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REPUBLIC OF TURKEY
ADANA SCIENCE AND TECHNOLOGY UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF NANOTECHNOLOGY AND ENGINEERING SCIENCES

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF BACTERIA LIVE IN EXTREME CONDITIONS

YAĞMUR ATAKAV

MSc THESIS

Adana 2017

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ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF BACTERIA LIVE IN EXTREME CONDITIONS

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ABSTRACT

Caves are formed by rocks, most commonly by calcareous rocks such as limestone and are natural, rocky cavities under the ground. Deeps of the cave receive no light therefore it depends other potential energy sources for microbial growth. Thus, each cave forms its own microbial community. Previous studies showed *Firmicutes, Actinobacteria* and *α- β- and γ- Proteobacteria* are three dominant phyla that inhabit cave environments. Furthermore, it is recently suggested that bacteria may also have a role in cave formations and depositions. In addition to that, the number of cave studies increase rapidly due to their potential to host microorganisms that can secrete secondary metabolites that may be of value to humans e.g. Enzyme, antibiotic and biofuel production.

In this study bacterial load of Gilindire Cave located in Mersin was investigated by using culture dependent and culture independent methods. Samples were taken by sterile swabs and submersed in distilled water and stored in +4°C degrees until culturing. For culturing the water that samples were submersed in was diluted 10^3 fold before plating. R2A media which is widely used for cave microbiology research was used as culture media and distinct looking colonies were picked from the petri dishes and isolated. After isolation, 16S rRNA PCR analysis was performed. 7 *Actinobacteria* (39%), 6 *Proteobacteria* (%33), 3 *Firmicutes* (17%) and 1 *Bacteriodetes* (5,5%) as well as 1 uncultured organism (5,5%) was found.

Keywords: *Cave, Microbiology, Isolation, Identification, 16S rRNA*

EKSTREM KOŞULLARDA YAŞAYAN BAKTERİLERİN İZOLASYONU, KARAKTERİZASYONU VE İDENTİFİKASYONU.

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ÖZET

Mağaralar yaygın olarak kireçtaşı gibi kalkerli kayalardan meydana gelen, yer altındaki doğal, taşsı boşluklardır. Mağara derinleri ışık almaz bu sebeple mikrobiyal büyüme için farklı enerji kaynaklarına bağımlıdır, bu da mağaraların kendilerine has bir mikrobiyal yük geliştirmesine sebep olur. Önceki çalışmalar *Firmicutes, Actinobacteria* ve *α- β- ve γ- Proteobacteria* filumlarının bu alanlardaki dominant üç filum olduğunu ortaya koymuştur. Ayrıca, yakın zamanda bakterilerin mağaradaki çökelti ve oluşumlarda rol alıyor olabilecekleri öne sürülmüştür. Buna ek olarak, mağara çalışmalarının sayısı enzim, antibiyotik ve biyoyakıt gibi önemli sekonder metabolit üreticisi mikroorganizmaların potansiyel barınma yerleri olması sebebi ile gittikçe artmaktadır.

Bu çalışmada kültür bağımlı ve kültür bağımsız teknikler kullanılarak Mersin ilinde bulunan Gilindire mağarasının bakteriyel yükü araştırılmıştır. Örnekler steril swablar ile alınmış, distile su içerisinde tutularak kültüre alınana kadar +4°C derecede saklanmıştır. Kültüre alımda ekim öncesinde swabların bekletildiği sular 10^3 kat seyreltilmiştir. Mağara mikrobiyolojisi çalışmalarında sıklıkla kullanılan R2A besiyeri kültür ortamı olarak kullanılmış farklı görünen koloniler seçilerek izole edilmişlerdir. İzolasyondan sonra 16S rRNA PCR analizleri yapılmıştır. 7 *Actinobacteria* (39%), 6 *Proteobacteria* (%33), 3 *Firmicutes* (17%) ve 1 *Bacteriodetes* (5,5%) ile 1 kültüre alınmamış organizma (5,5%) bulunmuştur.

Anahtar Kelimeler: *Mağara, Mikrobiyoloji, Izolasyon, İdentifikasyon, 16S rRNA*

ACKNOWLEDGEMENTS

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NOMENCLATURE

µl	Microliter
ml	Milliliter
L	Liter
sec	Second
min	Minutes
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
TAE	Tris Acetate EDTA
TSA	Tryptic Soy Agar
YGC agar	Yeast Extract Glucose Chloramphenical Agar

CHAPTER 1

1. INTRODUCTION

1.1. Caves and Their Formations

Caves are defined as a type of natural, rocky cavity under the ground that receives little to no light and also accessible to humans. They are formed by rocks such as gypsum, granite, quartzite, and talus but most commonly by calcareous rocks (e.g. Limestone) (Northup and Lavoie, 2001).

Caves are developed in soluble rocks and constitute a characteristic feature of carbonate (e.g. Limestone), and non-carbonate rock (e.g. Basalt, sandstone), and formed by environmental effects such as dissolution, volcanic activity, or even the melting of glacial ice (Engel, 2010). Although some caves are formed by erosions (erosion caves) or volcanic eruptions (volcanic caves), most of the caves are formed by dissolution of bedrocks because of the flowing water through or on the rock surface (Northup and Lavoie, 2001; Engel, 2010, 2015). Dissolution based formations are categorized as epigenic or hypogenic. Caves are formed by a flow of shallow meteoric water or stream, called epigenic while having formed by the solutions coming upward-flowing from great depth within the earth termed hypogenic. Epigenic caves, %75-80 of the total, are more common than hypogenic caves (Dublyansky, 2014; Engel, 2010; Palmer, 2011).

1.2. Energy Mechanisms in Caves

Autotrophy and heterotrophy are the two main mechanisms in caves that a microbe can obtain energy and nutrients. Autotrophs convert inorganic carbon to organic carbon for growth by photosynthesis or chemosynthesis. Heterotrophs take in the organic carbon that already exists in an ecosystem. Photosynthesis powered by sunlight which is uncommon in cave environment. Chemosynthesis is the use of energy released by conversion of inorganic carbon to organic carbon through redox reaction. Some of the compounds found in the cave rock and water such as iron, manganese, methane and sulfide are redox-sensitive and they act as energy sources for chemoautolithotrophs (or chemolithoautotrophs) or in another word self-feeding rock eaters (Engel, 2015).

Lee et al. (2012), divided zones of the cave into three based on their habitat; (a) Entrance zone, b) Twilight zone, and c) Dark zone (Engel, 2010; Lee et al., 2012). Zones are named after their exposure level to light penetration and intensity. In the dark zone, no light or plant species can be seen and the temperature is stable through the year, while there are still light and temperature changes both in the entrance and twilight zone (Lee et al., 2012). However, deep cave zones are usually considered to be extremely nutrient

poor (oligotrophic) environments which means having less than 2 mg of total organic carbon per liter, since many of the resources needed for surface-based ecosystems (e.g. Light and organic matter) are limited. Life that seen in the dark zone heavily rely on minimal allochthonous energy sources or chemoautolithotrophic activities (Mulec, 2008; Ortiz-Ortiz, 2012).

Despite the fact that deep cave zones are extremely nutrient poor environments because of the absence of photosynthetic activity due to the lack of sunlight and the geologic isolation (Pemberton, 2005); the cave environment presents variety of other potential energy sources for microbial growth. There are several routes for the microbial energy and nutrient acquisition in caves: (i) atmospheric gases (e.g. Nitrogen, CO_2, H_2S and aromatic hydrocarbons), (ii) soil-derived aromatic and polyaromatic compounds carried in the percolating water and (iii) reduced metal ions within the rock itself such as iron and manganese (Barton and Northup, 2007).

1.3. Cave Studies

Early studies on the microbial life of cave water and sediments showed the presence of both bacteria and fungi (Caumartin, 1963). However, it is possible to encounter fungi, microalgae, protozoa, cyanobacteria as well as bacteria (Mulec, 2008).

Caves habitat by a disparate bacteria that can live in extreme conditions can be seen as surface dots, unusual colorations, precipitations, corrosion residues or sticky looking biofilms on cave walls as well as shiny gold, white, pink, yellow white looking patches (Barton, 2006; Engel, 2015; Stewart, 2012).

Although, the presence of the microorganisms in the deep cave zones was thought to be carried by the wind , meteoric drip waters, surface streams, air currents or animals (Caumartin, 1963; Engel, 2010), individual cave bacteria have reported in various studies. These bacterial communities vary from one cave to another or even in different speleothems at the same cave (Ortiz-Ortiz, 2012). However, these studies also revealed that there is a consistent range of phyla found that can be interpreted as core set of cave phyla, depending on the geochemical and geographical conditions of the cave. *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteriodetes* are the most abundant phyla in oligotrophic cave environments.

The very first microbiological studies of cave and karst habitats, mostly based on the investigation of sediment and water by using microscopy or enrichment and isolation culture-based approaches (culture dependent studies), After its discovery, findings of the studies of Movile Cave in Romania (Sarbu et al., 1996) pointed out captivating nature of

extreme dark environments. Movile Cave was exceptional since it is sustained by chemoautolithotrophic microbial primary productivity and host dozens of unique animals (Engel, 2010; Sarbu et al., 1996). After that the microbial life of cave ecosystems began to draw more attention from the scientific field.

Beside Movile Cave, another cornerstone of this field was The Breakthroughs Conference held in 1994 which brought cave geologists and biologists together and gave them a new insight about how caves are formed and the role of microorganisms in these formations (Barton and Northup, 2007)

Caves are under the spotlight due to several reasons. First of all, extreme and dark conditions of caves represent ideal ecosystems for biological adaptation studies. The unique and not fully explored cave ecosystem provides an opportunity for the discovery of novel microorganisms and biological byproducts such as ethanol production, environmentally friendly enzymes, antibiotics and even cancer treatments (Barton, 2006; Onaga, 2001). Furthermore, cave research helps finding answers to unanswered questions about the role of microorganisms in cave formation as well as the relation between mineral surfaces and microbial mats, and the effect of human activities, such as tourism, on microbial cave ecology. Also, knowing more about the cave microbiology will lead us to understand other areas of speleology, including microbial impacts on water and contaminant flow into and through the subsurface, mineralogy, geochemistry, ecosystem nutrient flux and species adaptation and evolution (Engel, 2015). Caves are also subject to this studies due to their accessibility and stable environment which do not cause substantial physical variations in the system (Engel, 2010; Pemberton et al., 2005).

Limestone ($CaCO_3$) caves have mild, stable temperatures, high humidity, relatively neutral pH and are rich in oxygen and low nutrient conditions for growth. These conditions force bacteria to rely on alternative production strategies to complement the limited supplies of organic carbon sources from the surface. For instance, bacterial species that live in limestone caves have a higher rate of the ability to precipitate calcium carbonate. Additionally, compared to other environments, these species show an overall increase in total $CaCO_3$ precipitation levels (Banks et al., 2010).

