ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATION OF MICROBIAL DIVERSITY OF LAKE ACIGOL, A HYPERSALINE LAKE IN SOUTHERN TURKEY, AND THEIR INFLUENCE ON BIOMINERALIZATION IN THE LAKE

M.Sc. THESIS

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JANUARY 2012

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ACIGÖL'DEKİ MİKROBİYAL ÇEŞİTLİLİĞİN VE BU TÜRLERİN BİYOMİNERALİZASYON ÜZERİNDEKİ ETKİLERİNİN BELİRLENMESİ

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OCAK 2012

To my father Hasan MENEKSE...

FOREWORD

I would like to express my appreciation and thanks to my advisors, Assoc. Prof. Dr. Nevin GUL KARAGULER and Assoc. Prof. Dr. Nugül ÇELIK BALCI for giving me chance to involve in this study, for their continuos support and encouragement.

I would specially thank to Assis. Prof. Şeref Sönmez for XRD analyses, his encouragement and continous support during my studies.

I would like to thank my friends Havva Esra BIYIK, Nurcan VARDAR and Gizem Yılmaz HABIB for their endless support during laboratory works and also for making my life more enjoyable and livable. I would also thank to the each member of Protein Engineering Laboratory, MOBGAM, ITU.

Finally, I would like to express my love and thankfullnes especially to my father Hasan MENEKSE and my mother Nefise MENEKSE and also I would like thank my sisters and brother in laws Ümmügülsüm MENEKSE, Huriye TASDELEN, Eyüp TASDELEN; Hatice KORKMAZ, İsmail KORKMAZ for their endless moral support and encouragement throughout my life.

December 2011

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ABBREVIATIONS

DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide triphosphate
LB : Luria Bertani Broth
PCR : Polymerase Chain Reaction
TAE : Tris-Acetic acid-EDTA
X-gal : 5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside
UARR : Universal Amplified Ribosomal Region
MHBM : Modified Halophilic Bacterial Medium
SMHBM : Solid Modified Holophilic Bacterial Medium
XRD : X-ray Diffractions
SEM : Scanning Electrone Microscope

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SUMMARY

Investigations of extreme environments are important for the study of evolution relationships, discovery of new species and various ecological relations among organisms which survive extreme environments. New metabolites and metabolic pathways of organisms, discovery of new antibiotics can determine from extreme environments. Extreme habitats have highly specialized organisms which are called extremophiles. These organisms can adapt different stress conditions such as high temprature, pressure, alkalinity, salinity and low pH, temperature. Lake Acigol which is located between Afyon, Denizli and Burdur city boundaries, in Aegean region, Turkey, is an good example for extreme environments with its high saline (about 200g/L) concentration. It is very important for Na_2SO_4 production which comprises 85 % of Turkey production. Also, analyses indicate that mineralization is important point in Lake Acigol but biomineralization mechanism and relation among microorganisms have not found out in the lake yet. Therefore, study of Lake Acigol is very important for to be discovered microbial population and their potential applications of industry and also relation between microflora and biomineralization. The main purpose of study, investigate the microbial flora of Lake Acigol and microbial effects on biomineralization process. For our aim, sediment samples were collected from different part of the lake and they used for biomineralization experiments and determination of microflora. At first, sediments which were 1AGN and 3AGN were enriched in 6% and 3% NaCl containing Nutrient Broth (NB) to be obtaine high amount of halophiles. These enrichment cultures and sediment samples microflora were determined by 16S rDNA PCR method. Results indicated that flora was dominated by halophiles such as *Halomonas*, *Idiomarina*, *Virgibacillus* and Uncultured bacterium. Besides, biomineralization experiments were carried out with Modified Halophilic Bacterial Medium (MHBM) which was designed according to Lake Acigol cheamical conditions and used in biomineralization experiments. MHBM were prepared with four different Na_2SO4 concentrations (0, 14, 28, 56 mM) and inoculated by % 6 NaCl containing NB, incubated at 30°C and +4°C. Control experiments were carried out with uninoculated MHBM and inoculated by autoclaved enrichment culture. Any mineral formation was not determined in control experiments but mineral formation was observed in biomineralization experiments. Biominerals were characterized by XRD and SEM analysis.As a conclusion our studies show that Ca^{2+} and Mg^{2+} concentrations, metabolizable organic substrate and the type of bacteria, are all influential factors in the biomineralization process.

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ÖZET

Ekstrem çevrelerin araştırılması, evrimsel sürece ışık tutması, yeni türlerin ve türler arasındaki ekolojik ilişkinin keşfi açısından oldukça önemlidir. Ekstrem çevrelerden yeni metabolitler, yeni metabolik yol izleri, yeni antibiyotikler keşfedilebilir. Ekstrem çevrelerde, bu çevre koşullarına adapte olarak özelleşmiş türler bulunmaktadır, bu türler ekstremofiller olarak adlandırılmaktadır. Ekstremofiller; çok yüksek; sıcaklık, basınç, pH, tuz ve çok düşük; pH, sıcaklık gibi farklı stress koşullarına adapte olmuşlardır. Türkiye sahip olduğu eşsiz coğrafi özelliklerin yanı sıra ekestrem çevreleride barındırmaktadır. Ege Bölgesi'ndeki Afyon, Denizli ve Burdur il sınırları arasında yer alan yaklaşık 200 g/L tuz konsantrasyonuyla, Acıgöl Türkiye'deki ekstrem çevrelere örnek olarak verilebilir. Türkiye'nin % 85 $NaSO_4$ üretimi bu gölden karşılanmaktadır. Na_2SO_4 üretimi endüstride ham madde olarak kullanıldığından Acıgöl endüstri açısından da oldukça önemli bir göldür. Bunun yanında Acıgöl'ün dikkat çeken özelliklerinden bir diğeri de içerdiği minerallerdir. Bu minerallerin oluşum şekilleri, Acıgöl'ün bakteriyel topluluğu, mineraller ve mikroorganizmalar arasındaki ilişki hakkında literatürde bir bilgi bulunmamaktadır. Bu yüzden Acıgöl'ün çalışılıyor olması, mikrobiyal çeşitliliğin belirlenmesi, bunların mineralizasyonla ilişkilendirilmesi, ayrıca endüstride kullanılabilirliğinin ortaya çıkması açısından oldukça önemlidir.

Çalışmanın amacı, Acıgöl'ün bakteriyel topluluğunun belirlenmesi ve bu mikroorganizmaların biomineralizasyon sürecine etkisini ortaya koymaktır. Bu amaç doğrultusunda, Acıgöl'ün farklı bölgelerinden sediment örnekleri toplanmıştır. Bu sediment örnekleri, mikrobiyal çeşitliliğin belirlenmesi ve biyomineralizasyon deneylerinde kullanılmıştır. Alınan örneklerden 1AGN ve 3AGN örnekleri seçilerek %3 ve %6 NaCl'ür içeren sıvı Nutrient besiyerinde (NB) zenginleştirilme yapılmıştır. Bu zenginleştirilmiş kültürlerden ve sediment örneklerinden genomik DNA izolasyonu yapılmış ve bakteriyel topluluğun belirlenmesi amacıyla 16S rDNA polimeraz zincir reaksiyonu yönteminden yararlanılmıştır. Elde edilen genomik DNA'lardan korunmuş 16S rDNA bölgeri pA-F(5'-AGAGTTTGATCCTGGCTCAG-3') ve pH-R(5'AAGGAGGTGATCCAGCCGCA-3') primerleri kullanılarak polimeraz zincir reaksiyonuyla (PZR) çoğaltılmıştır. Elde edilen PZR ürünlerinin istenilen boyutta olup olmadığını anlamak için PZR ürünleri agaroz jel elektroforezi yapılmıştır. PZR ürünlerinin istenilen boyutta olduğu doğrulandıktan sonra, reaksiyondan geriye kalan enzim, primer, dNTP gibi fazlalıklardan kurtulmak için PZR ürünleri saflaştırılmıştır. Saflaştırılan PZR ürünleri klonlama ve transformasyon işlemleri için kullanılmıştır. Bu işlemler için ticari olarak alınan TOPO TA® Cloning Kit kullanılmıştır. PZR ürünü kitte belirtilen

şekilde vektör ile karıştırılarak klonlama reaksiyonu hazırlanmış ve bu reaksiyondan 2 µl alınarak One Shot Electrocompetent *E.coli* hücrelerinin bulunduğu tüpe aktarılmıştır ve bu karışımdaki vektörler elektroporatör yardımıyla One Shot Electrocompetent *E.coli* hücrelerine aktarılmıştır. Elektraporatör aracılığıyla transforme edilen vektörün içindeki PZR ürünleri Mavi/Beyaz Tarama Yöntemi (Blue/White Screening) ile taranmıştır. Bunun için X-gal ve Ampisilin antibiyotiği içeren LB agar üzerine transforme olmuş One Shot Electrocompetent *E.coli* hücreleri ekilmiştir. Vektörün içinde bulunan β-galaktosidaz kodlayan gen (*lacZ*), vektöre bir PZR ürünü girdiyse aktivitesini kaybeder ve bu vector+PZR ürününü taşıyan hücreler LB agar besiyerinde X-gal bileşiğini parçalayamadıkları için beyaz renkli koloni oluştururlar. Ancak herhangi bir PZR ürünü vektöre giremediyse *lacZ* geni activitesini kaybetmez ve bu vektörü taşıyan hücreler X-gal bileşiğini parçalayarak, mavi renkli koloniler oluştururlar. Bu şekilde yapılan tarama ile beyaz renkli koloniler seçilerek plasmid izolasyonu yapılmış ve her bir PZR ürünü tek tek elde edilebilmiştir. İzole edilen plasmidlerden M13-F(5'-TGTAAAACGACGGCCAGT-3') and M13-R(5'-GTTTTCCCAGTCACGAC-3') primerleriyle sekans PZR kurulmuş ve ardından saflaştırma işlemleri yapılarak ABI 3100 Avant otomatik sekanlama cihazıyla sekanlanmışlardır. Elde edilen nükleotid dizileri, NCBI veri tabanı kullanılarak, veri tabanındaki diğer dizilerle karşılaştırılarak en yüksek oranda benzerlik gösterdiği diziyle aynı tür olduğu kabul edilmiştir. Yapılan analizler sonucunda mikrobiyal çeşitlilik şu şekilde belirlenmiştir; %33,3 Gammaproteobacteria, %33,3 Firmucutes, %25 Uncultured bacterium %8,3 Bacteriodetes. *Halomonas, Idiomarina, Virgibacillus* gibi türlerin yanı sıra daha önce kültüre edilmemiş halofilik microorganizmaların göl populasyonunda bulunması Acıgöl'ün keşfinin önemini göstermektedir. Bu bilgiler doğrultusunda Acıgöl, içinde keşfedilmeyi bekleyen yeni türleri ve ekstrem koşullarda aktivite gösterebilecek metabolitleri barındırmaktadır diye bir çıkarım yapılabilir.

Tür analizlerinin yanı sıra, Acıgöl de gerçekleşen mineralizasyon olayına ışık tutması açısından biyomineralizasyon deneyleri tasarlanmıştır. Biyomineralizasyon deneyleri, Acıgöl'ün kimyasal koşullarına göre halofilik bakteriler için modifiye edilmiş (MHBM) besiyerinde (%1 yeast extract, %0,1 glikoz, %0,5 peptone, % 4,5 NaCl and 84 mM Mg^{+2}, 11 mM Ca^{+2} g/L) gerçekleştirilmiştir. Halofiller için modifiye edilmiş besiyeri % 4,5 NaCl ve mevsimsel Na_2SO_4 değişimini temsil edecek şekilde dört farklı Na_2SO_4 (0, 14, 28, 56 mM) konsantrasyonuyla hazırlanmıştır. Bu şekilde tasarlanan deney setleri % 6 NaCl içeren zenginleştirilmiş kültürlerle (1AGN ve 3AGN) inokule edilmiş ve 30°C, 180 rpm çalkalamalı inkübatörde inkübe edilmişlerdir. Aynı deney düzeneği agarla katılaştırılmış besiyerlerinde de gerçekleştirilmiştir. Biyomineralizasyon deneylerine paralel olarak kurulan kontrol deneyleri ise iki gruptan oluşmaktadır. Birinci gurupta Halofiller için modifiye edilmiş besiyeri, bakteri inokulasyonu yapılmadan inkübe edilirken, ikinci grupta ise Halofiller için modifiye edilmiş besiyeri, otoklavlanmış zenginleştirme kültürüyle inokulasyonu yapılarak inkübe edilmiştir. Her bir deney setinden periyodik olarak yedi günde bir 10 ml kültür örneği alınarak pH ve Ca^{+2}, Mg^{+2} iyon değişimleri belirlenmiştir. Ayrıca ayrılan kültür örneğinden inkübasyon süresinde oluşmuş olan biyomineraller ayrılarak, yıkanmış ve XRD ve SEM analizleri için hazırlanmıştır. pH ölçümleri sonucunda inkübasyondaki kültürün pH'nın periyodik olarak artarak pH 9,0'da sabitlendiği gözlenmiştir. pH'ın yükselmesi şu şekilde açıklanabilir; biyomineralizasyon deneyleri için kullanılan besiyerinin içeriğindeki pepton, yeast ekstrakt gibi organik maddeler bakteriler tarafından metabolize

