- [27] Boopathy,R.,“Factors limiting bioremediation Technologies”, *Bioresour.Technol*,74: 63–67, (2000).
- [28] Ron,E.Z., Rosenberg, E., “Enhanced bioremediation of oil spills in the sea”, *Curr.Opin. Biotechnol*, 27: 191–194, (2014).
- [29] Bhattacharya, D., Sarma, P.M., Krishnan, S., Mishra.S., Lal. B, “ Evaluation of genetic diversity among *Pseudomonas citronellic* strains isolated from oily sludge contaminated sites”, *Appl. Environ. Microbiol.*, 69: 1435-1441, (2003).
- [30] Head, I.M., Jones, D.M., Roling, W. F., “ Marine microorganisms make meal of oil”, *Nature Review of Microbiol*, 4: 173-182, (2006).
- [31] Joshi P. A., Shekhawat D. B., “Effect of carbon and nitrogen source on biosurfactant production by biosurfactant producing bacteria isolated from petroleum contaminated site”, *Appl. Sci. Res.*, 5(6): 159-164, (2014).
- [32] Varjani S.J. “Microbial degradation of petroleum hydrocarbons”, *Bioresource Technology*, 223: 277–286, (2017).

- [33] Varjani, S.J., Upasani, V.N., “A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants”, *Int. Biodeterior. Biodegradation*, 120: 71-83, (2017).
- [34] Rahman, K.S.M., Rahman, T.J., Kourkoutas, Y., Petsas, I., Marchant, R., Banat, I.M., “Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients”, *Bioresour. Technol.*, 90(2): 159-168, (2003).
- [35] Kavitha, V., Mandal, A.B., Gnanamani, A., “Microbial biosurfactant mediated removal and/or solubilization of crude oil contamination from soil and aqueous phase: an approach with *Bacillus licheniformis* MTCC 5514”, *Int. Biodeterior. Biodegrad*, 94: 24-30, (2014).
- [36] Sajna, K.V., Sukumaran, R.K., Gottumukkala, L.D., Pandey, A., “Crude oil biodegradation aided by biosurfactants from *Pseudozyma* sp. NII 08165 or its culture broth”, *Bioresour. Technol*, 191: 133–139, (2015).
- [37] Varjani, S.J., Rana, D.P., Bateja, S., Sharma, M.C., Upasani, V.N., “Screening and identification of biosurfactant (bioemulsifier) producing bacteria from crude oil contaminated sites of Gujarat, India”, *Int. J. Innovative Res. Sci. Eng. Technol*, 3(2): 9205–9213, (2014).
- [38] Karlapudi A. P. , Venkateswarulu T.C. , Tammineedi J., Kanumuri L., Ravuru B. K., Dirisala V., Kodali V. P., “Role of biosurfactants in bioremediation of oil pollution-a review”, *Petroleum* , 4 (3): 241-249, (2018).
- [39] Elumalai, P., Parthipan, P., Karthikeyan, O.P., Rajasekar, “A Enzyme-mediated biodegradation of long-chain n-alkanes (C32 and C40) by thermophilic bacteria”, *Biotech*, 7: 116, (2017).
- [40] Zargari, S., Ramezani, A., Ostvar, S., Rezaei, R., Niazi, A., Ayatollahi, S., “Isolation and characterization of gram-positive biosurfactant-producing halothermophilic bacilli from Iranian petroleum reservoirs”, *Jundishapur J Microbiol.*, 7(8), (2014).
- [41] Perfumo, A, Banat, IM, Marchant, R, Vezzulli, L., “Thermally enhanced approaches for bioremediation of hydrocarbon-contaminated soils”, *Chemosphere*, 66: 179–184, (2007).
- [42] Rahman, T.,J, Marchant R., Banat I.M., “Distribution and molecular investigation of highly thermophilic bacteria associated with cool soil environments”, *Biochem Soc Trans*, 32: 209–213, (2004).
- [43] Timmis, K.N.(ed.), Marchant, R., Banat, I.M., “21 The Genus *Geobacillus* and Hydrocarbon Utilization, *Handbook of Hydrocarbon and Lipid Microbiology*”, Springer-Verlag Berlin Heidelberg, (2010).
- [44] Logan, N.A., Vos, P. D. (eds.), “Endospore-forming Soil Bacteria”, *Soil Biology* 27, Springer-Verlag Berlin Heidelberg, (2011).
- [45] Al-Wahaibi, Y. M., Al-Bahry, S. N., Elshafie, A. E., Al-Bemani, A. S., Joshi, S. J., Al-Bahri, A. K., “Screening of minimal salt media for biosurfactant production by *Bacillus* spp.”, *World Academy of Science, Engineering and Technology International Journal of Environmental and Ecological Engineering*, 8(2), (2014).
- [46] Marchant, R., Banat, I.M, *The Genus Geobacillus and Hydrocarbon Utilization*. In: Timmis K.N. (eds) *Handbook of Hydrocarbon and Lipid Microbiology*, Springer Berlin, Heidelberg, (2010).