Even though it is not proven yet, many cave deposits are considered to have microbiological origins. For the last twenty years, the progression of the cultivation independent approaches has provided a myriad of information about bacterial community composition, the abundance of different taxa and their possible function in different habitats (Aydın, 2012). However, there are still so little known regarding the process

leading to the observed diversity in different ecosystems and how environmental factors contribute to shaping microbial communities (Aydın, 2012; Engel, 2010)

1.3.1. Culture-dependent method

Culture based analysis remain the most direct and effective ways to describe the metabolic processes and physiological requirements of different taxa (Engel, 2010). It requires cultivation, isolation and identification of bacteria. However, for the cultivation of microorganism from a cave ecosystem a drawback surfaces; majority of microorganisms cannot be cultured in regular petri plates which had led scientist to think that the cave environment was not as rich as the soil in the mean of livings (Engel, 2010, 2015). Cave microorganisms developed new metabolic strategies to obtain energy from low nutrient environments. Therefore, they cannot adapt this nutrient change in a short amount of time (Barton, 2006; Engel, 2015) In fact, it is estimated that 99% of microorganisms in an environment are not known in the culture which is explained as "great plate count anomaly" by Staley and Konopka(1985). Using nutrient-rich culture media, especially developed for medical studies and aim to cultivate organisms that are related to the human body, such as human pathogens, inhibits microbes to be able to shut down their metabolic pathways, leading them to die from osmotic pressure (Barton, 2006; Engel, 2015; Stewart, 2012).

To overcome this problem, several new media that are low in nutrients were improved to allow cultivation of environmental microbial populations (Joseph et al., 2003; Staley and Konopka, 1985) Mimicking the environment that the sample was taken is another strategy to develop new media suitable for the cultivation of the cave microbiome (Engel, 2015). R2A medium was developed to address oligotrophic microorganisms found in potable water and have a low nutrient content (Reasoner and Geldreich, 1985). The medium is now one of the most preferred media to cultivate cave bacteria (Curry et al., 2009; Ikner et al., 2007; Snider, et al., 2009). The development of new media for environmental samples has increased the number of the isolated colonies from cave and has allowed to identify metabolic activities such as calcium carbonate precipitation (Banks et al., 2010; Danielli and Edington, 1983; Sanchez-Moral et al., 2003) and antibiotic resistance mechanisms (Bhullar et al., 2012) in these microorganisms. Although there are still some cultivation problems caused by the medium such as the type and amount of the carbon preferred for the medium or the culture conditions (e.g. Temperature, pH, humidity) (Engel, 2015; Saiz-Jimenez and Groth, 1999).

Nevertheless, culture dependent methods rely on identification based on some biochemical tests and their interpretation according to the Bergey's Manual which is time and resource consuming versus using new technological advances (Barış, 2009).

1.3.2. Culture-Independent methods

It is possible to identify most of the living on a surface (e.g. Next generation sequencing) by using rRNA gene sequence which encodes ribosomal RNA with the developments in biotechnology. Bacterial and archeal ribosomes have three individual sequences for rRNA; 5S, 16S and 23S. 16S rRNA occur in the small ribosomal subunit and is highly conserved between different species of bacteria and archaea. Various techniques based on the gene sequences have been developed such as, Sanger sequencing, rRNA gene cloning, High throughput rRNA amplicon sequencing (or sometimes tag or pyrotag sequencing), Terminal restriction fragment length polymorphism (T-RFLP), Denaturing gradient gel electrophoresis(DGGE) and Fluorence in situ hybridization(FISH). Metagenomics is another method that is differ from previous methods due to its ability to analyze genomic material directly from a mixed microbial community. This technology helped to the researchers to detect the unculturable microbes and determine the relation and similarity between them. Molecular phylogenetic analysis may also give information about the available nutrients in an environment (Barton, 2006).

After this technology the abundance of microorganisms in the cave was proven. Molecular studies also presented a new way to search extremophilic bacteria from varying communities (e.g. Oligotrophic, acidophilic, sulfidophilic etc.) (Engel, 2010)

Although, instead of abandon culture dependent methods, using a combination of culture dependent and independent methods has preferred since 1997 (Engel, 2015) because molecular studies only reveal genetic combinations but the meaning of these combinations are not known most of the times. Also resulting phenotype of a genotype is not fully understood yet (Engel, 2015; Rinke et al., 2013).

1.4. Study Site

Gilindire Cave (36°07'58.08"N 33°24'11.04"E) is found by a shepherd in 2000 and opened for tourism in 2012. It is located 9km east of the district Aydıncık in Mersin, Mediterranean cost of Turkey (Nazik et al., 2000; Özşahin and Kaymaz, 2014). The cave is 555m long and has developed along NE-NW trending faults in limestone and dolomic limestone of Cambrian age. The cave is unique as being one and only representative cave of the transition phase after the last glacial climate change of the Quaternary Period, in the East Mediterranean Region (Özşahin and Kaymaz, 2014).

Gilindire has three major segments that represent disparate formation and development processes. The first part, from 22m of inside to the entrance, have developed and opened at the Thyrrenian period of erosion surface periods while the second part, the oldest part of the cave, have developed at the Pliocene period. The third, youngest and active part of the cave, have developed when the Mediterranean Sea was at the lowest level, the Wurm period. This part started to develop due to karstification however flooded with the sea water after rise of the sea level (Nazik et al., 2000). Formed stalactites, stalagmites, cave pillars and elephant's ears were protected by the raised sea level and remained unaffected by atmospheric changes to the present day(Anonym, 2014).

The cave also has a lake at the end which also gives the cave its name, Aynalıgöl (Mirrored-lake). The lake consists of brackish water (10m from the surface) and sea water below this depth. Sea water is thought to reach by leaking along the fractures and fissures into the lake from the sea (Nazik et al., 2000).

CHAPTER 2

2. LITERATURE REVIEW

Even though the increase in the last decade, cave microbiology studies are still in their infancies. Some of the related studies are presented according to the year of publishing.

Boquet et al. (1973) investigated calcium carbonate precipitation abilities of 210 bacteria that were isolated from soil and also were able to grow on B-4 medium. They found that all isolated bacteria could form crystals. Additionally they tested bacteria from their laboratory such as *Salmonella spp.*, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus* and more. They found that these bacteria were also capable of forming crystals on B-4 medium. As a result, they claimed that crystal formation is a general phenomenon that takes place when the media is used under suitable conditions.

Danielli and Edington (1982) investigated basic mechanisms of calcium carbonate formation by using several simple media from cave bacteria. Gran's medium and five other variation of Gran's medium that contain different calcium sources and nutrient broth were used as media. Their findings showed that calcium source does not affect the initiation of growth but it affects the overall rate of colony growth and calcium precipitation are essentially waste products of the bacteria. Calcium carbonate formation was observed more at lower pH (pH 6.8). Also they agreed that crystal formation is actually a general phenomenon as suggested by Boquet et al in 1973.

Mikell et al. (1995) used HCFU and R2A medium to culture bacteria from karst and sand aquifer spring with different plating methods. In terms of cell count HCFU and R2A were superior to TSA.

Groth et al. (1999) cultured *Actinomycetes* from deterioration of rock art in Altamira and Tito Bustillo Cave in order to investigate their role in biogeochemical processes. Culture dependent method was used in the study. Several pigment producer *Streptomyces* isolates were found and linked with the deterioration of the rock art. They also showed that metabolic activity of these organisms may have a role in the stalactite destruction by inoculating isolated *Streptomyces xanthophaeus* on a stalactite which led the dissolution of calcite.

Pemberton et al.(2005) studied the effect of the surface condition on the bacterial diversity in the Jack Bradley Cave, Kentucky. For this aim, 10 different medium varying from nutrient deficient to high nutrient were used. Culture dependent and independent

methods were used in order to isolate and identify colonies. They were able to isolate and identify 111 unique and undescribed species from 501 cultivars belonging to *Actinomycetes, Caulobacter* and *Pseudomonas.* The phenomenon of adaptation of cave microorganism to low nutrient environment was proven again by showing greater diversity of colonies on the nutrient deficient plates. This study also referred to the importance of using a number of different media in order to obtain diverse cultured species.

Barton et al. (2007) searched the effect of rock fabric and mineralogy on bacterial diversity by comparing two different sampling site chosen according to their geological location and bedrock in the Carlsbad Cavern, New Mexico. Their results showed that the lower the additional energy source of the rock the more the microbial communities rely on heterotrophic growth from allochthonous energy sources and in the case of additional energy sources provided by the environment due to the geochemical complexity, species were tend to use chemolithotrophic mechanisms for energy conservation. Also they suggested that the geochemistry of the bedrock may have an important effect on both diversity of the community and the type of energy conservation reactions of these communities.

Pasic et al. (2010) investigated the bacteria and archea responsible for the "cave silver" or "cave gold" formations which are named after their ability to reflect light in silver or gold color contrary to their transparent look. They used culture independent method to analyze communities. Eight different phyla were identified and *Proteobacteria* (51,4%), *Actinobacteria* (16,3%) and *Nitrospira* (15,2%) was found the most abundant phylum.

Rusznyak et al. (2011) aimed to interpret the bacterial diversity and activity as well as their possible contributions on carbonate mineralization with scanning electron microscopy (SEM), energy-dispersive X-Ray spectroscopy (SEM-EDX) and confocal laser scanning microscopy (CLSM) in newly found, karstic cave, Herrenberg Cave, Germany. Phylogenetic analysis, microscopic techniques and culture based methods were also used. It was shown that two of the isolates cultured on the B-4 produced mixtures of calcite, vaterite and monohydrocalcite. The most abundant bacterial communities from the stalactite samples were *Proteobacteria* and *Acidobacteria.* Stalactite community was found to be very similar those found on sediment samples hinting a connection between the surface environments.

Bhullar et al. (2012) investigated antibiotic resistance of the previously found cave bacteria which have never been exposed to any antibiotics, from Lechuguilla Cave, New

Mexico. On average 70% of the 93 in total strains were found to be resistant to 3-4 antibiotics while 3 of them were resistant to 14 antibiotics. Results suggested that antibiotic resistance has a long evolutionary past and is common and widespread in these communities and that cave bacteria have more resistance to natural antibiotics than the synthetic ones.

Legatzki et al. (2012) studied factors that effects bacterial community variations on 10 active calcite formations in oligotrophic Kartchner Caverns, USA. Culture independent methods were used to identify bacterial communities on the surfaces. They also analyzed physical (length, diameter, presence of drip water, surface color) and chemical (organic carbon concentration, elemental analysis) properties of the locations. It was found that elemental concentrations were unique for each speleothem and bacterial community structure was speleothem specific, also affected by the formation's location within the cave while dripping water was found the most influencing factor. However, organic carbon and elemental concentration were no significant effect on the community structure.

Cheeptham et al. (2013) searched antimicrobial activities of cave bacteria particularly *Actinomycetes* against various pathogens at Helmcken Falls Cave, Canada. Over 400 isolates, 26,5% presented inhibitory activity against *Klebsiella pneumoniae*, 10,25% against *Micrococcus luteus,*7,5% against *Candida albicans* proving novel compounds, especially to fight gram-negative antibiotic resistant bacteria, for developing new drugs can be obtain from cave bacteria. Like some other researchers in this review, Cheeptham et al. also analyzed chemical and physical characteristics of environmental samples pointing out the importance of surface properties on bacterial community as well as their potential to enlighten mineral-bacteria interactions and to identify optimal growth conditions and metabolite production for the interested bacteria.