edildiğinde NH^{+4} and PO_4^{-3} gibi bileşikler meydana gelir, bu bileşikler pH'ın yükselmesine sebep olurken aynı zamanda biyomineralizasyon oluşumu için ortam hazırlamaktadır. pH ölçümlerinin yanısıra yapılan Ca^{+2}, Mg^{+2} iyon konsantrasyonları ölçümleri Ca^{+2}, Mg^{+2} iyonlarının zamanla azaldığını göstermektedir. Bu değişim ise şu şekilde açıklanabilir; Bakterilerin hücre duvarlarını oluşturan peptidoglukan N-asatil glukozamin ve N-asatil müramik asit gibi negatif yüklü bileşiklerden oluşur ve bu bileşikler bakteri yüzeyinin negatif yükle yüklenmesine neden olur. Bakterinin içinde bulunduğu besiyerindeki pozitif yüklü iyonlar (Ca^{+2}, Mg^{+2} iyonları gibi) negatif yüklü bakteri yüzeyiyle elektrostatik etkileşime girerler ve bakteri üzerinde veya çevresinde birikmeye başlarlar. Bakteri bir çekirdek gibi davranarak mineral oluşumunu başlatmaktadır. Bu şekilde gerçekleşen mineralizasyon mekenizması biyolojik olarak uyarılmış mineralizasyon (Biologically induced mineralization) olarak adlandırılmıştır. Bu olayın bir sonucu olarak zamanla Ca^{+2}, Mg^{+2} iyonlarının konsantrasyonlarında düşüş gözlenmesi beklenen bir sonuçtur. Oluşan minerallerin karakterizasyonu XRD ve SEM analizleriyle gerçekleştirilmiştir ve analizlere göre dört farklı konsantrasyonda iki farklı zenginleştirilmiş kültürle inokule edilerek gerçekleştirilen biyomineralizasyon deneyleri sonunda mineraller şu şekilde karakterize edilmiştir; **0mM Na_2SO_4 1AGN;** Monohyrocalcite [$CaCO_3(H_2O)$], Dypingite [$Mg_5(CO_3)(OH)_2(H_2O)_5$]; **14mM Na_2SO_4 1AGN**; Monohyrocalcite, Dypingite, Struvite [$Mg_5(CO_3)(OH)_2(H2O)_8$]; **28mM Na_2SO_4 1AGN;** Struvite, Monohyrocalcite, Dypingite, Magnesiumsulfitehyrate [$MgSO_3(H_2O)_6$]; **56mM Na_2SO_4 1AGN;** Monohyrocalcite, Struvite, Dypingite; **0mM Na_2SO_4 3AGN;** Monohyrocalcite, Struvite, Dypingite; **14mM Na_2SO_4 3AGN;** Monohyrocalcite, Struvite, Hyromagnesite [$Mg_5(CO_3)(OH)_2(H_2O)_4$], Calciumacetate [$C_4H_6CaO_4$]; **28mM Na_2SO_4 3AGN;** Struvite, Monohyrocalcite, Dypingite; **56mM Na_2SO_4 3AGN;** Monohyrocalcite, Struvite, Dypingite. Diğer bir biyomineralizasyon deney grubu olan katı besiyerinde elde edilen minerallerin sıvı besiyerindeki minerallerden farklılık gösterdiği belirlenmiştir. Katı besiyerinde oluşan minerallerin Mg'ca zengin mineraller olduğu göze çarpmaktadır. Katı besiyerlerinde oluşan mineraller ise şu şekildedir; **0mM Na_2SO_4 1AGN;** Magnesium adipate glysinate ($C_8H_{13}MgNO_6$), Hyromagnesite; **14mM Na_2SO_4 1AGN**; Magnesium adipate glysinate, Dypingite; **28mM Na_2SO_4 1AGN;** Magnesium adipate glysinate, Hyromagnesite, Dypingite; **56mM Na_2SO_4 1AGN;** Magnesium glutarate glysinate ($C_7H_{11}MgNO_6$), Dypingite; **0mM Na_2SO_4 3AGN;** Magnesium adipate glysinate, Calciumchloride ($CaCl_2$), Calcium phosphate hydrate ($CaP_2O_712H_2O$); **14mM Na_2SO_4 3AGN;** Magnesium adipate glysinate, Hyromagnesite, Dypingite; **28mM Na_2SO_4 3AGN;** Magnesium adipate glysinate, Hyromagnesite; **56mM Na_2SO_4 1AGN;** Magnesium adipate glysinate, Hyromagnesite. Bütün bu verilerin yanı sıra kontrol gruplarının pH ve Ca^{+2}, Mg^{+2} iyon konsantrasyonlarında kayda değer bir değişiklik olmayıp bunun yanı sıra mineral oluşumları gözlenmemiştir. Bu da gösteriyor ki mineral oluşum mekanizmasında bakteriyel aktivite en önemli rolü üstlenmektedir.

Biyomineral oluşumunda zamanın ve farklı sıcaklıkların etkilerini gözlemleyebilmek için '14mM Na_2SO_4 3AGN' deney parametresi seçilmiştir. % 6 NaCl içeren NB'de zenginleştirilmiş 3AGN kültürü MHBM'a inoküle edilerek +4°C ve 30°C'de inkübe edilmişlerdir. Periyodik olarak pH'ları ölçülerek Ca^{+2}, Mg^{+2} iyon konsantrasyonları belirlenmiştir. +4°C denemelerinde pH değişimi ve Ca^{+2}, Mg^{+2} iyon konsantrasyonundaki değişim 30°C'de denemelerine göre daha yavaştır, bunun sonucu olarak da üretilen mineral miktarı daha düşüktür. Yapılan XRD analizlerine

göre ise uzun inkübasyon süresi ve farklı sıcaklık, oluşan minerallerin cinsi üzerinde bir etkiye sahip değildir. Zamana dayalı deneyler devam etmektedir.

Sonuç olarak yapılan analizler gösteriyor ki; Ca^{+2}, Mg^{+2} konsantrasyonu, besiyerinde bulunan, bakteriler tarafından metabolize edilen organik bileşikler ve gölün mikrobiyal topluluğunda yer alan microorganizmalar biyomineralizasyon sürecine etki eden faktörlerdir.

1. INTRODUCTION

Microbial growth can be observed in the wide range environments such as extreme environments. While they are growing, they regulate element distributions associated with biogeochemical cycles in those environments and causing various minerals formations. Extreme environments such as hypersaline lakes are considered one of the most interesting places that microorganism grow because of high salt concentration and their effects on microbial activity. Extreme environments have been getting more attention due to their unusual chemical/geochemical conditions. Recent studies have been focused on microbial diversity and biomineralization processes in these environments. The study of biomineralization in hypersaline environments is important: 1-it can extend our knowledge about the earliest microbial life on Earth which may have been halophilic 2-Because of the presence of hypersaline conditions on Mars, the analog environments in Earth may have implications for the possibility of life on Mars. 3- Biotechnological applications such as isolating unusual enzyme which may have industrial applications. Microorganisms thrive in hypersaline environments are called halophilic, salt lovers, microorganims. Among halophilic niches are found a variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes.

1.1Halophiles

Microorganisms found in the hypersaline environments usually need sodium ions for optimal growth and reproduction. These microorganisms are called halophiles. Halophiles are divided into three groups according to their ability to survive at different salt concentrations. *Mild halophiles* require 1-6 % NaCl, *moderate halophiles* require 7-15 % NaCl and *extreme halophiles* require 15-30 % NaCl (Figure 1.1) [1].

Halophiles may have different morphologies, from rod shape to cocci shape. They are gram negative, reproduce by binnary fission and do not form spores. Most of halophiles are nonmotile, a few strains are able to float by their gas vesicles [1]. Most halophiles are obligate aerobes but some of them grow anaerobically. Halophiles are generally distinguised with their reddish colour and because of this property, their environments seem reddish (Figure 1.2 and Figure 1.3).

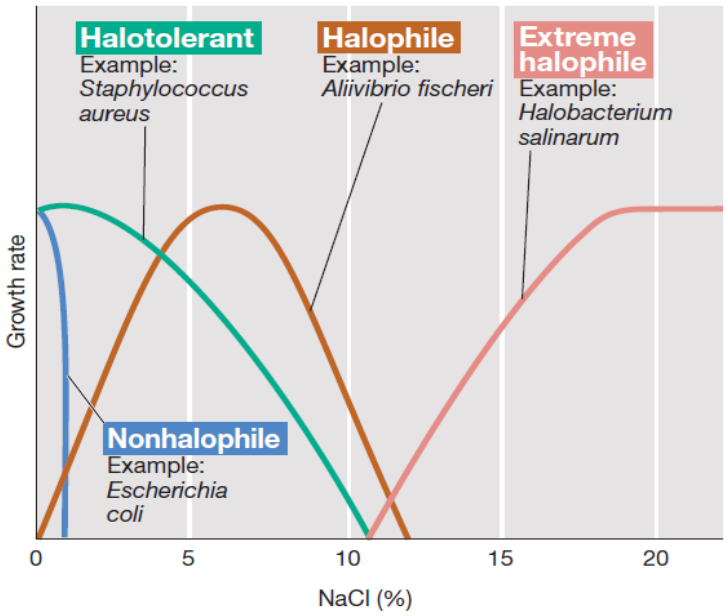


Figure 1.1 : Effects of NaCl concentration on growth of microorganisms of different salt requirements [1].

Figure 1.2: Lake Acigol, Turkey.

Figure 1.3: Lake Hamara, Egypt [1].

Adaptation mechanism of Halophiles

Halophilic microorganisms encounter with two stress factors that are high inorganic ion concentration and the low water potential, in saline environments. They have devoloped two principle mechanism to overcome these stress factors. Those mechanisms are called 'salt-in-cytoplasm' mechanism and the 'organic-osmolyte mechanism' [2].

'Salt-in-cytoplasm' mechanism protect the water potential of the cell by increasing KCl concentration in the cytoplasm [3,4]. The potassium gradient in the cell is maintained by sodium ion/proton antiporter and a potasium ion uniporter [5]. Figure 1.4 shows that ion transport mechanism of halobacteria on a model.

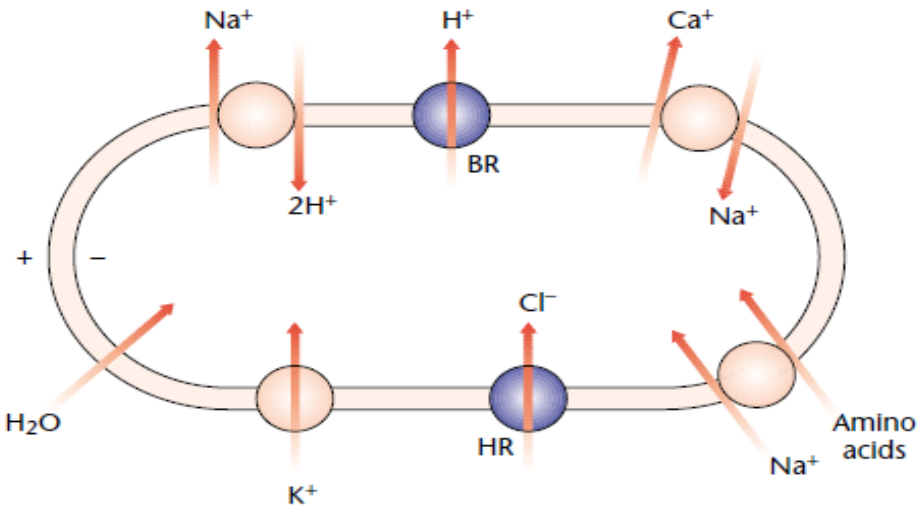
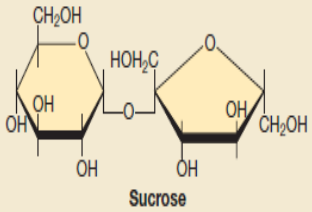


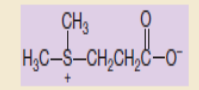
Figure 1.4: Flow of ions and molecules across the membrane of halobacteria. Bacteriorhodopsin (BR) is a membrane protein which absorb light energy and catalyze formation of a proton motif force, halorhodopsin (HR) is a cloride pump are shown in blue, the sodium/proton antiporter, potassium uniporter, sodium/amino acid symporter and calcium/sodium antiporter are shown in pink. The directionality of ions, amino acids and water is indicated by arrows and the electrical potential across the membrane is indicated by the – and + charges [5].

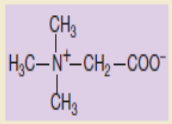
'Organic-osmolyte' mechanism is another mechanism for adaptation to the high salt content environments. Halophiles accumulate osmoprotective organic compounds, which are aminoacids and/or aminoacid derivatives in their cytoplasm to response the osmatic stress [6]. These non-ionic, higly water-soluble organic compounds

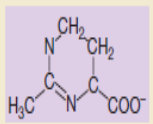
should be noninhibitory to biochemical process in the cell [7]. These organic compounds called compatible solutes. Compatible solutes can be synthesized by the microorganisms directly as *glycine betaine* or can be accumulated from the environment as *ectoine*. *Glycine betaine, ectoine* and a few compatible solutes are shown in Figure 1.5. Especially some extremely halophilic bacteria produce *ectoine* (Figure 1.5) which is a cyclic derivative of the amino acid aspartate.

Organism	*Major cytoplasmic solute(s)*	*Minimum a_w for growth*
Nonphototrophic *Bacteria*/freshwater cyanobacteria	Amino acids (mainly glutamate or proline[a])/sucrose, trehalose[b]	0.98–0.90
Marine cyanobacteria	α-Glucosylglycerol[b]	0.92
Marine algae	Mannitol,[b] various glycosides, dimethylsulfoniopropionate	0.92
Salt lake cyanobacteria	Glycine betaine	0.90–0.75
Halophilic anoxygenic phototrophic purple *Bacteria*	Glycine betaine, ectoine, trehalose[b]	0.90–0.75
Extremely halophilic *Archaea* and some *Bacteria*	KCl	0.75
Dunaliella (halophilic green alga)	Glycerol	0.75
Xerophilic and osmophilic yeasts	Glycerol	0.83–0.62
Xerophilic filamentous fungi	Glycerol	0.72–0.61









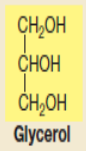


Figure 1.5: Compatible solutes of microorganisms.