- [47] Zhou, J.F., Gao, P.K., Dai, X.H., Cui, X.Y., Tian, H.M., Xie, J.J., Li, G.Q., Ma, T., "Heavy hydrocarbon degradation of crude oil by a novel thermophilic *Geobacillus stearothermophilus* strain A-2", *Int. Biodeterior. & Biodegradation*, 126: 224-230, (2018).
- [48] Atlas, R.M., "Microbial degradation of petroleum hydrocarbons: an environmental perspective", *Microbiol. Rev*, 45(1): 180-209, (1981).
- [49] Noordman, W.H., Janssen, D.B., "Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*", *Appl. Environ. Microbiol*, 68: 4502-4508, (2002).
- [50] Haghghat, S., Sepahy, A.A., Assadi, M.M., Pasdar, H., "Ability of indigenous *Bacillus licheniformis* and *Bacillus subtilis* in microbial enhanced oil recovery", *Int. J. Environ. Sci. Tech.*, 5: 385-390, (2008).
- [51] Elazzazy, A.M., Abdelmoneim, T.S., Almaghrabia, O.A., "Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia", *Saudi J Biol Sci.*, 22(4): 466-475, (2015).
- [52] Chen, C.C., Riadi, L., Suh, S.J., Ohman, D.E., Ju, L.K., "Degradation and synthesis kinetics of quorum-sensing autoinducer in *Pseudomonas aeruginosa* cultivation", *J. Biotechnol.*, 117: 1-10, (2005).
- [53] Tabatabaei, M., "Design and fabrication of integrated plasmonic platforms for ultra-sensitive molecular and biomolecular detections", *Electronic Thesis and Dissertation Repository, The University of Western Ontario*, 3161, (2015).
- [54] Agnes, M.N. "Screening, isolation and characterization of hydrocarbonoclastic bacteria from oil contaminated soils", *Biochemistry of University of Nairobi, MSc.Thesis*, p 119, (2017).
- [55] Batista, S.B., Munteer, A.H., Amorim, F.R., Totola, M.R., "Isolation and characterization of biosurfactant/bioemulsifier producing bacteria from petroleum contaminated sites", *Bioresour. Technol*, 97: 868-875, (2006).
- [56] Ron, E.Z., Rosenberg, E., "Enhanced bioremediation of oil spills in the sea", *Curr. Opin. Biotechnol*, 27: 191-194, (2014).
- [57] Varjani, S.J., Upasani, V.N., "Carbon spectrum utilization by an indigenous strain of *Pseudomonas aeruginosa* NCIM 5514: production, characterization and surface active properties of biosurfactant", *Bioresour. Technol*, 221: 510-516, (2016).
- [58] Kaczorek, E., Pacholak, A., Zdarta, A., Smulek, W. "The impact of biosurfactants on microbial cell properties leading to hydrocarbon bioavailability increase", *Colloid. Interface.*, 2(3): 35, (2018).
- [59] Chen, Q., Li, J., Liu, M., Sun, H., Bao, M., "Study on the biodegradation of crude oil by free and immobilized bacterial consortium in marine environment", *Plos One*, 12(3), (2017).
- [60] Chandra, S., Sharma, R., Singh, K., Sharma, A., "Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon", *Ann. Microbiol.*, 63: 417-431, (2013).
- [61] Varjani, S.J., Upasani, V.N., "Biodegradation of petroleum hydrocarbons by oleophilic strain of *Pseudomonas aeruginosa* NCIM 5514", *Bioresour. Technol.*, 222: 195-201, (2016).

- [62] Janssen, P. H., Yates, P.S., Grinton, B.E., Taylor, P.M., Sait, M., “ Improved cultivability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia ”, *Appl. Environ. Microbiol.*, 68: 2391-2396, (2002).
- [63] Alexander, M. ,*Advances in Microbial Ecology Vol 2*, Springer Science and Business Media, 297, (2012).
- [64] Marshall, K. C., *Advances in Microbial Ecology Vol 6*, Springer Science and Business Media, 240, (2012).
- [65] Schoenborn, L., Yates, P. S., Grinton, B. E, Hugenholtz, P., Janssen, P. H., “Liquid serial dilution is inferior to solid media for isolation of cultures representing the phylum-level diversity of soil bacteria”, *Appl. Environ. Microbiol.*, 70: 4363-4366, (2004).
- [66] Davis, K.E.R., Joseph, S.J., Janssen, P.H., “Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria”, *Appl Environ Microbiol.*, 71(2): 826–834, (2005).