Engel et al. (2015) reexamined cave enlargement process explained by the sulfuric acid speleogenesis in 1970s at Lower Kane Cave, USA. On the contrary to the original model which had claimed these formations were made by abiotic autoxidation of H_2S to sulphuric acid, their results revealed that subaqueous sulphur oxidizing bacterial communities colonized on carbonate surfaces, ϵ-*Proteobacteria* and γ-*Proteobacteria* particularly, consumes almost all H_2S and converts it to sulphuric acid as a metabolic byproduct. Within the study it was shown that small pH changes have a very low impact on calcite solution and that is, cave enlargement due to dissolution is microbially induced. Interpretation of the results suggest microbial catalysis may be responsible of the enlargement other carbonate surfaces such as oil-field reservoirs and aquifers.

Wu et al. (2015) conducted the first study to present evidence the presence of cave specific bacterial lineages in East Asian cave and examined bacterial abundance and composition in Jinjia Cave, China. Seven samples were taken from different spots through the cave 4 of them cave wall, 2 from sediment in pools, 1 from soil at the bottom of a sinkhole. They conducted elemental and molecular analyzes in order to reveal elemental composition and bacterial diversity in the cave. Results suggested that the diversity was similar in soil and sediment and significantly higher than cave deposits indicating mineral chemistry may impact bacterial community on the surface. In addition to that, phylum found between cave and surface communities were different from each other.

Lamprinou et al. (2015) studied the antimicrobial susceptibility of extracted lipids from cave cyanobacteria isolated from Francthi Cave, Greece against human pathogenic bacteria. Their findings showed that most of the extracted lipids of two isolates demonstrated antimicrobial activity against the tested gram positive bacteria indicating these active compounds may be used to develop new antibiotics.

Barış (2009) investigated bacterial contributions on formation of dripstone for the first time by using both culture dependent and independent methods in two different caves in Erzurum, Turkey. Potential isolates were chosen according to their ability to produce calcium carbonate crystals on B-4 medium and 64 bacteria were isolated, identified and characterized. Based on phenotypic and genotypic data 48 of the isolates were identified at the species, 5 isolates identified at the genus level. The remaining 11 isolates were unidentified.

Pektaş-Güleçal (2015) investigated bacterial diversity and composition in Oylat Cave, Turkey. Bacterial communities after Sanger Sequencing were identified as *Proteobacteria* (α, β, γ) as well as *Actinobacterium, Acidobacterium, Bacteriodetes, Gemmatimonodates, Verrucomicrobia, Firmicutes, Chloroflexi Planctomycetes* and *Nitrospirae* divisions. Pyrosequencing results revealed the presence of *Proteobacteria* (α, β, γ, δ). A second dominated phylum was *Actinobacteria*, followed by *Acidobacteria, Nitrospirae, Firmicutes, Bacteriodetes, Planctomycetes, Gemmatimonadete, Verrucomicrobia* and *Chloroflexi*. Both methods gave parallel results which were also consistent with other cave studies.

Pektaş-Güleçal and Temel (2015) studied the structure, diversity and abundance of bacteria communities on moon milk deposits with next generation sequencing in Oylat Cave, Turkey. Culture-independent techniques were applied to analyze the environment. At the deepest section of the cave *Proteobacteria* was found to be the dominant phyla. It

was followed by *Nitrospira, Actinobacteria* and *Firmicutes* respectively. Their results indicated that the entrance of the cave is saprophytic while further segments are oligotrophic.

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Materials

Samples were taken from Gilindire Cave (36°07'58.08"N 33°24'11.04"E), Mersin province in Turkey. Special media and chemicals were used to culture, isolate, identify and characterize bacteria from the samples. Additionally, universal primers and ready-to-use kits were used for molecular analysis.

3.1.1. Equipments

The equipments that were used during the experiments can be seen in Table 3.1.

Table 3.1. Equipments used in experiments

Equipment	Brand	Model
Analytical scale	Denver Instruments	TP-214
Autoclave	HMC Hirayama	HV-50L
Biosafety cabinet (Class II)	Nuaire	NU-425-400E
Centrifuge	Hettich	Universal320r
Electrophoresis System	ATTO	
Gel Imagining system	UVP	Constopower3000
Ice Machine	Hoshizaki	FM 80 KE
Incubator	Memmert	IN 55
Magnetic Stirrer	Ika	C-MAG HS 10
Microscope	Leica	DM750
Microwave	Kenwood	MW440
PCR	Agilent	Surecycler 8800
pH Meter	Mettler Toledo	Seven Compact S220
Refrigerator	Beko	
Shaker	Benchmark	Everlast Rocker 247
Ultralow Freezer(-86)	Nuaire	NU-96G8E
Vortex	Ika	Vortex 4 Basic
Water Bath	Daihan	WB-22

3.1.2. Preparation of solutions

The preparation of the solutions that were used during the experiments is given in the appendix A.

3.2. Methods

3.2.1. Sample collection

The required permissions were taken from the related government agencies prior to study. Samples were taken from cave walls and speleothem of The Gilindire Cave by using sterile swabs. Swabs were wetted with sterile tap water (pH = 7.2–7.8) and immersed to 3 mL of sterile tap water in capped test tubes, placed on ice, and transported back to the Adana Science and Technology University Bioengineering Laboratory for the same-day processing.
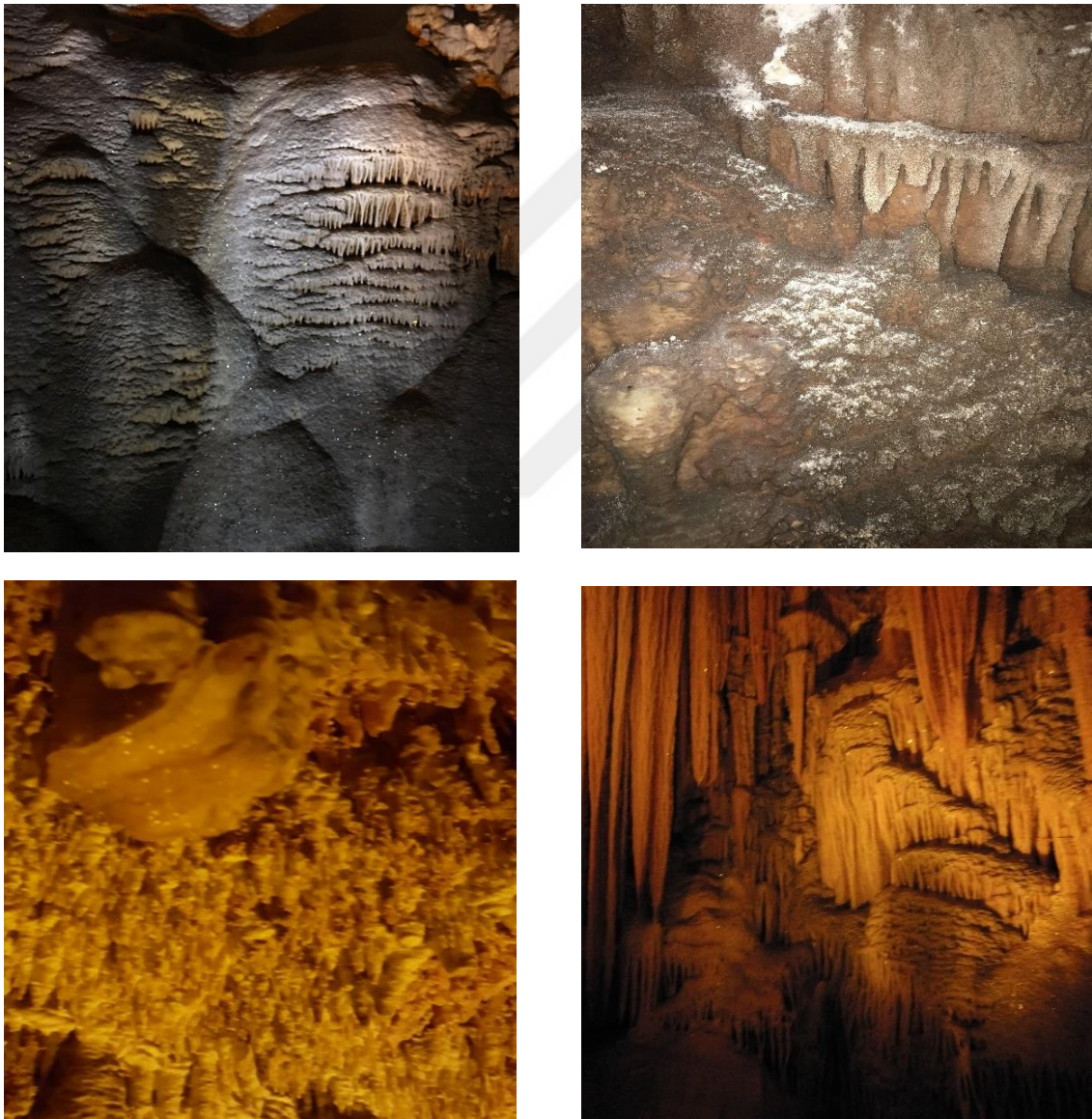

Figure 3.1. Sampling sites

3.2.2. Sample processing

In order to enumerate heterotrophs and to allow visual assessment of morphological diversity, samples were vortexed for 5 min and then 0.1 mL volume of five serial dilutions were plated on R2A medium in the laboratory. Plates were incubated at 30°C for two months.

3.2.3. Isolation, characterization and identification of bacteria

For the selection of unique bacteria for identification, first, during the 2-months period of incubation, morphologically distinct colonies were selected for isolation from each dilution series (10^{-1} to 10^{-6}) as they appeared. This was repeated 3 times after which a single colony was transferred to liquid R2A medium until pure colonies were obtained.

R2A is not a specific media therefore yeast and mold cultures were also grown on the plates. In order to differ these colonies, YGC agar plates were used. The colonies were plated on YGC agar plates and the ones who were able to grow were eliminated since only mold and yeast able to grow on YGC plates and the focus of this study is only bacteria. Bacterial isolates were also confirmed by gram staining, which was carried out according to the standard protocol, according to their morphology by optical microscope (Prescott et al., 2002). The isolates that have similar bacterial and colony morphology were also eliminated.

3.2.4. Determination of morphologic characteristics

Bacterial cell morphology can only be seen with microscopes after treated with special stains. Isolated bacteria have to be cultured on suitable medium such as agar plates or liquid broths for staining. Bacterial shape (coccus, vibrio, rod, spiral etc.) and cell wall type (gram positive, negative) was determined by investigation of morphological characteristics (Prescott et al., 2002).

3.2.4.1.   Determination of cell wall type and cell morphology

A thin smear was prepared for each isolate on a clean glass slide. One loop full of sterile PBS was placed on the slide, a sterile loop was slightly touched on one colony and then mixed with PBS drop. A known gram negative (*Escherichia coli*) and gram positive (*Bacillus subtilis*) bacteria were also prepared on the same slide as controls.