Halophiles have able to survive under non-standard conditions in extreme environments and enzymes of these extremophilic microorganisms have been optimized for extreme conditions. Indeed, previous experimental studies in which enzymes from those microorganisms are isolated and characterized support this assumption [7]. The extremozymes, extremophilic enzymes, which have unique properties, can be used novel applications in industrial processes. Some examples for enzymes from halophiles are shown in Table 1.1

Table 1.1: Industrial applications of extremozymes [7].

Type	Growth characteristic	Enzymes	Applications
Thermophiles (hypertermophile) (thermophile)	Temp>80°C 60-80°C	Proteases	Detergents, hydrolysis in food and feed, brewing, baking
		Glycosyl hyrolases amylases, glucosidases, cellulases, xylanases	Starch, cellulose, chitin, pectin processing, textiles
		Xylanases	Paper bleaching
		Lipases, esterases	Detergents, stereo-specific reactions(e.g. trans esterification, organicbiosynthesis)
		Chitinases	Chitin modification for food and health products
Psychrophiles	Tem< 15°C	Proteases	Detergents, food application (dairy products)
		Amylases	Detergents and bakery
		Cellulases	Detergents, feed and textiles
		Dehydrogenases	Biosensors
		Lipases	Detergents, food and cosmetics
Halophiles	Hih salt, (e.g. 2-5 M NaCl)	Proteases	Peptide synthesis
Alkaliphiles	pH > 9	Proteases, cellulases	

Table 1.1 (continued): Industrial applications of extremophiles' enzymes [7].

Acidophiles	pH < 2-3	Amylases, glucoamylases	Starch processing
		Proteases, cellulases	Feed componenets
		Oxidases	Desulfurization of coal
Piezophiles	Pressure-loving up to 130MPa	To be defined	Food processing and antibiotic production

In recent years, importance of halophilic microorganisms for biotechnological applications is increasing because of its extraordinary properties. Industrial products obtained from halophiles can be categorized into different groups according to their properties [8]. First, proteins of halophiles are both resistant to high salt concentrations and require salts for activity [4]. Halophilic enzymes such as amylase, nuclease and protease have been experienced for different biotechnological applications [8]. Second, some compatible solutes that are organic osmotic stabilizer are produced by halophiles for example; ectoines used in dermopharmacy as anti-ageing agents in skin creams, as components of shampoos, as adjuvant for vaccines [9]. Third, halophilic microorganisms have the ability to produce valuable products such as β-hyroxyalkanoate, which is biologically degradable plastic material and this compound can be obtained with high yield from halophiles. Lysis of the halophilic cells in the absence of salt provides advantages for biotechnological production processes [8]. Furthermore, potential biotechnological uses of halophilic microorganisms are shown in Table 1.2.

1.2 Biomineralization Mechanisms

Biomineralization is the deposition of inorganic solids by organisms. More than 40 different mineral types are known to be formed by organisms from all the kingdoms. Silicates in algae and diatoms, carbonates in invertebrates, calcium phosphates and carbonates in vertebrates are important examples (More examples are shown in Table 1.3) [11].

Table 1.2 : Potential biotechnological uses of halophilic microorganisms [8,9,10].

Product	**Producing organism**	**Uses and present status of technology**
β- carotene	*Dunaliella* species	Produced as antioxidant and food coloring agent
Different carotenoid pigments	Members of the *Bacteriaceae*	Light absorption
Ectoine and hydroxyectoine	*Halomonas elongata Marinococcus M52 Chromohalobacter salexigens*	Produced as enzyme stabilizier, moisturizer in cosmetics
Poly-β-hydroxyalkanoate	*Haloferax meditarrenei*	Organism has high potencial for PHA* production
Salt tolerant enzymes	Different halophilic Bacteria and Archea	Not yet industrially exploited
Bacteriorhopsin	*Halobacterium salinarum*	Potencial uses photoelectric converts but in the experimental stage
Extracellular polysaccharides	*Haloferax meditarrenei*	Potential for the production polysaccharides, Not yet industrially exploited
Halophile cell biomass for cosmetics.	*Duniella* species	Used as an additive in cosmetic antiwrinkle preparation

*Ployhdyroxyalkanoat

Biomineralization include the selective extraction and uptake of elements from the local environment and also involves their incorporation into functional structures under strict biological control [12]. Over the past three decades, researchers focused on biomineralization studies. The general aims of biomineralization studies includes the structures and compositional characterization of biominerals, understanding the functional properties of biominerals, making clear of the processes through which organic macromolecules and organic structures control the synthesis, construction and organization of inorganic mineral – based materials [12].

Table 1.3: The main inorganic solids' types and functions in biological systems [11].

Mineral	Formula	Organisms/Function
Calcium Carbonate		
Calcite	$CaCO_3$	Algae/ exoskeletons Trilobites/ eye lens
Aragonite	$CaCO_3$	Fish /gravity device Mollusks/ exoskeleton
Vaterite	$CaCO_3$	Ascidians / spicules
Amorphous	$CaCO_3.nH_2O$	Plants/ Calcium store
Calcium Phosphate		
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	Vertebrates/ endoskeletons, teeth, Ca store
Octacalcium phosphate	$Ca_8H_2(PO_4)_6$	Vertebrates/ precursor phase in bone
Amorphous		Mussels/ Ca store Vertebrates/ precursor phase in bone
Calcium Oxalate		
Whewellite	$CaC_2O_4.H_2O$	Plants/ Ca store
Weddellite	$CaC_2O_4.2H_2O$	Plants/ Ca store
Group 2A(IIA) Metal Sulfates		
Gypsum	$CaSO_4$	Jellyfish larvae/ gravity device
Barite	$BaSO_4$	Algea/ gravity device
Celestite	$SrSO_4$	Acantharia/cellular support
Silicon Dioxide		
Silica	$SiO_2.nH_2O$	Algae/ Exoskeletones
Iron Oxides		
Magnetite	Fe_3O_4	Bacteria/ Magnetotaxis Chitones/ teeth
Geothite	α-FeOOH	Limpets/ teeth
Lepidocrocite	γ- FeOOH	Chitones(mollusca)/ teeth
Ferrihydrite	$5Fe_2O_3.9H_2O$	Animals and plants Few storage proteins
Iron Sulfides		
Greigite	Fe_3S_4	Bacteria/ magnetotaxis

Biomineralization process can be categorized according the process which can be intentional and intracellular or a byproduct of metabolism. Biomineralization processes are divided two groups as biologically controlled mineralization (BCM) and biologically induced mineralization (BIM) [13].

1.2.1 Biologically controlled mineralization (BCM)

Biologically controlled mineralization (BCM) is a highly regulated process. Bones, shells and teeth are some examples for BCM. These materials (minerals) have specific biological functions. Furthermore, these minerals are directly synthesized at a specific location within or on the cell and the process is only occurred under certain conditions [10]. These biominerals are distinguished by reproducibility and species – specific crystallochemical property and their structures and compositions are well defined [12]. In the microbial world, the most characterized example of BCM is magnetosome formation by Magnetotactic bacteria. These organisms produce magnetite (Fe_3O_4 and Fe_3S_4) nanoparticles with species – specific shapes [14]. Magnetotactic bacterium is shown in Figure: 1.6.

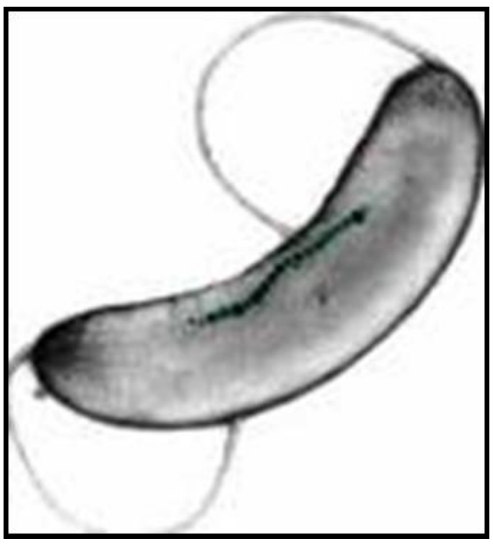


Figure 1.6: Magnetotactic bacterium (image crated by: Frankel R.).

1.2.2 Biologically induced mineralization (BIM)

Minerals formed by biologically induced mineralization processes generally nucleate and grow extracellularly as a result of metabolic activity of the microorganisms. These microorganisms secrete metabolic byproducts that react with ions and compounds in the environment and after that this phenomena mineral particules are constituted. Therefore BIM is a unintended and uncontrolled consequence of metabolic activities. Minerals produced in BIM are characterized by poor

crystallinity, broad particle-size and lack of specific crystal morphologies. In some cases, the metabolic products diffuse away and minerals form from solution. However, bacterial surfaces such as cell walls or polymeric materials which are (exopolymers) exuded by bacteria, can act as important sites for the adsorption of ions and mineral nucleation and growth [14,15,16]. BIM is especially important for bacteria in anaerobic environments. Because under anaerobic conditions, many bacteria respire with sulfate or various metals including iron. After these metabolic activities, metabolic products are produced such as metal ions and sulfide which are reactive and participate in subsequent mineral formation [14].

Bacterial cell surfaces and the surfaces of exopolymers can be especially important in BIM processes. Negative charges on most cell which orginate in properties of cell wall (peptidoglycan) of microorganisms and exopolymer surfaces can result in binding of cations by non-specific electrostatic interactions. These electrostatic interactions help stabilize the surfaces of nascent mineral particales, crystal-nucleus formation. Two surface BIM processes, known as passive and active, have been identified [14]. Passive mineralization is the simple, non specific binding of cations and solution anions, resulting in surface nucleation and growth of minerals. Active mineralization takes place through the formation of cationic or anionic byproducts of metabolic activities that form minerals on the bacterial surfaces.

Halophilic microorganisms are good examples for both active mineralization and passive mineralization. Biologically induced mineralization has been described for carbonate and phosphate minerals by halophilic microorganisms [17,18,19,20,21,22]. Ca^{+2} and Mg^{+2} ions naturally exist in microenvironments where halophiles live. When the bacteria pump Ca^{+2} towards the exterior of the cell, Mg^{+2} towards the interior and Ca^{+2} is adsorbed with greater intensity than Mg^{+2} by the negatively charged surface of bacteria. These bacteria serve as a nucleus for biologically induced mineralization. In addition, microbial metabolic activity is very important for mineralization of Ca^{+2} and Mg^{+2} minerals. By the metabolic activity some by products, which are CO_3^{-2}, NH_4^{+} and PO_4^{-3} ions are produced. These ions change the pH in their environment, influence cell surface charge and these changes lead to carbonate mineralization (Figure 1.7) [17,23,24].

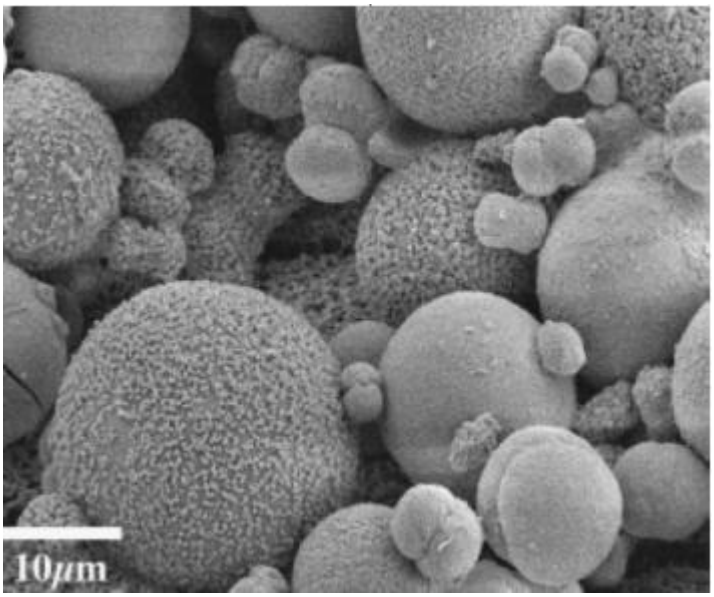


Figure 1.7: Calcified bacterial cells [17].

In this thesis, the bacterial diversity of Lake Acigol, a unique hypersaline lake in southwest of Turkey, have been investigated by integrating geochemical and microbiological approaches: 1- to determine bacterial diversity in the lake which have not been investigated 2- elucidate effect of bacterial activity on recent biomineralization occurring in the lake 3- isolating enzyme which may involve biomineralization processes and may have biotechnological usage.

1.3 The Study Area

Lake Acigol, located between Afyon, Denizli and Burdur city boundaries in the SW of Turkey, is a perennial lake and was evolved in graben basins of Miocene to Pliocene age. Its surface area changes significantly through the seasons, 65 km^2 in late spring and 55 km^2 in the late summer with a maximum depth of 1.63 m. The lake's altitude is about 836 m. The lake is fed primarily by high-sulfate springs its water chemistry is controlled by the weathering, and chemical leaching of the ultramafic–dolomitic bedrock with evaporitic occurrences in the basin's catchment area. As a result the lake water has high concentration of Na, Mg; Ca and CI causing deposition of various minerals such as Mg-carbonates and Na-SO_4 in the lake. Na_2SO_4 production from Lake Acigol comprises 85 % of Turkey’s production.

Sodium sulfate (Na_2SO_4) is a row material for different industries such as paper, detergent and glass industry [25].

Extreme chemical conditions such as high salinity (ca.200 g/L) and alkali pH: (8,0 - 8, 6) of the lake make this environment very attractive for interdisciplinary studies such as biogeochemistry and biominerals formation.

Figure 1.8: Lake Acigol is a hypersaline lake in SW Turkey.

2. MATERIALS AND METHODS

2.1 Materials

Materials are listed in Appendix A.

2.1.1 Laboratory equipments

Laboratory equipments are listed in Appendix B.

2.2. Methods

2.2.1 Field measurements and sampling

Field measurements and sampling were conducted in June 2008. pH, temperature, and salinity were measured with pH and conductivity probes insitu. Concentrations of Fe ($Fe+^{2}$), sulfate, phosphate, nitrite, and nitrate were measured by field colorimetric Hach kits (DR2800). Water samples from the lake and the salt ponds around the lake were collected with 50-ml sterile centrifuge tubes. Surface sediment samples (depth of 10 to 12 cm) were collected using a sterile spatula. After collection, all the samples were immediately stored in a refrigerator at 4°C and were carried to Geomicrobiology-Biogeochemistry Laboratory in Department of Geological Engineering at İstanbul Technical University. Microbiological analysis were only carried on the sediment samples. Two sediment samples, selected 1AGN (salt pond sediments), 3AGN (lake bed sediments 10 to 12 cm), were geochemically and microbiologically analyzed. The analyses included mineralogy of the lake by X-ray diffraction (XRD), and microbial diversity by 16S rRNA gene analysis. Enrichments and isolations were performed for 1AGN, 3AGN.

2.2.2 Enrichment of Samples

Enrichment experiments were performed for the two sediment samples (1AGN, salty pond and 3AGN lake sediment) with a modified Nutrient broth medium for halophiles. Modified NB was used for general heterotrophs in the lake water. The enrichment experiments were performed in 250 ml Erlenmeyer flasks with 10 g

sediment sample incubated at 30 °C for 7 days by horizontal shaking at 180 rpm. The pH value for media was 7.2.

Gradients of NaCl salt (3%, 6%, 10%) were used to obtain target halophiles with the modified Nutrient Broth. Positive enrichments were transferred three times. The enrichment cultures were streaked onto agar plates consisting of the original enrichment medium supplemented with 2% agar. The plating step was repeated three times for isolates to ensure purity. The colonies from the final set of agar plates were grown in liquid medium, preserved in 80% glycerol, and frozen at –80°C for later analyses.

2.2.3 Determination of Microbial Population

2.2.3.1 Genomic DNA isolation from enrichment culture

Genomic DNA from enrichment culture was isolated using MoBio UltraClean™ Microbial DNA Isolation Kit (Catalog # 12224-50).

1. 1.8 ml of each of the enrichment culture was taken into a 2 ml tube, centrifuged at 10000 rpm for 5 min.
2. Supernatant was discarded; cell pellet was resuspended with 300 µl MicroBead solution. Resuspended cells were transferred to MicroBead tube.
3. 50 µl of MD1 solution was addedto MicroBead tube.
4. Samples were vortexed for 10 min at maximum speed using the special aparatus for microbead tubes.
5. MicroBead tube was centrifuged for 30 sec at 10000 g. Supernatant was transferred to a 2 ml collection tube.
6. 100 µl of MD2 solution was added, vortexed for 5 sec and incubated at 4 °C for 5 min.
7. Tube was centrifuged at 10000 g for 1min. Supernatant was transferred to a new collection tube.
8. 900 µl of MD3 solution was added, vortexed for 5 sec.

9. 700 µl was transferred to spin filters and centrifuged for 30 sec. Filtrate, collected in collection tube, was discarded and remaining solution added to same filter, centrifuged again and filtrate was discarded.
10. 300 µl of MD4 solution was added to filter and centrifuges at 10000 g for 30 sec. Filtrate was discarded. Empty filter was centrifuged at 10000 g for 1 min again to dry.
11. Spin filter tube was replaced in a new collection tube. 50 µl of MD5 solution was added and centrifuged at 10000 g for 30 sec.
12. DNA solution in the collection tube was stred at – 20 °C.

2.2.3.2 Genomic DNA isolation from sediment samples

Fast DNA Spin Kit for Soil (Catalog # 6560-200, MPBio) was used for DNA isolation from sediment samples following the protocol described below.

1. Approximately 1 g of sediment sample was washed with 500 µl PBS, centrifuged at 14000 rpm for 10 min and supernatant was discarded.
2. Up to 500 mg of washed sample added to Lysing Matrix E tube.
3. 978 µl sodium phophate buffer and 128 µl MT buffer was added to the sample in the tube.
4. Sample containing tubes were secured in FastPrep Instrument and processed for 40 seconds at a speed of 6.
5. Sample was centrifuged at 14000 g for 5 min.
6. Supernatant was transferred to a clean tube and 250 µl protein precipitation solution (PPS) was added and mixed by shaking the tube 10 times.
7. Sample was centrifuged at 14000 g for 5 min to pellet precipitate. Supernatant was transferred to a clean 2 ml tube.
8. Binding matrix solution was resuspended and 1 ml of it was added to the supernatant.
9. Solution is mixed by inverting by hand for 2 min to allow binding the DNA.
10. Tube is placed on a rack for 3 min to allow settling the silica matrix.

11. About 500 µl of the upper solution was discarded and the remaining mixture was resuspended.
12. 600 µl of the mixture was transferred to SPIN Filter, centrifuged at 14000 g for 1 min, catch tube was empitied and the remaining mixture was added and centrifuged again.
13. 500 µl of SEWS-M (EtOH) was added to the fitler and centrifuged at 14000 g for 1 min to wash the DNA.
14. Centrifuge at 14000 g for 2 minutes to "dry" the matrix of residual SEWS-M wash solution. Replace SPIN Filter in a clean catch tube.
15. Binding matrix was resuspended gently with 50 µl DNase-Pyrogen-Free Water (DES). Tube was incubated at 55 °C for 5 min for increasing the DNA yield.
16. A clean tube was placed under spin filter and centrifuged at 14000 g for 1 min.
17. DNA solution in the tube stored at – 20 °C.

2.2.3.3 Universal amplified ribosomal region (UARR) PCR

Microbial diversity of Lake Acigol was determined by 16 s rDNA UARR PCR method. The genomic DNA, which is isolated from enrichment culture and sediment samples, was used as template DNA. Two different primer set were used for amplification. Approximately 1500bp of 16S rRNA gene fragment was amplified using the universal primer pairs pA-F (5'-AGAGTTTGATCCTGGCTCAG-3') and pH-R (5'-AAGGAGGTGATCCAGCCGCA-3') Reaction components and PCR conditions are shown in Table 2.1.

Table 2.1: Chemicals used for UARR PCR.

Chemical	Amount
10X PCR Buffer	5 µl
10 mM dNTP	1 µl
Forward primer(pA-F&8F2-F)	1 µl
Reverse primer(pH-R&907R2-R)	1 µl
Taq polymerase	1 µl
25 mM $MgCl_2$	6 µl
Template DNA	1 µl
Water	34 µl
Total	50 µl

Table 2.2: UARR PCR condition.

Temperature	Duration	
95 °C	5 min	
95 °C	45 sec	35cycle
55 °C	45 sec	
72 °C	60 sec	
72 °C	5 min	
4 °C	∞	

2.2.3.4 Agarose gel electrophoresis and PCR product purification

After the amplification, PCR products were confirmed by 1 % agarose gel electrophoresis. 0.4 g agarose was boiled in 40 ml of TAE buffer in a microwave oven for 1 min. Agarose solution was poured onto horizontal gel system. After agarose solution gets cooled, DNA samples were mixed with 5X Amesco DNA Loading Dye & Buffer and loaded in to wells. PCR products were electrophoresed under 8V/cm and visualized by UV Transilluminator. After the presence of DNA band about 1500bp and 800bp long was confirmed, all of the PCR products were purified by ROCHE – High Pure PCR Product Purification Kit (Catalog # 11 732 668 001). Purification procedure is explained below.

1. After amplification is completed, 500 µl Binding Buffer were added to PCR reaction tube and mixed well.
2. High pure filter tube was inserted into one collection tube, the sample from step 1 was transferred to the filter tube and filter tube was centrifuged 60 sec. at maximum speed.
3. After centrifugation solution was discarded and filter tube was inserted into the same collection tube.
4. 500 µl Wash Buffer was added to the filter tube and centrifeged 1 min. at maximum speed.
5. Solution and collection tube were discarded and filter tube was inserted into a clean 1.5 ml microcentrifuge tube.
6. 50 µl Elution Buffer was added to the upper reservoir of the filter tube and centrifuged 1 min. at maximum speed.
7. Pure PCR product in the tube stored – 20 °C.

2.2.3.5 Cloning and transformation of PCR products

TOPO TA® Cloning Kit (Catalog # K4500-01, Invitrogen) was used for cloning PCR products. Chemicals used in cloning reaction are given in Table 2.3.

Table 2.3: Chemicals for clonnig reaction.

Chemicals	Amount
Salt Solution	1 µl
TOPO TA Vector	1 µl
PCR Product	2 µl
Water	2 µl
Total	6 µl

1. All chemicals were mixed in 1.5 ml tube and incubated 15 minutes at room temperature.
2. 2 µl of cloning mixture was added in to One Shot Electrocompetent *E.coli* (50 µl).

3. The new mixture was transferred carefully to 0.1 cm electroporation cuvette. Avoiding formation of bubbles is important in this step. The sample was electroporated at 1800 V using electroporator (EPPENDORF).
4. Immediately after electroporation, 250 µl SOC medium was added and mixture was transferred to a sterile 1.5 ml tube.
5. Mixture was incubated at 37 °C for 40 min to allow the antibiotic resistance genes to be expressed.
6. After the incubation cells were spread into LB agar plates containing 100 mg/ml ampicilline and 40 µl of X-gal stock solution.
7. Agar plates were incubated at 37 °C for 16 hours.

2.2.3.6 Blue/White screening and plasmid isolation

White colonies on LB agar, which is selective agar containing 100 mg/ml ampicillin and 40 µl of X-gal stock solution, were picked and inoculated 15 ml tubes containing 3 ml of LB broth and 3 µl of ampicillin stock solution. Cells were inoculated at 37 °C for overnight with shaking. After incubation plasmids were isolated by Roche High pure Plasmid Isolation Kit (Catalog # 1754785) and described below.

1. Each 3 ml cell culture was centrifuged at 6000 rpm for 1 min to pellet the cells. Supernatant was discarded.
2. 250 µl of Suspension + Rnase Buffer was added to the pellets and resuspended.
3. 250 µl of Lysis Buffer was added to the mixture and tubes were inverted 6-8 times and incubated for 5 min. at room temperature.
4. 5 minutes later 350 µl of pre-chilled Binding Buffer was added to the mixture and the mixture was incubated on ice for 5 min. after mixing gently.
5. Samples were centrifuged at 13000 g for 10 min.
6. High Pure Filter tubes were inserted into collection tubes and entire supernatant from step 5 was transferred to the filter.
7. Samples were centrifuged at 13000 g for 10 min. Liquid in the collection tube was discarded.
8. Filter was washed with 500 µl of Wash Buffer I and with 700 µl of Wash Buffer II, respectively. Sample was centrifuged at 13000 g for 1 min. after each wash.

9. Empty filter tube was centrifuged at 13000 g for 2 min to dry completely.
10. Filter was transferred to a clean 1.5 ml tube. 50 µl of Elution Buffer was added to the filter tube and centrifuged at 13000 g for 1 min.
11. Plasmids were stored at – 20 °C until usage.

2.2.3.7 Sequence PCR and sequence PCR purification

Sequence PCR was set to amplify the insert region within the plasmid vector. Big Dye Terminator Sequencing Kit was used for this purpose. Chemicals used in sequence PCR reaction and the PCR conditions are shown in Table 2.4 and Table 2.5.

Table 2.4: Sequence PCR components.

Chemicals	Amount
ABI-RR 100 Dye	2 µl
ABI 5X PCR Buffer	2 µl
M13 F/RPrimers	1 µl
Template	1 µl
Water	4 µl
Total	10 µl

Table 2.5: Sequence PCR conditions.

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

1. After terminated of sequence PCR, PCR products were purified with regard to the following procedure.
2. Each of PCR products were transferred to 1.5 ml tubes.
3. 25 µl ice-cold 95 % EtOH and 1 µl sodium acetate (3 M, pH 5.2) were added onto tubes.
4. Tubes were incubated on ice for 15 min.

5. After the incubation, tubes were centrifuged at 14000 rpm for 15 min and after that supernatant were discarded.
6. 250 ml of ice- cold 70 % EtOH were added onto pellets and tubes were centrifuged at 1400 rpm for 15 min. After centrifugation supernatant was discarded.
7. Excess EtOH was evaporated at 95 °C for 2-3 min.
8. 20 µl formamide were added onto pellets and pellets were dissolved. Followed by DNA was denaturated at 95 °C for 3 min.
9. Samples were analysed by ABI 3100 Avant automized sequencer.

2.2.3.7 Phylogenetic analysis

Sequence analysis results were compared in NCBI database using Basic Local Alignment Tool (BLAST). Acording to comparisons, best similarities of sequences were accepted. Sequence library is transferred to MEGA program (Molecular Evolutionary Genetics Analysis, version 5.0) to compare with each other and phylogenetic tree is drawn.

2.2.4 Biomineralization experiments

Many studies proved the effect of microorganisms in biomineralization processes. In order to understand and elucidate the biomineralization processes occurring in Lake Acigol, various set of experiments were carried out under the similar conditions with the lake. For these experiments, Modified Halophilic Bacteria Medium (MHBM) was designed based on the physicochemical properties of Lake Acigol. Modified Halophilic Bacteria medium contain he following composition, (g/ml): 1 % yeast extract, 0.1 % glucose, 0.5 % peptone, 4.5 % NaCl and 84 mM Mg^{+2}, 11 mM Ca^{+2} were added [21] (Table 2.6). Four different concentration of Na_2SO_4 which are 0, 14, 28, 56 mM, were added in the main medium to represent seasonal sulfate concentration in the Lake. In addition to liquid media experiments, biomineralization experiments with solid media were also carried out. To obtain a solid media 15 g/L agar was added. The pH was adjusted to 7.2 with 0.1 M KOH. 100 ml steril media was transferred to 250 ml Erlenmeyer flasks. Medium were sterilized at 121°C for 20 min. 0.1 % steril glucose was added after the sterilization.