After the smears dried, they were heat fixed by passing the slide through the flame three times. The smears were covered with crystal violet for one minute before washing with water. Iodine was used as mordant. After one minute of iodine treatment, smears were washed with 50:50 ethanol acetone decolorizing solution until it ran clean. Smears then covered with safranin, the counter-stain, and waited for one minute in order to

complete staining procedure. Under 1000x magnification purple colonies were interpreted as gram positive while pink colonies were gram negative. Morphological properties were also observed with this method (Prescott et al., 2002).

3.2.5. Biochemical tests

3.2.5.1. Catalase Test

Catalase test was used in order to determine the presence of the enzyme cytochrome oxidase in the isolated colonies. This enzyme only presents in aerobic or facultative microorganisms and converts hydrogen peroxide (H_2O_2) that formed through aerobic respiration into water (H_2O) and oxygen (O_2) creating visible bubbles on the sample. For the test, one loop full of 18 to 24 hour colony were taken and placed on a clean glass slide. One drop of 5% H_2O_2 solution was added to the sample. Visible bubble formation was considered as a positive result (Prescott et al., 2002).

3.2.5.2. Urease test

Urease test was used to determine the colonies that can able to hydrolyze urea using the enzyme urease. Hydrolyzation of urea results 2 moles of ammonia, a weak base, and one mole of carbon dioxide which increase pH of the media. The pH change turns indicator phenol red from yellow to pink which interprets positive result for this test. For the test, bacteria were inoculated on Christensen agar at 33°C degrees for 1 to 5 days (Prescott et al., 2002)

3.2.5.3. Amylase test

Amylase test were performed to show the bacteria that have the ability to hydrolyze starch using amylase enzyme. Isolates were cultured on starch agar and cultured for 3 to 7 days. After incubation iodine was added on to medium and color change was observed. As the indicator of starch, iodine changes its yellow color to black in the presence of starch on the media (Prescott et al., 2002).

3.2.6. Antimicrobial activity test

The antimicrobial activities of isolated colonies were tested against *Escherichia Coli, Staphylococcus Aureus, Aspergillus Niger and Candida Albicans* with the disk diffusion assay. In the test, 19µl of liquid culture were inoculated on blank disc and placed on the media. TSA and PDA were used to culture bacteria and yeast-fungi respectively. Beside *Aspergillus* which was kept at room temperature, all microorganisms were incubated at 37°C for 3 days. At the end of the 3rd day, antimicrobial activity was evaluated by measuring the zone of inhibition against test microorganisms.

3.2.7. Calcium carbonate crystal precipitation abilities

B-4 media is traditional test medium and being used widely to investigate calcite precipitation and crystal production of the bacteria. Calcite precipitation may indicate that bacteria have a role in the cave formation. In order to see the crystal formation, isolates were cultured on the B-4 medium and the media was observed under light microscope (Baskar et al. 2006; Boquet et al., 1973)

3.2.8. Molecular analysis of the isolates

DNA was extracted by using Fast DNA Spin Kit (MP Biomedicals; Solon, OH, USA) according to the manufacturer's instruction for molecular analysis of the isolates. Agarose gel electrophoresis was performed in order to confirm the extraction of DNA. Wells on the prepared gel were loaded with 5µl of DNA 5µl of dH20 and 2µl 6X loading dye (ThermoScientific; Waltham, Massachusetts, USA). After the conformation of DNA on gel, the amount of DNA extracted from the isolates was measured with Qubit 2.0 (Invitrogen) by using Qubit dsDNA BR Assay Kit (Invitrogen; Waltham, Massachusetts, USA).

Table 3.2. Reagents for PCR reaction

Brand/Cat.No.	Reagent	Volume(µl)	Concentration
NEB-M0486S	Buffer(2x)	25	
Promega- P1193	Water	20.75	
NEB-N0447S	dNTP	1	10 mM
Macrogen	Forward Primer	1	10 µM
Macrogen	Reverse Primer	1	10 µM
NEB-M0480S	Enzyme	0.25	
	DNA	1	

Universal bacterial primers 27F (forward 5'- AGA GTT TGA TCC TGG CTC AG - 3'), and 1492R (reverse 5'- GGT TAC CTT GTT ACG ACT T -3') (Park et al., 2006) were used as primers for the PCR. PCR performed with the reagents from New England Biolabs (NEB; Ipswich, Massachusetts, USA), the amount of the reagent that was used in the experiment was shown in Table 3.2.

Extracted genes were amplified using 95ºC for 2 min for initial denaturation, then 35 cycles of 95ºC 30 sec, 52ºC 40 sec, 72ºC 1,30 min, with an extension period of 72ºC for 5 min as illustrated in Table 3.3.

Table 3.3. The program for PCR reaction

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	30 sec	
Annealing	52°C	40 sec	35 times
Extension	72°C	1.30 min	
Final Extension	72°C	5 min	1
Hold	4°C	∞	∞

PCR products was examined by gel electrophoresis, wells were loaded with 5µl of PCR product without a loading dye. PCR products purified and concentrated with the DNA Clean & Concentrator™- 100 (Zymo; Irvine, California, USA), according to the manufacturer's instructions. One exception was made through the instruction; 8µl of nuclease free water was used instead of DNA elution buffer to concentrate amplified DNA at the last step. Concentration of each sample was measured with Qubit dsDNA BR Assay Kit (Invitrogen; Waltham, Massachusetts, USA) and fixed at 50ng DNA per microliter by dilution. Cleaned and concentrated products were sent for sequence analysis in order to identify isolates.

Sequencing of the almost complete 16S rRNA gene was performed by the company. Reverse and forward gene sequences were compiled manually. Closely related 16S rRNA gene sequences identified by using the BLASTN algorithm (http://ncbi.nlm.nih.gov/blast).

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1. Bacterial Isolation

58 isolates were obtained after cultivation however further investigation was completed on 30 individual isolate due to elimination of isolates (See 3.2.3).
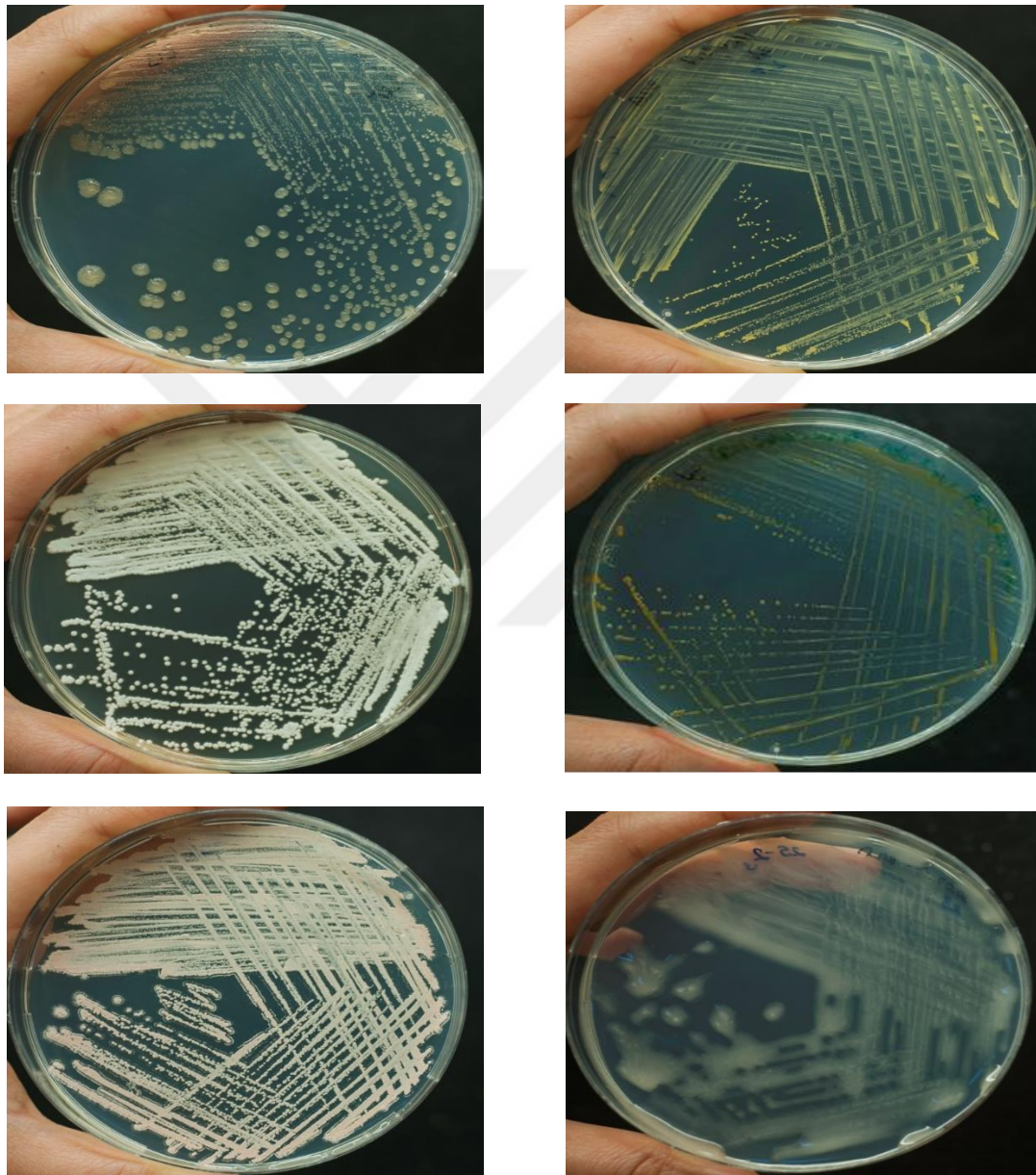

Figure 4.1 Isolated colonies on R2A media. Left to right sample 4, 2, 11, 27, 19 and 25 respectively.

4.2. Characterization of the isolates

Phenotypic properties of the isolates were determined by gram staining and observing motility from the samples taken liquid media. Morphologic characteristics of the isolates are shown in the Table 4.1.

Table 4.1. Characteristics of colony morphologies

Sample	Colony Morphology					
	Shape	Margin	Elevation	Texture	Optical Property	Pigmentation
1	Circular	Entire	Raised	Smooth	Opaque	Yellow
2	Punctiform	Entire	Raised	Smooth	Opaque	Yellowish
3	Irregular	Undulate	Flat	Glistening	Translucent	Whitish
4	Irregular	Curled	Umbonate	Wrinkled	Transparent	-
5	Irregular	Lobate	Flat	Smooth	Opaque	White
6	Irregular	Undulate	Flat	Glistening	Translucent	Whitish
7	Punctiform	Entire	Convex	Smooth	Opaque	Cream
8	Irregular	Undulate	Flat	Glistening	Translucent	Yellowish
9	Circular	Entire	Raised	Smooth	Translucent	Yellowish
10	Filamentous	Filamentous	Raised	Powdery	Opaque	White
11	Filamentous	Filamentous	Raised	Powdery	Opaque	White
12	Irregular	Lobate	Flat	Smooth	Opaque	White
13	Irregular	Lobate	Flat	Smooth	Translucent	Whitish
14	Irregular	Undulate	Raised	Smooth	Opaque	Cream
15	Filamentous	Filamentous	Umbonate	Powdery	Opaque	White
16	Filamentous	Filamentous	Flat	Powdery	Opaque	White
17	Irregular	Undulate	Flat	Glistening	Translucent	Whitish
18	Punctiform	Entire	Pulvinate	Smooth	Opaque	Cream
19	Filamentous	Filamentous	Pulvinate	Powdery	Opaque	Pinkish
20	Irregular	Entire	Raised	Smooth	Opaque	Orange
21	Punctiform	Undulate	Flat	Rough	Opaque	Whitish
22	Circular	Entire	Raised	Smooth	Translucent	Yellowish
23	Circular	Entire	Raised	Smooth	Opaque	Cream
24	Punctiform	Undulate	Flat	Rough	Opaque	Whitish
25-2	Irregular	Undulate	Flat	Glistening	Translucent	Whitish
26	Punctiform	Entire	Raised	Smooth	Opaque	Red
27	Circular	Entire	Raised	Smooth	Opaque	Orange
28	Circular	Entire	Raised	Smooth	Opaque	Orange
29-1	Irregular	Lobate	Flat	Smooth	Opaque	Cream
29-2	Irregular	Entire	Pulvinate	Glistening	Transparent	-

According to the gram staining, 15 of the isolates were found to be gram negative while 13 of them were gram positive. 2 of the isolates could not be stained with gram staining. Motility was observed but it was seen that, motility was not prevalent among the isolates which was also suggested by Ortiz-Ortiz (2012). Only 9 isolate showed motility as shown in Table 4.2.

Table 4.2. Morphologic features of isolates

Sample	Cell Morphology	Gram Stain	Motility
1	Sarcinae	+	-
2	Single cocci	+	+
3	Rods in pairs	-	+
4	Diplococci	-	+
5	Cocci in clusters	-	+
6	Rods in pairs	-	+
7	Filamentous	+	-
8	Coccobacilli	-	-
9	Diplobacilli	+	-
10	Diplobacilli	+	-
11	Filamentous	+	-
12	Single rods	-	+
13	Single rods	-	+
14	Single rods	+	-
15	Branched Filamentous	-	-
16	Single Rods	+	-
17	Diplobacilli	-	+
18	Filamentous rods	+	-
19	Filamentous	+	-
20	Short rods	S	-
21	Filamentous	+	-
22	Filamentous	+	-
23	Filamentous	-	-
24	Filamentous	+	-
25-2	Diplobacilli	-	+
26	Ovoid diplococci	-	-
27	Long diplobacilli	-	-
28	Diplococci	-	-
29-1	Rods in pairs	-	-
29-2	Ovoid	S	-

S:Does not stained

Catalase, amylase and urease tests were done for each sample. The results are shown in Table 4.3. Samples of each tests are presented in Figure 4.2.

Table 4.3. Characteristic features of isolates

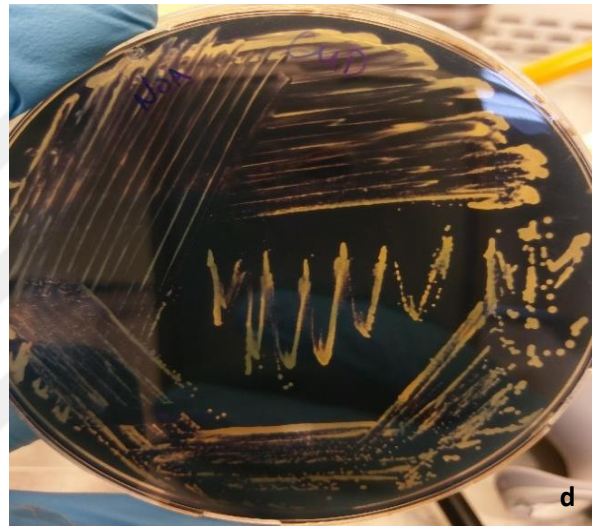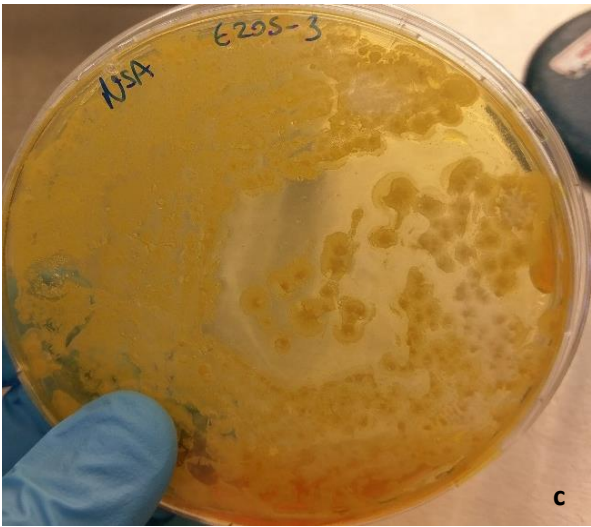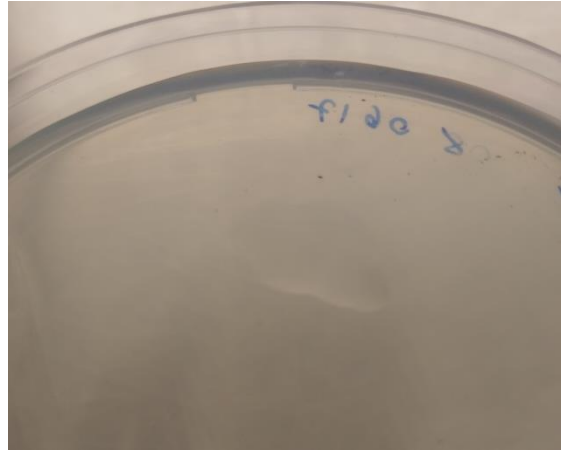| Sample	Catalase	Amylase	Urease
1	+	+	-
2	+	-	+
3	+	-	+
4	+	+	-
5	+	-	-
6	+	-	-
7	+	-	+
8	W+	-	-
9	+	+	-
10	+	-	+
11	+	+	+
12	+	-	+
13	+	-	+
14	+	-	-
15	+	-	+
16	+	+	+
17	W+	-	+
18	+	-	+
19	+	+	W+
20	+	-	+
21	W+	+	+
22	+	+	-
23	+	+	W+
24-3	W+	+	W+
25-2	W+	-	++
26	+	-	-
27	+	N	-
28	W+	N	+
29-1	+	-	-
29-2	+	+	-

W+: Weak positive

N: No growth
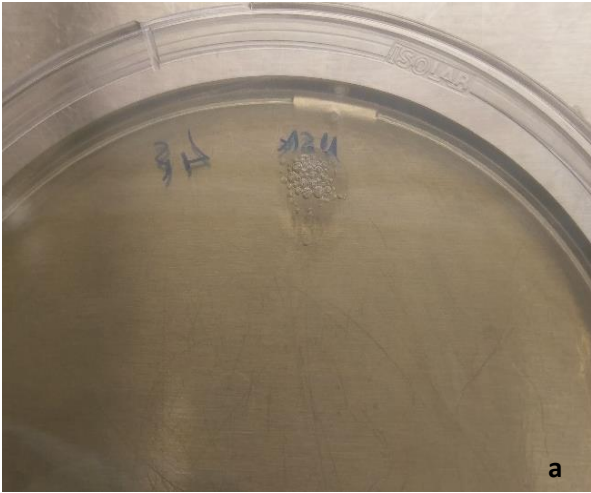
Figure 4.2. Samples of biochemical tests. a. Catalase positive, b. Catalase negative, c. Amylase positive, d. Amylase negative, e. Urease positive, f. Urease negative

Crystal formation was investigated with B-4 media and it was seen that 3 of the isolates was unable grow on this media while the rest 27 were able to grow successfully. Crystal formation observed on 21 isolates while 6 isolates were not capable of producing crystals on the same conditions. Crystal formations are given in Figure 4.3 and the date of formation observed can be seen in Table 4.4.

Boquet et al. (1973) suggested crystal formation is a general phenomenon among bacteria and is depend on suitable conditions. Therefore the bacteria that cannot produce crystals may also produce these formations under a more favorable condition. During the culturing, the amount of the bacteria plated on the media was seen significant in order to obtain colonies. Some isolates were not able to grow when plated minimum amount while with the increase in amount, the same isolates were able to grow. This phenomenon may be explained by osmotic pressure (Barış, 2009; Barton and Jurado, 2007).
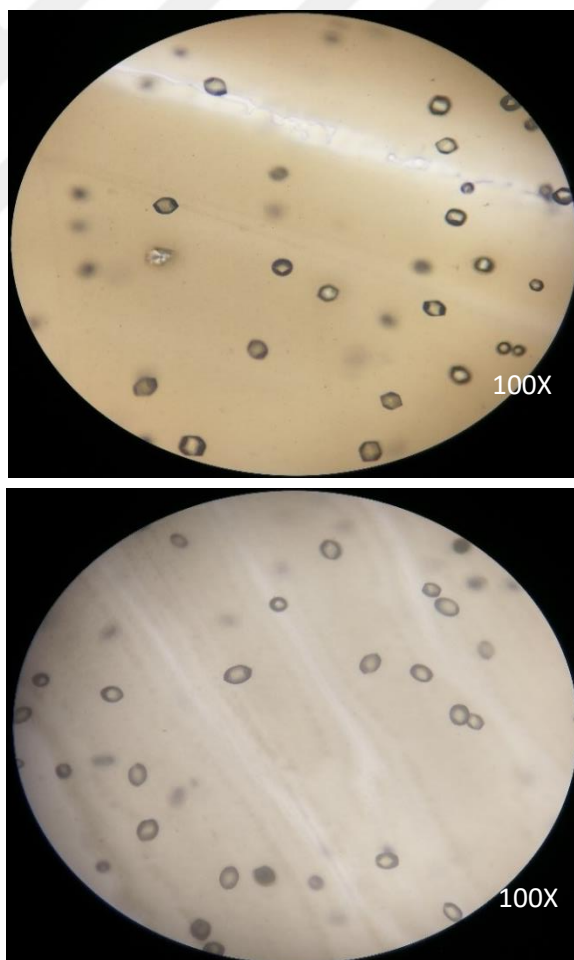
Figure 4.3. Crystal formations of two different isolates a. Isolate 28 and b. Isolate

3

Table 4.4. The date of crystal formation

Sample	B-4 Cultivability	Crystal Formation	The day of formation
1	+	+	9
2	+	-	-
3	+	+	7
4	+	+	31
5	+	+	5
6	+	+	7
7	+	+	8
8	-	-	-
9	+	+	6
10	+	+	7
11	+	+	7
12	+	+	2
13	+	+	2
14	+	+	6
15	+	+	6
16	+	-	-
17	+	+	29
18	+	+	8
19	-	-	-
20	+	+	3
21	+	+	9
22	+	+	6
23	+	+	3
24	+	+	8
25-2	+	+	34
26	+	-	-
27	+	-	-
28	+	-	-
29-1	+	+	7
29-2	-	-	-

4.2.1. Antimicrobial activity

Antimicrobial activity was not observed in any of the isolates. Even though one isolate showed a weak activity, it faded away after 5 days of incubation. Some samples of the result are given in Figure 4.4.