Table 2.6 : MHBM for Biomineralization experiments.

Birim(w/v)	$Na_2SO_4^{2-}$	Mg^{2+}	Ca^{2+}	Yeast exstract	Peptone	Glucose
1.group	0	84mM	11mM	1 %	0.5%	0.1%
2.group	14mM	84mM	11mM	1%	0.5%	0.1%
3. group	28mM	84mM	11mM	1%	0.5%	0.1%
4. group	56mM	84mM	11mM	1%	0.5%	0.1%

Biomineralization experiments were carried out in 250 ml Erlenmeyer flasks, which were sterilized according to the standard microbiological techniques and contained 100 ml MHBM, were inoculated by 10 ml of enrichment culture, containing 6% NaCl, and incubated at 30°C and 4°C shaking at180 rpm . In addition, 100 µl of enrichment culture was spreaded on solid MHBM and incubated aerobically at 30°C. Control experiments were set up without enrichment culture and autoclaved enrichment culture and were incubated in the same conditions with biomineralization experiments.

2.2.4.1 Analysis of biominerals

During the biomineralization experiments, 10 ml solutions were periodically withdrawn from each of medium to determine variation in pH of the solution, concentration of Ca, Mg along with characterize biominerals.Ca and Mg concentration were measured by using atomic absorption spectrometer (AAS, Perkin-Elmer 1100B). Each of 10 ml solution were centrifuged at 5000 rpm for 15 min. After the centrifugation, supernatants transferred in steril 15 ml tubes. Tubes are stored at 4°C until medium for analysis. Besides, pellets and solid medium were prepared for characterization of biominerals. Pellets were washed by distilled water three times and after that, they were dried at room temperature. Solid medium were dissolved in boiling water bath. Supernatants were discarded and the sediments were washed by distilled water three times. After washing they are dried at room temperature. Characterization studies of biominerals were carried out with X-ray diffraction (XRD) and scanning electron microscope (SEM).

The phase changes of biominerals were examined by X-ray diffractometer (XRD, PAN alytical PW3040/60 with a Cu Kα radiation). XRD scans of powders were

conducted between 5 and 90 degrees in order to characterize any formation, XRD studies carried out according to JCPDS and ASTM criteria [26]. XRD analyses are carried out by Asist. Prof.Dr. Şeref Sönmez from Metalurgical and Materials Engineering Department, ITU. In addition SEM analyses were carried out by Tubitak Marmara Research Center (MAM).

3. RESULTS AND DISCUSSION

3.1 Genomic DNA Isolation

Sediment samples 1AGN and 3AGN collected different part of Lake Acigol (Figure 3.1 and Figure 3.2) were used for Genomic DNA isolation by using Fast DNA Spin Kit for Soil and MoBio UltraClean™ Microbial DNA Isolation Kit for enrichment cultures. Following the isolation of DNAs, they were analyzed on 1 % agarose gel as shown in Figure 3.3 and Figure 3.4.

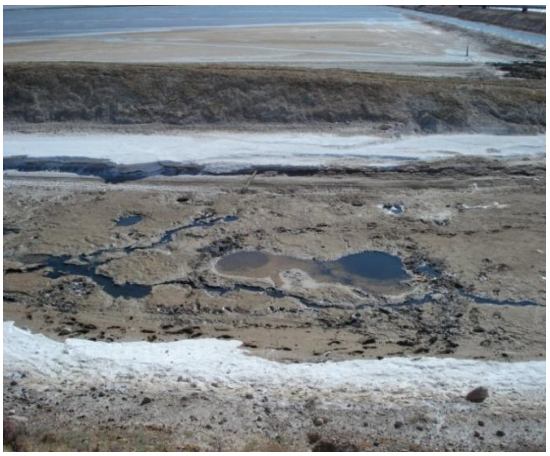


Figure 3.1: 1AGN.

Figure 3.2: 3AGN.

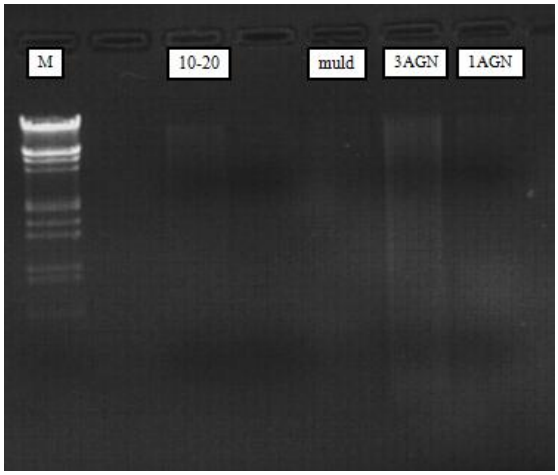


Figure 3.3: gDNA isolation from the samples. Lane1: Marker3 (21226, 5148,4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 bp) Lane2: 10-20, Lane3: sediment/mud, Lane4: 3AGN, Lane5: 1AGN.

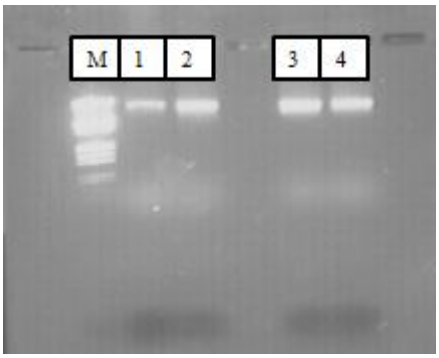


Figure 3.4: gDNA isolation from enrichment cultures. Lane1: Marker 3 (21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 bp) Lane 2 (1): 1AGN, 3 % NaCl enrichment, Lane 3 (2): 3AGN, 3 % NaCl enrichment, Lane 4 (3): 1AGN, 6 % NaCl enrichment, Lane 5 (4): 3AGN, 6 % NaCl enrichment.

UARR PCR amplification and PCR product purification

According to 16S rDNA UARR PCR method, approximately 1500 bp rDNA fragments were amplified by using pA-pH primers. PCR products were controlled on 1% agarose gel shown in Figure 3.5. After the control, all PCR products were purified by using ROCHE – High Pure PCR Product Purification Kit to clean the excess primers, dNTP and Taq polymerase.

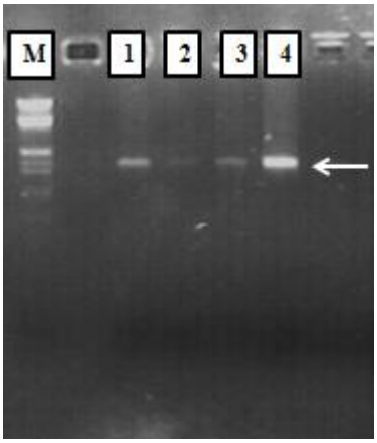


Figure 3.5: Purified PCR product. Lane1: 1AGN, 3% NaCl enrichment, Lane2: 1AGN, 6% NaCl enrichment, Lane3: 3AGN, 3% NaCl enrichment, Lane4: 3AGN, 6% NaCl enrichment, Lane5: Marker3 (21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 bp), arrow indicate that 1500 bp.

Transformation of PCR products

TOPO TA vector was used for transformation of purified PCR pruducts. TOPO TA vector is a plasmid which has ampicilline resistance gene and β-galactosidase coding

gene called *lacZ*. (TOPO TA vector map was shown in Appendix C). *lacZ* gene is a kind of selection marker which provide blue/white screening on X-gal containinig plate. In addition, the poly (A) tail added by *taq polymerase* to the end of the rDNA fragments is required for ligation to the vector. When these fragments ligated to the vector, *lac Z* gene loses its activity. When these products are transformed into TOP 10 bacterial cells, they form white colonies on X-gal containing ampicilline agar plate. Each of the white colonies contains different rDNA fragments and these white colonies constitute our cloning library. On the other hand, vector which hasn't rRNA fragment can produce β-galactosidase and cell gain blue colour due to the hydrolysis of X-gal. Blue/white screening plate was shown in Figure 3.6. Approximately 20 colonies were selected for plasmid isolation from each sample.

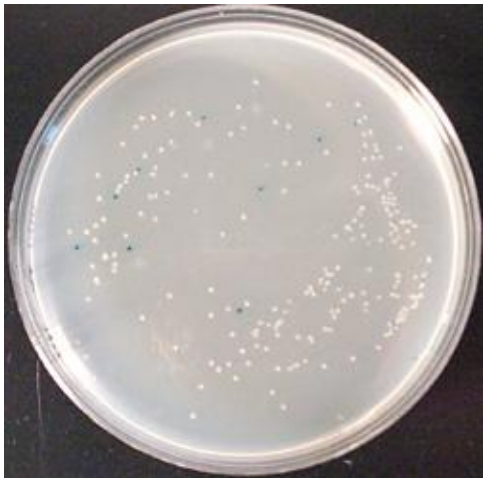


Figure 3.6 : Blue/White screening plate.

Sequencing and Phylogenetic Analysis

Target rDNA fragments, which are located in plasmid, were amplified by using M13-F/M13-R primers. After the amplification, sequence PCR products were purified by ethanol precipitation and they were sequenced by using the ABI Prism 3100-Avant automated sequencer at the Molecular Biology and Genetic Department, ITU. Partial rDNA gene sequences which were obtained by sequencing were compared with NCBI database using BLAST-N [27]. Results of enrichment cultures sequence analysis are shown in Table 3.1, 3.2 and phylogenetic tree is shown in Figure 3.7.

Table 3.1: Sequence analyses of 3AGN.

Clone Enrichment 3AGN	**Closest match microorganism**	**Max. Identification**	**Taxanomy**
1.clone	***Uncultured bacterium*** *clone SSW53Au*	99%	Bacteria; Environmental samples.
4.clone	***Cyclobacterium sp.*** *HMD3055*	97%	Bacteria; Bacteroidetes/Chlorobi group; Bacteroidetes; Cytophagia; Cytophagales; Cyclobacteriaceae; Cyclobacterium
5.clone	***Bacillus sp***. *SK47*	99%	Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus
6.clone	***Uncultured bacterium clone*** *BE326ant14g05*	96%	Bacteria; environmental samples.
8.clone	***Idiomarina sp***. *TBZ29*	99%	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; Idiomarina.
10.clone	***Idiomarina seosinensis*** *strain CL-SP19*	99%	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; Idiomarina
11.clone	***Bacillus selenitireducens*** strain M1S6-17	98%	Bacteria;Firmicutes; Bacillales; Bacillaceae; Bacillus

Table 3.2: Sequnce analyses 1AGN.

Clone Enrichment 1AGN	**Closest match microorganism**	**Max. Identification**	**Taxonomy**
1.clone	***Halomonas saccharevitans strain AJ275***	91%	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Halomonadaceae; Halomonas
2.clone	***Salinicoccus roseus strain DSM 5351***	98%	Bacteria; Firmicutes; Bacillales; Salinicoccus.
3.clone	***Uncultured bacterium clone A5_10.1_2***	98%	Bacteria; Environmental samples.
4. clone	***Halomonas alimentaria strain L7B***	98%	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Halomonadaceae; Halomonas
5.clone	***Virgibacillus marismortui strain TPA3-3***	98%	Bacteria; Firmicutes; Bacillales; Bacillaceae; Virgibacillus

According to results of the sequence analysis, frequency of phylogenetic groups is as follows: 33.3% Gammaproteobacteria, 33.3% Firmucutes, 25% Uncultured bacterium 8.3% Bacteriodetes (Table 3.1). The results indicate that *Halomonas*, *Idiomarina* genus and uncultured clones were major microorganisms of Lake Acigol. Especially *Halomonas* genuses used in various biotechnological applications such as exopolysaccharite production is very common [28,29] Virgibacillus which is an another genus in Lake Acigol's population is also suitable for biotechnological applications. Compatible solute synthesis [30] and thermostable serine alkaline protease synthesis [31] are examples for potential applications. On the other hand, uncultured clones consist of major part of the microbial population. Sequence results

indicate that Lake Acigol has a big potential for new species that have not been discovered yet. These uncultured species may have biotechnological potential.

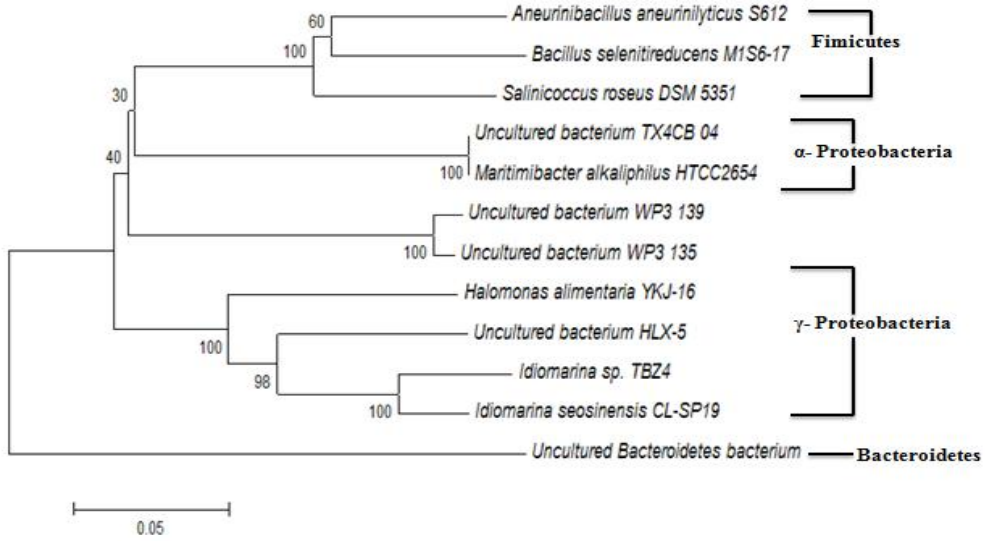


Figure 3.7: Phylogenetic tree of enrichment cultures.