Figure 4.4.Samples of antimicrobial activity test

4.2.2. PCR Results

Amplified DNA extracts were loaded on the gel and it was confirmed all PCR products gave bands on the 1500bp length since 27F-1492R primers give products approximately at 1500bp as seen in Figure 4.5. The results of sequencing are given in the appendix B.
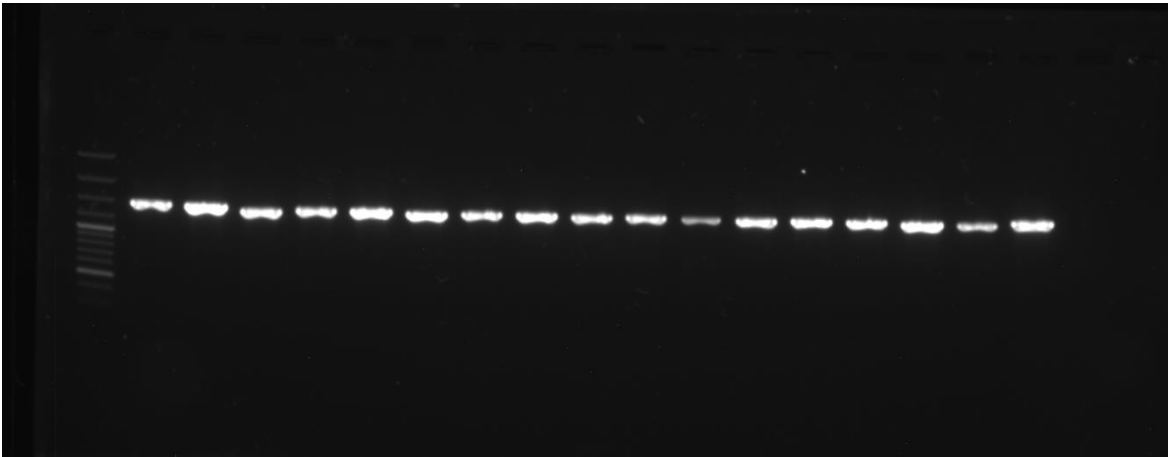

Figure 4.5. Gel image of PCR products. Left to right; Marker, 1, 2, 3, 4, 5, 6, 7, 8, 9, 16,17, 18, 19, 20, 25, 26,27 and negative control respectively.

4.2.3. Sanger Sequence Analysis

Twenty of the isolates were chosen and send to analysis. Among the isolates; 7 *Actinobacteria* (39%), 6 *Proteobacteria* (%33), *3 Firmicutes* (17%) and 1 *Bacteriodetes* (5,5%) as well as 1 uncultured organism (5,5%) was found, two of the isolates did not give usable results, for this reason number of the samples are accepted as eighteen. Four isolates of the *Proteobacteria* were grouped with the α-, while the remaining two were grouped with the β- and γ-*Proteobacteria*.

Previous studies showed that *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteriodetes* are the dominant phyla in these environments (Gulecal-Pektas and Temel, 2015; Ikner et al., 2007; Ortiz-Ortiz, 2012; Ortiz et al., 2014). Ikner et al. (2007) found *Firmicutes* (66%), *Actinobacteria* (19%) and *Proteobacteria* (15%) from the rock samples in Kartchner Caves on R2A media. Ortiz et al. (2014) identified *Proteobacteria* (52%) and *Actinobacteria* (13%) in Kartchner Caves in another site of the cave. Gulecal-Pektas and Temel (2015) found *Actinobacteria* (46-50%), *Proteobacteria* (22-25%) and *Acidobacteria* phyla (6-7%) in Oylat Cave. On the contrary, the study conducted by Barton et al. (2007) showed dominant *Actinobacteria* phylum with 80% of the total community of bacteria. Even though percentages of the phyla are changing, our results are compatible with the

findings of other researchers and also supports Porca et al. (2012) who suggested there is a core group of microorganisms in caves even though each cave is unique in terms of bacterial diversity. Ortiz-Ortiz, (2012) also stated that hypogenic caves, such as limestone caves, are tend to show variability in terms of found dominant groups which also explains different dominant groups found in our study.

Actinobacteria phylum was found to be consisted of *Micrococcus, Rhodococcus, Microbacterium, Streptomyces, Mycobacterium* and *Nocardia* genus (Table 4.5). Two of the isolates are members of the same genus, *Rhodococcus*, however their morphologies were seen different. *Actinobacteria* are common soil heterotrophs and are isolated by culture dependent methods with vast variety in cave environments (Groth et al. 1999; Saiz-Jimenez, and Groth, 1999). They present diverse physiological and metabolic properties. Extracellular enzyme production and secondary metabolite production are important features of this phylum (Snel et al., 2002). *Actinobacteria* are known to produce 75% of the antibiotic compounds which are the most important secondary metabolite of microorganisms (Zhang and Demain, 2005). *Actinobacteria* are responsible of decomposition of the organic matter, also some strains are able to fix free nitrogen. Nitrogen fixation is thought to have a significant role in caves (Engel, 2015).

Table 4.5. The list of isolates identified as *Actinobacteria*

Sample	Phylum	Order	Family	Genus
1	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Micrococcus*
7	*Actinobacteria*	*Corynebacteriales*	*Nocardiaceae*	*Rhodococcus*
9	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*
15	*Actinobacteria*	*Corynebacteriales*	*Nocardiaceae*	*Nocardia*
18	*Actinobacteria*	*Corynebacteriales*	*Nocardiaceae*	*Rhodococcus*
19	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces*
20	*Actinobacteria*	*Corynebacteriales*	*Mycobacteriaceae*	*Mycobacterium*

Ikner et al. (2007) stated that *Proteobacteria* are more sensitive to pH, temperature, nutrient or water stress. Therefore *Proteobacteria* are uncommon to found in cave environments while *Firmicutes* are known to be highly resistant to stress factors mentioned above hence expected to able to live in such conditions. Nevertheless our findings, as most of the cave researches conducted, are not supporting that statement since the *Proteobacteria* have been shown one of the dominant phyla of cave microbiota.

Also their results suggested that *Proteobacteria* dominate where human contact is high. Gilindire Cave is open for tourism over a decade and high *Proteobacteria* rates may hint human contact but Gulecal-Pektas and Temel (2015) showed high *Actinobacteria* (46-50%) at the entrance of the touristic cave, Oylat.

Proteobacteria that were found in our study were consisted of *Bosea, Azotobacter, Bradyrhizobiaceae, Hydrogenophaga* and *Sinorhizobium* group (Table 4.6). *Proteobacteria* are a major research topic since they show extreme metabolic diversity and constitute bacteria known for medical, industrial and agricultural significance (Madigan, 2012; Marin, 2011). Members of *Bosea, Azotobacter, Bradyrhizobiaceae* and *Sinorhizobium* distinguish by their ability to fix nitrogen (Marcondes de Souza et al., 2014). Most of the *Hydrogenophaga* members are known for their ability to use H_2 and CO_2 as their energy and carbon source respectively.

Table 4.6. The list of isolates identified as *Proteobacteria*

Sample	Phlum	Class	Order	Family	Genus
3	*Proteobacteria*	*α-proteo bacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	*Bosea*
4	*Proteobacteria*	*γ-proteo bacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Azotobacter*
6	*Proteobacteria*	*α-proteo bacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	
8	*Proteobacteria*	*β-proteo bacteria*	*Burkholderiales*	*Comamonadaceae*	*Hydrogenophaga*
17	*Proteobacteria*	*α-proteo bacteria*	*Rhizobiales*	*Rhizobiaceae*	*Sinorhizobium/Ensif er group; Ensifer.*
25	*Proteobacteria*	*α-proteo bacteria*	*Rhizobiales*	*Rhizobiaceae*	*Sinorhizobium/Ensif er group; Ensifer.*

Staphylococcus, Viridibacillus and Bacillus genus are found in Firmicutes phylum. Firmicutes are best known for their fermentative growth and can degrade complex polymers such as cellulose, pectin, xylan, chitin and lignin (Bernardet and Nakagawa, 2006; Vos et al., 2011). The ability to degrade complex compound give the bacteria the role of breaking down macromolecules entering the cave such as fungal matter, dead animal or plants thus, provide other energy and carbon sources for the microbial community of cave (Engel, 2015).

Engel (2015) pointed out Firmicutes and Actinobacteria are in an inverse correlation at the same community. The number Firmicutes are increasing when the number of Actinobacteria decreasing and vice versa. Our results are also present an example to this statement since there is a gap between the percentages of these two phyla.

Table 4.7. The list of isolates identified as Firmicutes

Sample	Phlum	Class	Order	Family	Genus
2	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus
5	Firmicutes	Bacilli	Bacillales	Planococcaceae	Viridibacillus
16	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus

Bacteriodetes are also found in caves by Ortiz-Ortiz (2012), Pektaş-Güleçal (2015), Pektaş-Güleçal and Temel (2015), Ikner et al. (2007) and Rinke et al. (2013).

Pedobacter was found in the Bacteriodetes phylum in Gilindire Cave. Sphingobacteria class is one of the classes of environmental Bacteriodetes (Thomas et al., 2011). Sphingobacteria along with other environmental Bacteriodetes are responsible of degradation of macromolecules such as polysaccharides and proteins (e.g. Algal, plant or animal compounds) (Church, 2008).

Table 4.8. The list of isolates identified as Bacteriodetes

Sample	Phlum	Class	Order	Family	Genus
26	Bacteroidetes	Sphingo bacteria	Sphingobacteriales	Sphingobacteriaceae	Pedobacter

CHAPTER 5

5. CONCLUSION

Gilindire Cave is microbiologically unexplored and unique place since its formation shows three different geological period of earth. Cave studies are increasing around the world with the hope to find novel microorganisms that are not discovered yet. These studies aim not only to find biotechnologically important microorganisms but also to present microbial diversity of the environment.

Present study aimed to show bacterial diversity of Gilindire Cave and their contributions to cave formations as well as to find unknown species. In order to achieve this aim, isolates were obtained by culture dependent methods and identified with culture independent method. Results of this study showed a variety of genus dominated by *Actinobacteria*. Also calcite formation, which is a phenomena suggested to have contribution on cave formations, has shown by the most of the isolates.

This is the first study performed in Gilindire Cave and one of few in Turkey. It is expected that these results will provide some insights about the Gilindire Cave not only about the diversity but also the potential energy dynamics of the cave. Additionally, the findings of soil associated bacteria in the Gilindire Cave give us the insight about the connection between the surface environment and the cave walls.

Many different research area and experiments are left for the future due to lack of time and resources. Future work could include a NGS Sequence Analysis of the samples to reveal whole bacterial community. Also the use of different media could result more diverse and broad community. Changing the pH of the media, temperature and humidity of the environment may differ the results and show new characteristics of the isolates. Additionally, different carbon sources and aromatic compounds might be added to culture media in order to find preferred carbon source and the use of these compounds.