Not only enrichment cultures bacterial population were determined, but also Lake Acigol sediment samples which were collected from pound around the lake and inside of the lake, bacterial populations were determined. Results of sediment samples sequence analysis are shown in Table 3.3 and phylogenetic tree is shown in Figure 3.8.

Table 3.3 : Sequence analyses result of sediment samples.

Sediment samples	**Closest match microorganism**	**Max.Identification**	**Taxonomy**
1.clone	***Idiomarina sp. TBZ4***	96 %	Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Idiomarinaceae; Idiomarina
2.clone	***Uncultured bacterium****, clone R105*	98 %	Bacteria; Environmental samples
3.clone	***Uncultured Bacteroidetes bacterium*** *clone D-16S-66*	90%	Bacteria; Bacteroidetes; Environmental samples

Table 3.3 (continued): Sequence analyses result of sediment samples.

4.clone	***Uncultured bacterium*** *clone TX4CB_04*	97%	Bacteria; Environmental samples
5.clone	***Unculturededdelta proteobacterium*** *clone MPD-41*	98%	Bacteria; Proteobacteria; Deltaproteobacteria; Environmental samples.
6.clone	***Uncultured Bacillus sp.*** *clone DGG2*	94%	Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; Environmental samples.
7.clone	***Uncultured bacterium clone*** *BE326ant14g05*	96%	Environmental samples.
8.clone	***Cyclobacterium lianum strain HY9***	94%	Bacteria; Bacteroidetes; Cytophagia; Cytophagales; Cyclobacteriaceae; Cyclobacterium
9.clone	***Uncultured bacterium*** *clone SINH924*	94%	Bacteria; Environmental samples
10.clone	***Clostridiisalibacter sp. SOL3f37***	97%	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridiisalibacter
11.clone	***Planococcus maritimus isolate LLQ***	97%	Bacteria; Firmicutes; Bacillales; Planococcaceae; Planococcus
12.clone	***Planococcus rifietoensis strain M8***	99%	Bacteria; Firmicutes; Bacillales; Planococcaceae; Planococcus
13.clone	***Uncultured bacterium*** *clone 1103200832078*	98%	Bacteria; Environmental samples

According to results of the sediment sequence analysis, frequency of phylogenetic groups is as follows: 38.8% Uncultured Bacterium, 30.8% Firmucutes, 15.3% Bacteriodetes, 7.6% Gammaproteobacterium and 7.6% Deltaproteobacterium (Table 3.2). These results indicate that Uncultured bacterium clones and Firmicutes are dominant in the sediment samples.

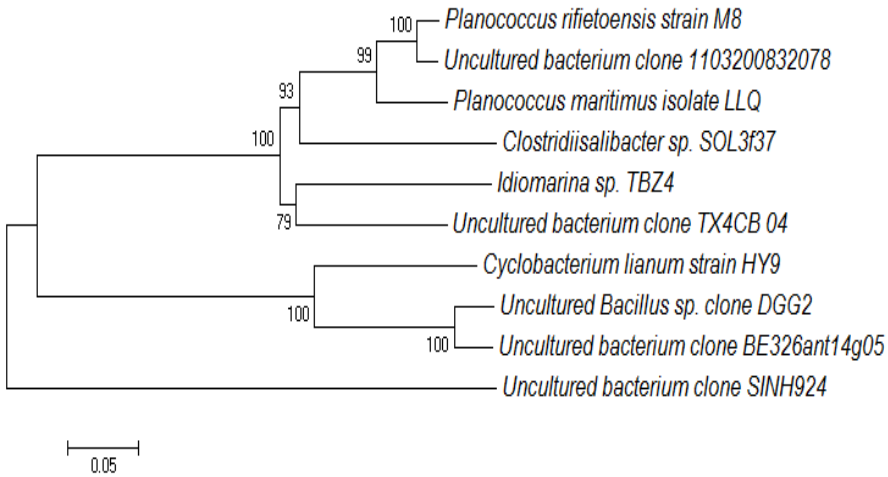


Figure 3.8: Phylogenetic tree of sediment samples.

3.2 Biomineralization Experiments

Enrichment cultures obtained from 1AGN and 3 AGN sediment samples were used in biomineralization experiments. Before the biomineralization experiments, 1AGN and 3AGN samples were enriched in 6 % NaCl Nutrient broth. Biomineralization experiments were carried out in Modified Halophilic Bacterial Medium with 4.5 % NaCl at 30°C.

The minerals formed in all the culture media were recovered and purified. The precipitates on solid media were removed by cutting out agar blocks and placing them in water until the agar dissolved. The precipitates from the liquid media were centrifuged. The supernatants were decanted and the sediments resuspended and washed in distilled water until the precipitate were free of impurities. In all cases, the washed crystals were then air-dried at room temperature.

pH values, Ca^{2+} and Mg^{2+} concentration through the experiments were monitored and results were shown in Table 3.4, Table 3.5, Table 3.6. Table 3.4 is indicated changing Ph values of biomineralization experiments and control experiments versus time. The pH results of control experiments which were carried out under the identical conditions with biological experiments except that bacteria did not add and autoclaved bacterial cell added were also presented in Table 3.4.

Table 3.4: pH values of experiments set.

	1AGN at 30°C				**3AGN at 30°C**			
Days	0 mM Na_2SO_4	14 mM Na_2SO_4	28 mM Na_2SO_4	56 mM Na_2SO_4	0 mM Na_2SO_4	14mM Na_2SO_4	28 mM Na_2SO_4	56 mM Na_2SO_4
0	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
7	8.3	8.3	8.3	8.3	8.3	8.0	8.3	8.3
14	8.5	8.5	8.5	8.6	8.5	8.5	8.5	8.6
28	8.8	8.9	8.8	8.9	8.8	8.9	8.9	8.9
35	9.0	9.0	9.0	9.0	9.2	9.1	9.1	9.1
control	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1

pH values showed increasing trend with time in all biological experiments but control pH didn't change during incubation period. Increasing trend in pH values during biomineralization experiments could have occurred due to degrading organic compounds, such as peptone, yeast extract and glucose which were used in the medium. Degrading these organic compounds produce NH^{+4} and PO_4^{-3} ions leading to increase of pH and in turn making favorable conditions for precipitation minerals. Increasing pH values could also be due to the fact that bacteria could accumulate Ca^{2+} and Mg^{2+} ions in the microenvironment at or around cell surfaces causing increase in alkalinity [17,18,19,20,21].

Table 3.5 and 3.6 show the concentrations of $Ca^{2+,}$ Mg^{2+} and Mg^{2+}/Ca^{2+} ratio through the biomineralization experiments. As expected, concentration of Ca^{2+} and Mg^{2+} ions decreased during the incubation period. However, Ca^{2+} concentration decreased more

Table 3.5: The concentration of Ca^{2+} and Mg^{2+} ions in 1AGN biomineralization experiments.

Days	0 mM Na2SO4 1AGN			14 mM Na2SO4 1AGN			28 mM Na_2SO_4 1AGN			56 mM Na_2SO_4 1AGN		
	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/Ca^{2+}	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/Ca^{2+}	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/Ca^{2+}	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/Ca^{2+}
0	430	2041	4.8	430	2041	4.8	430	2041	4.8	430	2041	4.8
7	221	2335	10.6	275	2244	8.2	229	2253	9.8	328	2286	6.9
14	629	1291	2.1	593	1891	3.2	449	1639	3.7	338	1029	3.1
28	335	462	1.4	340	535	1.6	296	222	0.8	220	571	2.6
35	21	260	12.3	27	220	8.2	25	185	7.3	26	192	7.4

Table 3.6: Concentration of Ca^{2+} and Mg^{2+} ions in 3AGN biomineralization experiments.

Days	0 mM Na2SO4 3AGN			14 mM Na2SO4 3AGN			28 mM Na_2SO_4 3AGN			56 mM Na_2SO_4 3AGN		
	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/Ca^{2+}	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/Ca^{2+}	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/Ca^{2+}	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/Ca^{2+}
0	430	2041	4.8	430	2041	4.8	430	2041	4.8	430	2041	4.8
7	654	2317	3.6	770	2015	2.6	472	1884	4.0	430	2301	5.4
14	353	1256	3.6	445	1350	3.0	360	1405	3.9	280	2057	7.4
28	16	191	12.1	23	351	15.4	24	294	12.3	25	390	15.7
35	29	155	5.4	39	197	5.1	38	170	4.5	35	197	5.6

drastically than Mg^{2+}. Decreasing concentration of Mg^{2+} and Ca^{2+} indicate that these positive charged ions were interacting with negative charged bacterial cell surface. As a result of these interactions Mg^{2+} and Ca^{2+} may have started to accumulate on bacterial cell surface. These accumulations serve as a nucleus and first step for biomineralization [17,18,19,20,21,22,23,24].

Table 3.7 and Table 3.8 indicate that characterization of biominerals by XRD analysis.

Table 3.7: Biominerals from 1AGN experiments in MHBM.

1AGN in Modified Halophilic Bacterial Medium			
O mM Na2SO4	**14 mM Na_2SO_4**	**28mM Na_2SO_4**	**56mM Na_2SO_4**
Monohydrocalcite $CaCO_3(H_2O)$	Monohydrocalcite $CaCO_3(H_2O)$	Struvite $MgNH_4PO_4(H_2O)_6$	Monohydrocalcite $CaCO_3(H_2O)$
Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Struvite $MgNH_4PO_4(H_2O)_6$	Monohydrocalcite $CaCO_3(H_2O)$	Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$
	Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Struvite $MgNH_4PO_4(H_2O)_6$
		Magnesiumsulfitehydrate MgSO3(H2O)6	

Table 3.8: Biominerals from 3AGN experiments in MHBM.

3AGN in Modified Halophilic Bacterial Medium			
O mM Na_2SO_4	**14 mM Na_2SO_4**	**28mM Na_2SO_4**	**56mM Na_2SO_4**
Monohydrocalcite $CaCO_3(H_2O)$	Monohydrocalcite $CaCO_3(H_2O)$	Struvite $MgNH_4PO_4(H_2O)_6$	Monohydrocalcite $CaCO_3(H_2O)$
Struvite $MgNH_4PO_4(H_2O)_6$	Struvite $MgNH_4PO_4(H_2O)_6$	Monohydrocalcite $CaCO_3(H_2O)$	Struvite $MgNH_4PO_4(H_2O)_6$
Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Hydromagnesite Mg5(CO3)(OH)2(H2O)4	Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$
	Calciumacetate $C_4H_6CaO_4$		

Table 3.7 indicate that biominerals from Modified Halophilic Bacterial Medium (MHBM) which were inoculated by enrichment culture of 1AGN. According to results; Monohydrocalcite and Dypingite were obtained from 0 mM Na_2SO_4 in MHBM, Monohydrocalcite, Struvite, Dypingite were obtained from 14mM Na_2SO_4 in MHBM, Monohydrocalcite, Struvite, Dypingite and Magnesiumsulfitehydrate were obtained from 28 mM Na_2SO_4 in MHBM, Monohydrocalcite, Struvite and Dypingite were obtained from 56 mM Na_2SO_4 in MHBM and also Table 3.8 indicate that biominerals from Modified Halophilic Bacterial Medium (MHBM) which were inoculated by enrichment culture of 3AGN. As seen in table; Monohydrocalcite, Struvite and Dypingite were isolated from 0 mM Na_2SO_4 in MHBM, Monohydrocalcite, Struvite, Dypingite and Calciumacetate were isolated from 14 mM Na_2SO_4 in MHBM, Monohydrocalcite, Struvite and Dypingite were isolated from 28 mM Na_2SO_4 in MHBM and Monohydrocalcite, Struvite and Dypingite were isolated from 56 mM Na_2SO_4 in MHBM.

As see in Table 3.7 and Table 3.8 Monohydrocalcite, Dypingite and Struvite are the common mineral in all experiment conditions. According to the sequence analyses (Table 3.1 and Table 3.2) 6 % of enrichment cultures from 1AGN and 3AGN are different from each other. However identical minerals were formed in the both experiments.

On the other hand, Table 3.9 and Table 3.10 indicate that biominerals from Solid Modified Halophilic Bacterial Medium (SMHBM) which were inoculated by enrichment cultures of 1AGN and 3AGN. Table 3.9 indicate that biominerals from SMHBM which were inoculated by enrichment culture 1AGN, according to XRD analysis results; Magnesium adipate glycinate and Hydromagnesite were obtained from 0mM Na_2SO_4 in SMHBM, Magnesium adipate glycinate and Dypingite were obtained from 14mM Na_2SO_4 in SMHBM, Magnesium adipate glycinate, Dypingite and Hydromagnesite were obtained from 28mM Na_2SO_4 in SMHBM and Magnesium glutarate glycinate and Dypingite were obtained from 56mM Na_2SO_4 in SMHBM otherwise Table 3.10 idicate that biominerals from Solid Modified Bacterial Medium (SMBM) which were inoculated by enrichment cultures of 3AGN. As is seen in Table 3.10; Magnesium adipate glycinate, Calcium chloride and Calsium phosphate hydrate were characterized from 0mM Na_2SO_4 in SMHBM,

Magnesium adipate glycinate, Hyromagnesite and Dypingite were characterized from 14mM Na_2SO_4 in SMHBM, Magnesium adipate glycinate and Dypingite were characterized from 28mM Na_2SO_4 in SMHBM and Magnesium adipate glycinate and Hydromagnesite were characterized from 28mM Na_2SO_4 in SMHBM.