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APPENDIX A

Preparation of the solutions and media

a. **PBS solution:** 9,55 g PBS (Sigma-Aldrich) was dissolved in 1L of distilled water and autoclaved at 121°C for 15 minutes before use.

b. **0,85% NaCl solution:** 8,5 g of NaCl was weighed and dissolved in 1L of distilled water before autoclaved at 121°C for 15 minutes.

c. **Crystal violet:** 2 g of crystal violet was dissolved in 20 ml of 95% ethanol. 0,8 g of ammonia oxalate monohydrate was dissolved in 80 ml of deionized sterile water. Both solution were mixed together and filtered with a filter paper no.1. Stored in amber glass bottle in +4°C.

d. **Lugol's iodine:** 0,825 g of iodine (I2), 1,65 g of potassium iodide (KI) were weighed and dissolved in 250 ml of deionized sterile water. Stored in amber glass bottle in +4°C.

e. **Safranin:** 2,5 g of Safranin O was weighed and dissolved in 10 ml of 95% of ethanol and 90 ml of deionized sterile water was added to the solution. Stored in amber glass bottle in +4°C.

f. **R2A:** 18,12 g of powdered media (Hi-Media) were added into 1 L of deionized water and boiled until solution becomes crystal clear then autoclaved at 121°C for 15 minutes. After cooled down to 55-60°C it was poured into the petri plates.

g. **B-4:** 2,5 g of calcium acetate, 4 g of yeast extract, 10 g of glucose and 18 g of agar were weighed and dissolved in 1 L of deionized water and autoclaved. After cooled down to 55-60°C it was poured into the petri plates.

h. **TSA:** 40 g of powdered media (Sigma-Aldrich) was added into 1 L of deionized water autoclaved. After cooled down to 55-60°C it was poured into the petri plates.

i. **PDA:** 39 g of powdered media in 1 liter of distilled water. Bring to the boil to dissolve completely.

j. **Primer Preparation:** Lyophilized primers were diluted with nuclease free water with the volume of written on the information sheet to make 100 pmol/μl primer. Aliquots were prepared from this stock solution. Each aliquot were added 50 μl of stock primer and 450 μl of water resulting 10 pmol/μl last concentration.

k. **Glycerol Stock:** 80 ml of glycerol and 20 ml of water were mixed. 500 μl of stock solution were mixed with 500 μl of 18-24 hour liquid culture or 500 μl of broth mixed with a single colony (18-24 hour) from the agar plate. Stored in -86°C

l. **Agarose Gel:** 0,25 grams of agarose was weighed and poured into 25 ml of 1X TAE buffer, sealed with a stretch film that put some holes on and heated in the

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microwave at medium heat 2 minutes until it started to boil and dissolve completely. After it was cooled down 2,5 µl SYBR Safe added and mixed slowly with caution and poured into gel tray.

APPENDIX B

Results of gene sequences are given below.

Sample 1.

TGCAGTCGAACGATGAAGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGT
AACACGTGAGTAACCTGCCCCTTAACTCTGGGATAAGCCTGGGAAACTGGGTCTAATA
CCGGATAGGAGCGTCCACCGCATGGTGGGTGTTGGAAAGATTTATCGGTTTTGGATG
GACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGG
TAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGA
CGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAG
CGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGG
CGGTTTGTCGCGTCTGTCGTGAAAGTCCGGGGGCTTAACCCCGGATCTGCGGTGGGT
ACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAAT
GCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGA
CGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATG
CCGTAAACGTTGGGCACTAGGTGTGGGGACCATTCCACGGTTTCCGCGCCGCAGCT
AACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAA
GAACCTTACCAAGGCTTGACATGTTCTCGATCGCCGTAGAGATACGGTTTCCCCTTT
GGGGCGGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTMRTGGTGG
GGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGAGGACGACGTCAAA
TCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGG
TTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGG
TCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTA
ACACCCGAAGCCGGTGGCCTAAC

Sample 2

CAAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTG
AGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTA
ATACCGGATAACATATTGAACCGCATGGTTCAATAGTGAAAGGCGGCTTTGCTGTCA
CTTATAGATGGATCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGC

AACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGA
CGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATCAG
GGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTGATCAGAAAGCC
ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG
AATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCAC
GGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAG
TGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCG
AAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGT
TTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGAC
CGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTC
TAGAGATAGAGTYTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTT
AGTTGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTAC
AATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCAAGCAAATCCCATAAAGTTGT
TCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATC
GTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCAC
ACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCA

Sample 3

CGAACGGGCACTTCGGTGCTAGTGGCAGACGGGTGAGTAACACGTGGGAACGTACC
TTTCGGTTCGGAATAATTCAGGGAAACTTGGACTAATACCGGATAAGCCCTTCGGGG
GAAAGATTTATCGCCGATAGATCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAAT
GGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGG
ACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG
GGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAA
GCTCTTTTGTCCGGGAAGATAATGACTGTACCGGAAGAATAAGCCCCGGCTAACTTC
GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGC
GTAAAGGGCGCGTAGGCGGACTCTTAAGTCGGGGGTGAAAGCCCAGGGCTCAACC
CTGGAATTGCCTTCGATACTGAGAGTCTTGAGTTCGGAAGAGGTTGGTGGAACTGCG
40

AGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGGCGAAGGCGGCCA
ACTGGTCCGATACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGCCGTTGGGGTGCATGCACCT
CAGTGGCGCAGCTAACGCTTTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTA
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGCAGAACCTTACCAGCTTTTGACATGTCCGGTTTGATCGACAGAGAT
GTCTTTCTTCAGTTCGGCTGGCCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCCTAGTTG
CCATCATTCAGTTGGGAACTCTAGGGGGACTGCCGGTGATAAGCCGCGAGGAAGGT
GGGGATGACGTCAAGTCCTCATGGCCCTTACAGGCTGGGCTACACACGTGCTACAA
TGGCGGTGACAATGGGCAGCGAAAGAGCGATCTGGAGCTAATCCCAAAAAGCCGTC
TCAGTTCAGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTAATCG
TGGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC
ACCATGGGAGTTGGGTTTACCCGAAGGCGTCGCGCTAACCG

Sample 4

GGTGCTTGCACCCTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCC
GATAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGA
GAAAGTGGGGGCTCTTCGGACCTCACGCTATCGGATGAGCCTAGGTCGGATTAGCT
AGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATG
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TCTTCGGATTGTAAAGCACTTTAAGTCGGGAGGAAGGGCTGTAAGCGAATACCTTGC
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AATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTG
GTTTGGTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACT
GCCTGACTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCG
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TGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGTCGACTAGCCGTTGGGCTCCTTGAGAGCTTAGTGGCGCAGCTAACGCA
TTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG
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CCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGMACTCTA
AGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG

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CCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAG
TCGCGAGGCGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAA
CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAAT
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AAGTAGCTAGTCT

Sample 5

CGAGCGAATGATGAAGAAGCTTGCTTCTTCTGATTTAGCGGCGGACGGGTGAGTAAC
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TCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCG
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Sample 6

CAGTCGAACGGGCACTTCGGTGCTAGTGGCAGACGGGTGAGTAACACGTGGGAAC
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GTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACA
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GGGCGTAAAGGGCGCGTAGGCGGACTCTTAAGTCGGGGGTGAAAGCCCAGGGCTC
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TCGTGGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT
CACACCATGGGAGTTGGGTTTACCCGAAGGCGTCGCGC

Sample 7

GCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTG
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AGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGA
GGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGG
ATGAAGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGTGACTGTA
CCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT
GCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTC
GTGTGTGAAATCCCGCAGCTCAACTGCGGGCTTGCAGGCGATACGGGCAAACTTGA
GTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGG
AGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAACGA

AAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGG
GCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCTAACGCATTAAGCG
CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGGCC
CGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTG
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GGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTGTCTTATGTTGCCAGCACGTAATGGTGGGGACTCGTAAGAGAC
TGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCCTTAT
GTCCAGGGCTTCACACATGCTACAATGGTCGGTACAGAGGGCTGCGATACCGTGAG
GTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACC
CCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCC
GGTGGC

Sample 8

GCAGTCGAACGGTAACAGGCCGCAAGGTGCTGACGAGTGGCGAACGGGTGAGTAA
TGTATCGGAACGTGCCCAGTCGTGGGGGATAACGCAGCGAAAGCTGCGCTAATACC
GCATACGATCTATGGATGAAAGCGGGGGGACCGTAAGGCCTCGCGCGATTGGAGCG
GCCGATATCAGATTAGGTAGTTGGTGGGGTAAAGGCTCACCAAGCCAACGATCTGTA
GCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGC
CGCGTGCAGGAAGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAACGG
TCCTGGTTAATACCTGGGGCTAATGACGGTACCGTAAGAATAAGCACCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGGCGTGAAATCCCCGGGCTTAACCT
GGGAATGGCGCTTGTGACTGCAAAGCTGGAGTGCGGCAGAGGGGGGATGGAATTCC
GCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATC
CCCTGGGCCTGCACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCCTAAACGATGTCAACTGGTTGTTGGGTCTCTTCTGACT
CAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTG
AAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGTTTAATTCG
ATGCAACGCGAAAAACCTTACCCACCTTTGACATGTACGGAATTTGCCAGAGATGGC
TTAGTGCTCGAAAGAGAGCCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACG
AAAGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT
CAAGTCCTCATGGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCCGGTACAA

AGGGTCGCAAACCCGCGAGGGGGAGCTAATCCATCAAAGCCGGTCGTAGTCCGGAT
CGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGCAT
GTCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGC
GGGTCTCGCCAGAAGTAGTTAGCCTAACCGC

Sample 9

GAAGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGCAAC
CTGCCCCTGACTCTGGGATAAGCGCTGGAAACGGCGTCTAATACTGGATACGAACAA
GAATCGCATGGTTACTTGTTGGAAAGATTTTTTGGTTGGGGATGGGCTCGCGGCCTA
TCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAG
AGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGAGG
GATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAGAGTGACGG
TACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCG
TCTGCTGTGAAATCCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTA
GAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCA
GGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGC
GAAAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTT
GGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGTGACGCAGCTAACGCATTAAGT
TCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGACC
CGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAA
GGCTTGACATATACGAGAACGGGCCAGAAATGGTCAACTCTTTGGACACTCGTAAAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATAC
TGCCGGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTAT
GTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGCAGCGATACCGTGAG
GTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGACC
TCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTC
CCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCTGAAGCCG
GTGGCCTAAC

Sample 15

AMCATGCAAGTCGAGCGGTAAGGCCCTTCGGGGTACACGAGCGGCGAACGGGTGA
GTAACACGTGGGTGATCTGCCTCGTACTTCGGGATAAGCCTGGGAAACTGGGTCTAA
TACCGGATATGACCTTCGGATGCATGTCTGAGGGTGGAAAGATTTATCGGTACGAGA