Table 3.9: Biominerals of sample 1AGN in SMHBM.

1AGN in Solid Modified Halophilic Bacterial Medium			
O mM Na_2SO_4	**14 mM Na_2SO_4**	**28mM Na_2SO_4**	**56mM Na_2SO_4**
Magnesium adipate glycinate $C_8H_{13}MgNO_6$ Hydromagnesite $Mg_4(OH)_2(CO_3)_33H_2O$	Magnesium adipate glycinate $C_8H_{13}MgNO_6$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Magnesium adipate glycinate $C_8H_{13}MgNO_6$ Hydromagnesite $Mg_4(OH)_2(CO_3)_33H_2O$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Magnesium glutarate glycinate $C_7H_{11}MgNO_6$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$

Table 3.10 : Biominerals of sample 3AGN in SMHBM.

3AGN in Solid Modified Halophilic Bacterial Medium			
O mM Na_2SO_4	**14 mM Na_2SO_4**	**28mM Na_2SO_4**	**56mM Na_2SO_4**
Magnesium adipate glycinate $C_8H_{13}MgNO_6$ Calciumchloride $CaCl_2$ Calcium phospate hydrate $CaP_2O_712H_2O$	Magnesium adipate glycinate $C_8H_{13}MgNO_6$ Hydromagnesite $Mg_4(OH)_2(CO_3)_33H_2O$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Magnesium adipate glycinate $C_8H_{13}MgNO_6$ Hydromagnesite $Mg_4(OH)_2(CO_3)_33H_2O$	Magnesium adipate glycinate $C_8H_{13}MgNO_6$ Hydromagnesite $Mg_4(OH)_2(CO_3)_33H_2O$

Solid Modified Halophilic Bacterial Medium (SMHBM) experiments showed that magnesium rich minerals were formed, despite all biomineralization experiments were inoculated with the same enrichment cultures. Hydromagnesite was only formed in the solid experiments whose enrichment cultures were obtained the lake sediment. Lake sediments represent less salinity relative to those taken from salt pans. Dypingite was the common mineral in all experiments especially in more salty ones. These results may indicate that Dypingite formation favor saltier water relative

hydromagnesite.These results indicate that both microbial activity and composition of microenvironments can affect variety and morphology of minerals.

Morphological analyses of biominerals were determined by SEM. 3AGN from 28 mM Na_2SO_4 MHBM experiments , 1AGN from 28 mM Na_2SO_4 MHBM experiments, 3AGN in 14 mM Na_2SO_4 SMHBM were selected for SEM analyses. Amorphous Monohyrocalcite crystals (Figure 3.9), spherical Hydromagnesite crystals and polyhedral Struvite crystals (Figure 3.10) were observed.

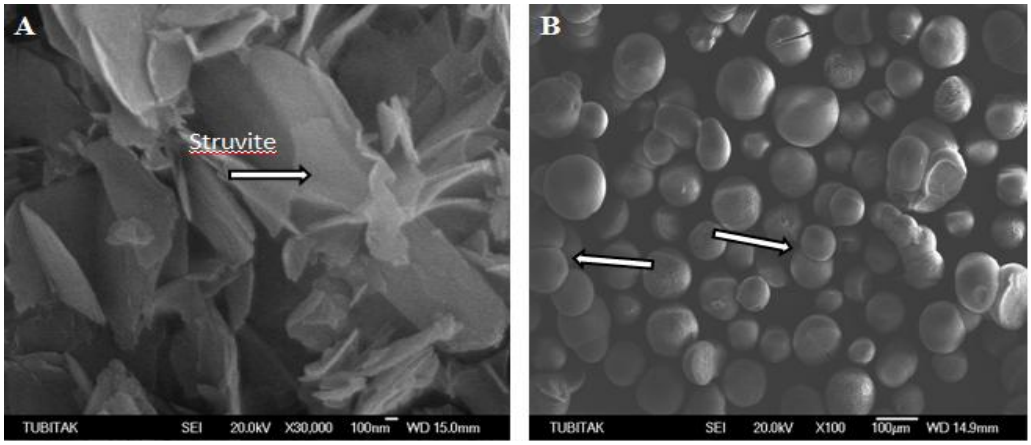


Figure 3.9: Morphology of minerals were observed by Scanning Electron Microscopy (SEM). (A): 28 mM Na_2SO_4 MHBM biomineralization with 3AGN enrichment, arrow points to polyhedral Struvite crystal. (B): 14 mM Na_2SO_4 SMHBM biomineralization with 3AGN enrichment, arrows point dumbbell-shape crystals which are Hyromagnesites.

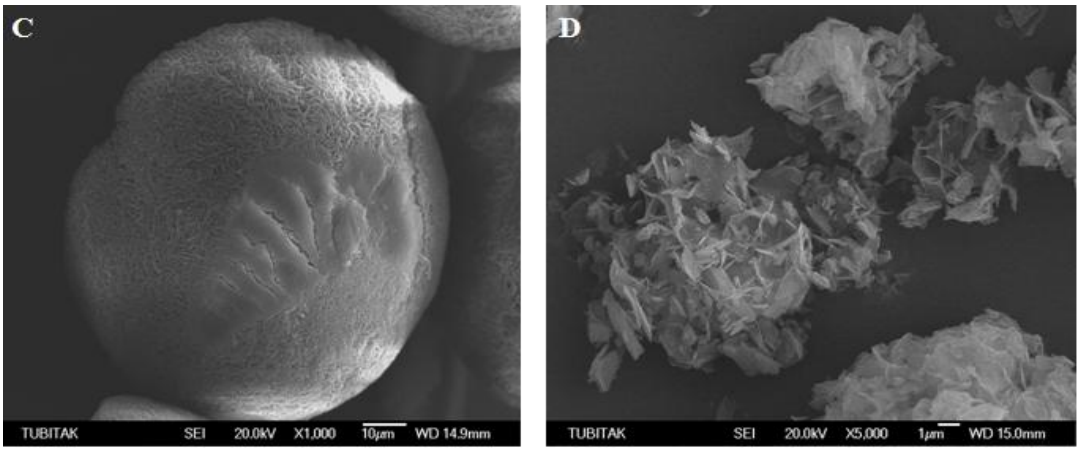


Figure 3.10: Morphology of minerals were observed by Scanning Electron Microscopy (SEM). (C): close up photo (B) spherical Hyromagnesite crystal. (D): 28 mM Na_2SO_4 MHBM biomineralization with 1AGN enrichment, amorphous Monohyrocalcite crystals.

Furthermore, long term biomineralization experiments with 14 mM Na_2SO_4 MHBM inoculated with 6% NaCl containing enrichment cultures from 3AGN, was carried out to understand how long incubation period's effect on biominerals. During the incubation, changing pH values along with Ca^{2+} and Mg^{2+} ions concentrations were measured and listed in Table 3.11, Table 3.12 respectively.

Table 3.11: pH values of 14 mM 3AGN in MHBM.

Days	**14 mM 3AG N (30°C)**	**14 mM 3AGN (+4°C)**
0	7.2	7.2
7	8.9	8.1
14	8.9	8.4
21	9.0	8.8
28	9.1	8.7
35	9.2	9.0
42	9.2	9.0
control	7.1	7.1

Table 3.11 indicates that pH value increased periodically at 42 days and after that it did not show any changing. In addition, there was no change in pH of control experiments but increase in pH values during +4°C experiment was slower than at 30°C experiments.

Table 3.12: Ca^{2+} and Mg^{2+} ions concentrations during long term biomineralization experiments.

	14mM Na_2SO_4 3AGN (+4°C)			**14 mM Na_2SO_4 3AGN (30°C)**		
Days	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/ Ca^{2+}	Ca^{2+}(ppm)	Mg^{2+}(ppm)	Mg^{2+}/ Ca^{2+}
0	430	2041	4.75	430	2041	4.75
7	198.30	1850	9.33	43.60	1840	42.20
14	95.40	1885	19.76	12.50	400	32.00
21	75.10	1800	23.97	23.30	360	15.45
28	32.20	1750	54.35	16.00	280	17.50
35	19.10	1750	91.62	15.80	280	17.72
42	18.00	1735	96.39	16.10	310	19.25

Changing in Ca^{2+} and Mg^{2+} ions concentrations were shown in Table 3.12. As seen in Table 3.12 Ca^{2+} and Mg^{2+} ions decreased in both of the experiments but decrease

of Ca^{2+} and Mg^{2+} ions at +4°C experiment was slower than those at 30°C experiments and the amount of mineral production is less than 30°C experiment.

On the other hand, biominerals were determined by XRD analyses. Identified minerals listed in Table 3.13. XRD results of different temperature and long term experiments showed that the same minerals were formed in both temperatures. Temperature and incubation time may not have effect on the type of mineral but these experiments have not terminated yet.

Table 3.13: XRD results of 30°C and +4°C experiments.

Days	**14 mM 3AG N at 30°C**	**14 mM 3AG N at +4°C**
7	Struvite $MgNH_4PO_4(H_2O)_6$ Monohydrocalcite $CaCO_3(H_2O)$	Monohydrocalcite $CaCO_3(H_2O)$ Struvite $MgNH_4PO_4(H_2O)_6$
14	Struvite $MgNH_4PO_4(H_2O)_6$	Struvite $MgNH_4PO_4(H_2O)_6$ Monohydrocalcite $CaCO_3(H_2O)$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$
21	n.d	Monohydrocalcite $CaCO_3(H_2O)$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$
28	Monohydrocalcite $CaCO_3(H_2O)$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Monohydrocalcite $CaCO_3(H_2O)$ Struvite $MgNH_4PO_4(H_2O)_6$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$
35	Monohydrocalcite $CaCO_3(H_2O)$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Monohydrocalcite $CaCO_3(H_2O)$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$
42	Monohydrocalcite $CaCO_3(H_2O)$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$	Monohydrocalcite $CaCO_3(H_2O)$ Dypingite $Mg_5(CO_3)(OH)_2(H_2O)_5$ Struvite $MgNH_4PO_4(H_2O)_6$

n.d: no data

4. CONCLUSION

In these research we aimed to identify microbial population of Lake Acigol, a hypersaline lake, in order to understand the role of microorganism especially bacteria on recent biomineralization occurring in the lake. For these purposes, samples were collected from Lake Acigol and these samples were enriched in 3% and 6% Nutrient Broth medium and after that biomineralization experiments were performed by Modified Halophilic Bacterial Medium (MHBM), which mimicked Lake Acigol chemical conditions. In addition control experiments with no culture and autoclaved culture were carried out to elucidate the role of bacteria in the biomineralization processes more precisely. . Various minerals formed in all MHBM which were inoculated by enrichment cultures but no precipitations were observed in any control experiments. These results strongly indicated that mineral formation in the lake is primarily controlled by microbial activities. In addition biomineralization experiments, bacterial population of Lake Acigol were determined by 16S rDNA method. Bacterial population were determined both enrichment cultures and sediment samples. According to sequences analysis *Halomonas*, *Idiomarina, Vigibacillus* genus and uncultured bacteria were dominant species in Lake Acigol. These microorganisms represent microbial populations in Lake Acigol.

Overall, our results show that Ca^{2+} and Mg^{2+} concentrations, metabolizable organic substrate and the type of bacteria, are all influential factors in the biomineralization process studied.

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APPENDICES

APPENDIX A: Materials

APPENDIX B: Laboratory Equipment

APPENDIX C: pCR®2.1.-TOPO® Vector Map

APPENDIX D: XRD Graphics

APPENDIX A : Materials

Bacterial strains

E.coli TOP 10 strain F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ- (One Shot TOP10 Electrocompotent cells,Invitrogen) was used in cloning 16S ribosomal genes for species identification procedure.

Cloning vector

pCR®2.1-TOPO® Cloning vector was obtained from Invitrogen and used in species identification procedure.

Enzyme

Taq DNA polymerase

Taq DNA polymerase is used in amplification of DNA fragments. It is obtained from Fermantas

DNA molecular weight markers

DNA Molecular weight markers are obtained from Fermantas.

Oligonucleotides

Oligonucleotides are synthesized by Genova using Applied Biosystems 308A DNA synthesizer. Oligonucleotides are as follows.

pA – F 5’- AGAGTTTGATCCTGGCTCAG 3’

pH – R 5’- AAGGAGGTGATCCAGCCGCA 3’

M13 – F 5’ – GTAAAACGACGGCCAG 3’

M13 –R 5’ – CAGGAAACAGCTATGAC 3’

Culture Medium

Luria-Bertani (LB) Medium

10g tryptone (Merck), 5g yeast extract(Labo), 5g NaCl (Carlo Erba) were dissolved in distilled water and completed up to 1 lt. The pH was adjusted to 7.0 with 10M NaOH and sterilized for 15 minutes under 1.5 atm at 121 °C. The medium was stored at room temperature.

LB Agar Medium

10g tryptone, 5g yeast extract, 5g NaCl and 15 g agar (Merck) were dissolved in distilled water and completed up to 1 lt. The pH was adjusted to 7.0 with 10M NaOH and sterilized for 15 minutes under 1.5 atm at 121 °C.After sterilization 100 mg/ml ampicillin were added to the warmed LB agar media and poured onto plates.

SOC Medium

20 g tryptone, 5 g yeast extract and 0.5 g NaCl were dissolved in distilled water. 10 ml of 250 mM KCl was added to the solution and the pH was adjusted to 7.0 with NaOH. Volume was adjusted to 1 lt with distilled water and the solution was autoclaved. 10mM $MgCl_2$ and 20 mM glucose were added just before the usage.

Nutrient Broth

8 g nutrient broth (Merck) was dissolved in distilled water and completed up to 1 lt. Three different concentrations (30 g/lt, 60 g/lt, 100 g/lt) of NaCl were added to the nutrient broth and sterilized for 15 minutes under 1.5 atm at 121°C. The medium was stored at room temperature.