45

TGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACG
GGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGC
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCGACAGGGACGA
AGCGCAAGTGACGGTACCTGTAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTTGTA
GGCGGTTCGTCGCGTCGTTCGTGAAAACTTGGGGCTCAACCCCAAGCTTGCGGGCG
ATACGGGCGGACTAGAGTACTTCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAA
ATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGAAGTAACT
GACGCTGAGAAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGGTGGGTACTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAG
CTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCG
AAGAACCTTACCTGGGTTTGACATACACCGGAAACCTGCAGAGATGTAGGCCCCCTT
GTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATCTTATGTTGCCAGCGCGTAATGGCGGG
GACTCGTGAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGT
CATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCGGTACAGAGGGC
TGCGATACCGTGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGGG
TCTGCAACTCGACCCCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGCTG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGT
AACACCCGAAGCCGGTGGCCTAACCCTTGTGGAGGGAGCCGT

Sample 16

CATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGT
GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGC
TAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTA
CCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCA
AGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG
TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTG
TTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG
TCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTCCTTAAGTCTGATGTGAAAG
CCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGA
GGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA

GTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGG
AGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGT
TAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG
AGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTC
TGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA
CACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCC
CACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAA
TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTA
TGGAGCCAGCC

Sample 17

CATGCAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGTAACGCGTGGGA
ATCTRCCCTTTTCTACGGAATAACGCAGGGAAACTTGTGCTAATACCGTATGAGCCCT
TCGGGGGAAAGATTTATCGGGAAAGGATGAGCCCGCGTTGGATTAGCTAGTTGGTG
GGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCA
CATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTG
GACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGG
GTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCG
GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAAT
TACTGGGCGTAAAGCGCACGTAGGCGGACATTTAAGTCAGGGGTGAAATCCCAGAG
CTCAACTCTGGAACTGCCTTTGATACTGGGTGTCTAGAGTATGGAAGAGGTGAGTGG
AATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGG
CGGCTCACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTCGGGCAGTTTA
CTGTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAA
GATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATCCCGATCGCGGATTACG
GAGACGTTTTCCTTCAGTTCGGCTGGATCGGAGACAGGTGCTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTA
GTTGCCAGCATTTAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGA
AGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCT

ACAATGGTGGTGACAGTGGGCAGCGAGACCGCGAGGTCGAGCTAATCTCCAAAAGC
CATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTA
ATCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCATGGGAGTTGGTTCTACCCGAAGGTAGTGCGCTAACCGCAAGGAGGCAG
CT

Sample 18

TCGAGCGGTAAGGCCCTTCGGGGTACACGAGCGGCGAACGGGTGAGTAACACGTG
GGTGATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATAT
GACCTTCGGCTGCATGGCCGTTGGTGGAAAGGTTTACTGGTGCAGGATGGGCCCGC
GGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGA
CCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGC
GTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGAAAG
TGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTT
GTCGCGTCGTCTGTGAAAACTCGAGGCTCAACCTCGAGCTTGCAGGCGATACGGGC
AGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAG
ATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGA
GGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
MCGGTGGGCGYTAGGTGTGGGTTTCYTTCCACGGGATCCGTGCCGTAGYTAACSCA
TTAAGCGCCCCSCCTGGGGAGTACGGCCGCAAGGYTAAAAYTCAAAGGAATTGACG
GGGGCCCGCMCAAGCGGCGGASCATGTGGATTAWTTSGATGCAACGSGAAGAMCC
TTMCCTGGKTTTGACATATMCCGGAAAGCKGCAGARAKGKGSCCCCCCTTGTGGTC
GGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTCTTATGTTGCCAGCACGTAATGGTGGGGACTCG
TAAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATG
CCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCGGTACAGAGGGCTGCGATA
CCGTGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAA
CTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCYGTCACGTCATGAAAGTCGGTAACACCC
GAAGCCGGTGGCCTAACCCCTCGTGGGAGGGAGCCGT

Sample 19

GCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCT
TCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACCGGCCTCCG

48

CATGGGGGGCCGGTTGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGC
TTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGG
CGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATG
ACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACC
TGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCG
GGTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATCCGATACGGGCAGGCTAGA
GTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGG
AGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGG
GAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTC
CCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAG
GCTTGACATATACCGGAAAGCATTAGAGATAGTGCCCCCCTTGTGGTCGGTATACAG
GTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCAC
AGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATG
CCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATG
CCGCGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAA
CTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCC
GAAGCCGGTGGCCCAACCC

Sample 20

AGTCGAACGGACCCTTTGGGGGTTAGTGGCGAACGGGTGAGTAACACGTGGGTGATC
TGCCCTGCACTTTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATAGGACTACG
CACTGCATGGTGTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCGCGGCCTAT
CAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAG
AGGGTGACCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGG
GATGACGGCCTTCGGGTTGTAAACCTCTTTCGCCAGGGACGAAGCGCAAGTGACGG
TACCTGGAGAAGAAGGACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GTCCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCG
TTGTTCGTGAAAACTCACAGCTTAACTGTGGGCGTGCGGGCGATACGGGCAGACTG
GAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCA

49

GGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGC
GAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGT
GGGTACTAGGTGTGGGTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGT
ACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCT
GGGTTTGACATGCACAGGACGCCGGCAGAGATGTCGGTTCCCTTGTGGCCTGTGTG
CAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCTCATGTTGCCAGCACGTTATGGTGGGGACTCGTGAGA
GACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCT
TATGTCCAGGGCTTCACACATGCTACAATGGCCGGTACAAAGGGCTGCGATGCCGT
GAGGTGGAGCGAATCCTTTCAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTC
GACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGA
AGCCGGTGGCCTAACCCTTGTGGAGGGAGCCGT

Sample 25

GCAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGTAACGCGTGGGAATC
TRCCCTTTTCTACGGAATAACGCAGGGAAACTTGTGCTAATACCGTATGAGCCCTTC
GGGGGAAAGATTTATCGGGAAAGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGG
GTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACA
TTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTT
GTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCT
AACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTAC
TGGGCGTAAAGCGCACGTAGGCGGACATTTAAGTCAGGGGTGAAATCCCAGAGCTC
AACTCTGGAACTGCCTTTGATACTGGGTGTCTAGAGTATGGAAGAGGTGAGTGGAAT
TCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCG
GCTCACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTCGGGCAGTTTACT
GTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGA
TTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATCCCGATCGCGGATTACGGA
GACGTTTTCCTTCAGTTCGGCTGGATCGGAGACAGGTGCTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGT
TGCCAGCATTTAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAG
GTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTAC

50

AATGGTGGTGACAGTGGGCAGCGAGACCGCGAGGTCGAGCTAATCTCCAAAAGCCA
TCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAAT
CGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCATGGGAGTTGGTTCTACCCGAAGGTAGTGCGCTAACCGCAAGGAGGCAGCT
A

Sample 26

ACATGCAAGTCGAACGATACCGGAGAGCTTGCTTTCCGGGAAAGTGGCGCACGGGT
GCGTAACGCGTATGCAACCTACCTTAATCAGGGGGGATAGCCTCTCGAAAGAGAGATT
AACACCGCATAACATCATTTTACGGCATCGTCGAATGATCAAATATTTATAGGATTAA
GATGGGCATGCGTGTCATTAGTTAGTTGGTGAGGTAATGGCTCACCAAGACGATGAT
GACTAGGGGATCTGAGAGGATGACCCCCCACACTGGTACTGAGACACGGACCAGAC
TCCTACGGGAGGCAGCAGTAAGGAATATTGGTCAATGGAGGCAACTCTGAACCAGC
CATGCCGCGTGCAGGAAGACGGCCCTATGGGTTGTAAACTGCTTTTATACGGGAATA
AACCTCTCTACGTGTAGGGAGTTGAATGTACTGTAAGAATAAGGATCGGCTAACTCC
GTGCCAGCAGCCGCGGTAATACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTT
AAAGGGTGCGTAGGCGGCCTGTTAAGTCAGGGGTGAAAGACGGTGGCTCAACCATC
GCAGTGCCCTTGATACTGATGGGCTTGATTACACTTGAGGTAGGCGGAATGTGACAA
GTAGCGGTGAAATGCATAGATATGTCACAGAACACCAATTGCGAAGGCAGCTTACTA
AGGTGTCAATGACGCTGAGGCACGAAAGCGTGGGGATCAAACAGGATTAGATACCC
TGGTAGTCCACGCCCTAAACGATGAATACTCGATGTTAGCGATATACAGTTAGCGTC
AAAGCGAAAGCGTTAAGTATTCCACCTGGGGAGTACGCCCGCAAGGGTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGAGGAGCATGTGGTTTAATTCGATGATA
CGCGAGGAACCTTACCCGGGCTTGAAAGTTACTGAATAACTCAGAGATGAGTTAGTC
CGCAAGGACAGGAAACTAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATGTTTAGTTGCCAGCATGTGATGG
TGGGGACTCTAAACAGACTGCCTGTGCAAACAGAGAGGAAGGAGGGGACGACGTCA
AGTCATCATGGCCCTTACGTCCGGGGCTACACACGTGCTACAATGGGCAGTACAGA
GGGCAGCTACCTGGTAACAGGATGCCAATCTCAAAAAGCTGTTCACAGTTCGGATAG
AGGTCTGCAACTCGACCTCTTGAAGTTGGATTCGCTAGTAATCGCAGATCAGCAATG
CTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCCATGGAAGTT
GGGGGTACCTGAAGTACGTAACCGCAAGGAGCG

Sample 27

CGGGCTCTTCGGAGCTAGTGGCGGACGGGTGAGTAACACGTGGGAACGTGCCTTTA
GGTTCGGAATAAGCCCGGGAAACTGGGTCTAATACCGGATGTGCCCTTCGGGGGAA

51

AGATTTATCGCCTTTAGAGCGGCCCGCGTCTGATTAGCTAGTTGGTGGTGTAATGGA
CCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACT
GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGG
CGAAAGCCTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATT
CTTTCACCGGGGACGATAATGACGGTACCCGGAGAAGAAGCCCCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTACTGGGCGTA
AAGGGAGCGTAGGCGGACATTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCTCGG
AACTGCCTTTGATACTGGGTGTCTTGAGTGTGATAGAGGTATGTGGAACTCCGAGTG
TAGAGGTGAAATTCGTAGATATTCGGAAGAACACCAGTGGCGAAGGCGACATACTGG
ATCATTACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGATTGCTAGTTGTCGGGGTGTTTACACCTCGGTGA
CGCAGCTAACGCATTAAGCAATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA
CGCGCAGAACCTTACCACCTTTTGACATGCCTGGACCGCGTGAGAGATCACGCTTTC
CCTTCGGGGACTAGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCATTAGTTGCCATCATTCAGT
TGGGAACTCTAATGGGACTGCCGGTGCTAAGCCGGAGGAAGGTGGGGATGACGTCA
AGTCCTCATGGCCCTTACAGGGTGGGCTACACACGTGCTACAATGGCGACTACAGA
GGGTTAATCCTTAAAAGTCGTCTCAGTTCGGATTGTCCTCTGCAACTCGAGGGCATG
AAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCTACCCGAAGGCGCTGCG
CTGACCGCA

VITA

Yağmur ATAKAV was born in Izmir, Turkey. She received her Bachelor of Science degree from the Bioengineering Department at Fırat University in 2014. She has been working as a Research Assistant in the Bioengineering Department at Adana Science and Technology University since 2015. Her research interests are microbiology and enzyme technologies.