Stock solution

Ampicillin stock

1g ampicillin was dissolved in 10 ml distilled water, sterilized by filtration (filter pore size is 0.22 μm) and stored at -20°C.

X-gal stock

400 mg X-gal (5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyronoside) was dissolved in 10 ml dimethyl formamide (DMF). Solution was stored in dark at -20°C.

Glycerol stock

80 ml glycerol (Riedel-de-Haen) and 20 ml distilled water were mixed to give a 80 % (v/v) solution. It was sterilized for 15 min at 121 °C.

Buffer solutions

Sodium Acetate Buffer

2.46 g sodium acetate (Reidel-de-Haen) was dissolved in 7 ml distilled water and pH was adjusted to 5.2. Volume was completed to 10 ml to give a 3M concentration and the solution was stored in 4 °C.

50X TAE Buffer

242 g Tris base, 57.1 ml glacial acetic acid and 18.6 g EDTA was dissolved in distilled water up to 1 lt.

Phospahate Buffered Saline (PBS)

8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 were dissolved in 800 ml distilled water and the pH was adjusted to 7.4. Distilled water was added to adjust the volume to 1 lt. Buffer was sterilized by fitration.

APPENDIX B : Laboratory Equipment

Analytical Balances: Precisa BT 610C

Autoclave: 2540 ML benchtop autoclave, Systec GmbH Labor-Systemtechnik.

Centrifuges: Microfuge 18, Beckman Coulter; AvantiJ30I, Beckman Coulter.

Deep freezes and refrigerators: Ultra low freezer MDF-U4086S, Sanyo; Ultra low freezer MDF-U333, Sanyo; 1061 M refrigerator, Arcelik.

Electrophoresis equipment: Horizon 11.14, Whatman, Biometra Gel Casting System, Horizon 20-25, Whatman,Biometra Gel Casting System.

Electroporator: Electroporator 2510, Eppendorf

Gel Documentation System: UVIpro GAS7000, UVItec Limited.

Ice machine: AF 10, Scotsman.

Laminar flow cabinet: Faster Laminar Flow BH-EN 2003.

Magnetic stirrer: Heidolph Standard

Orbital shaker: Forma orbital shaker, Thermo Electron Corporation.

pH meter: Inolab pH level 1, Wissenschaftlich-Technische Werkstätten GmbH & Co KG

Pipettes: epResearch Pipettes, Eppendorf.

Sonicator: Sonoplus, Bandelin

Thermal Cycler: Biometra Thermal Cycler

Thermomixer: Thermoshaker Ts1, Biometra.

Vortex: SI-D256 Daigger

Water Bath: Memert

APPENDIX C: pCR®2.1.-TOPO® Vector Map

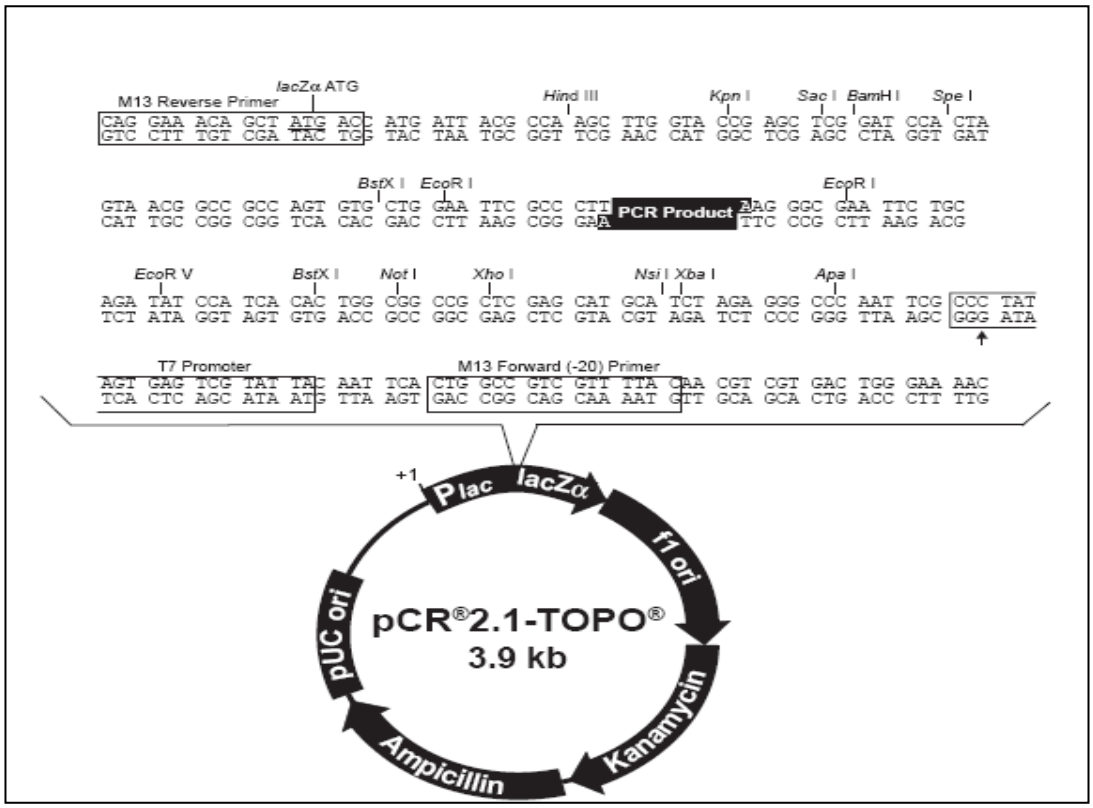

Figure C.1: Vector map of pCR®2.1-TOPO plasmid.

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

APPENDIX D: XRD Graphics

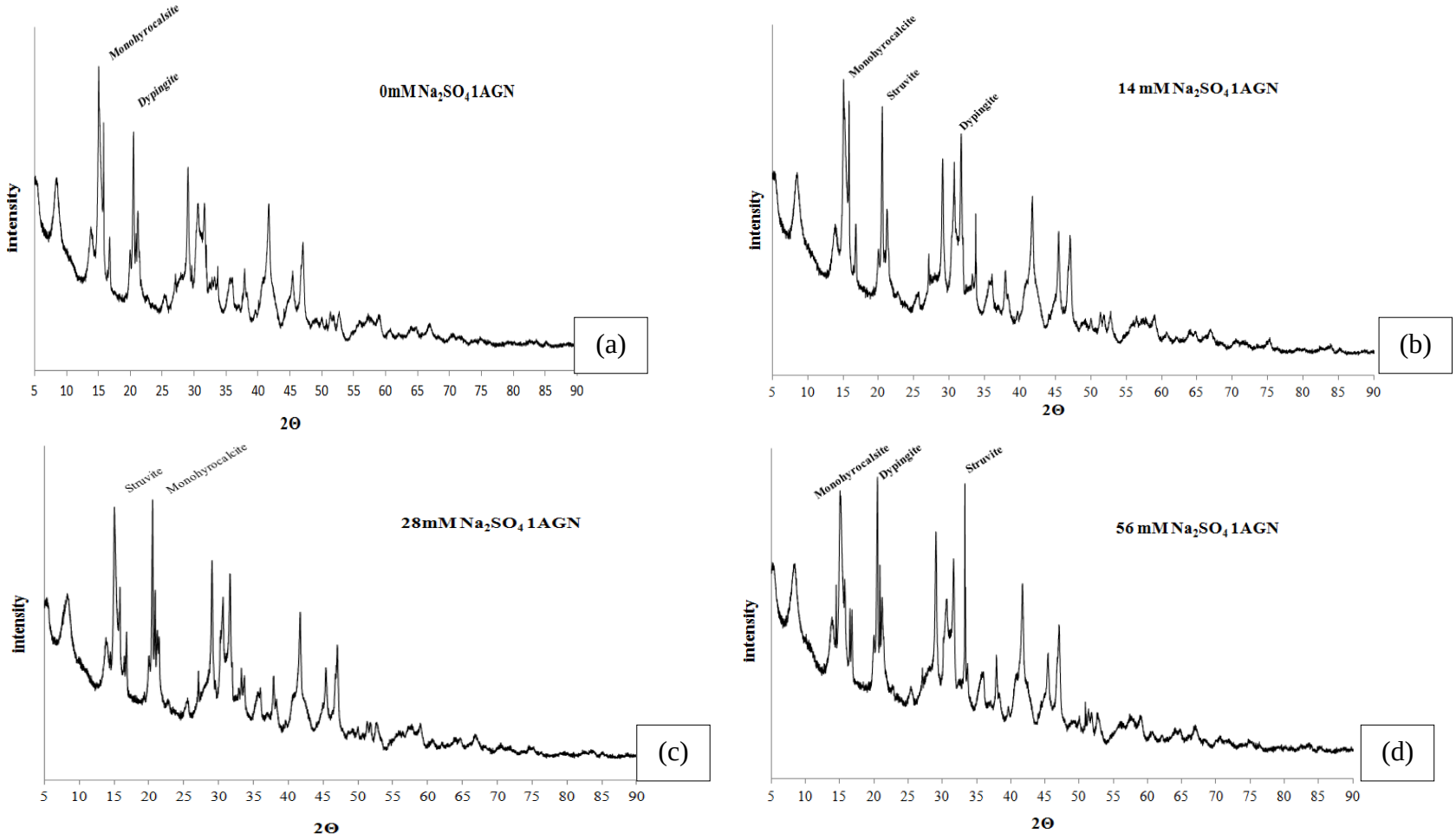

Figure D.1: XRD results of biominerals: (a) 0mM Na_2SO_4 containing media for 1AGN. (b) 14mM Na_2SO_4 containing media for 1AGN. (c) 28m Na_2SO_4 containing media for 1AGN. (d) 56mM Na_2SO_4 containing media for 1AGN**.**

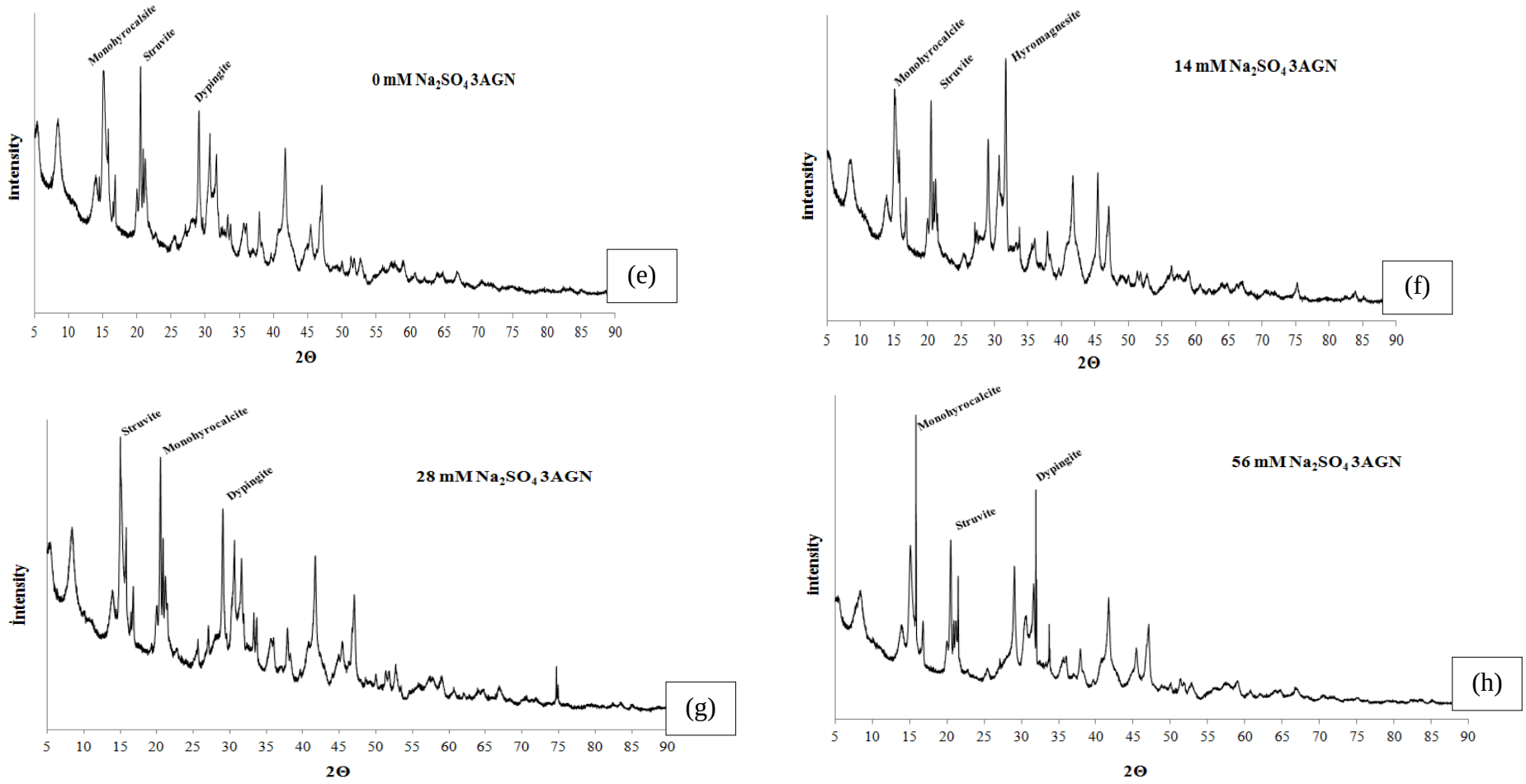

Figure D.2: XRD results of biominerals:(e) 0mM Na_2SO_4 containing media for 3AGN. (f) 14mM Na_2SO_4 containing media for 3AGN. (g) 28mM Na_2SO_4 containing media for 3AGN. (h) 56mM Na_2SO_4 containing media for 3AGN.

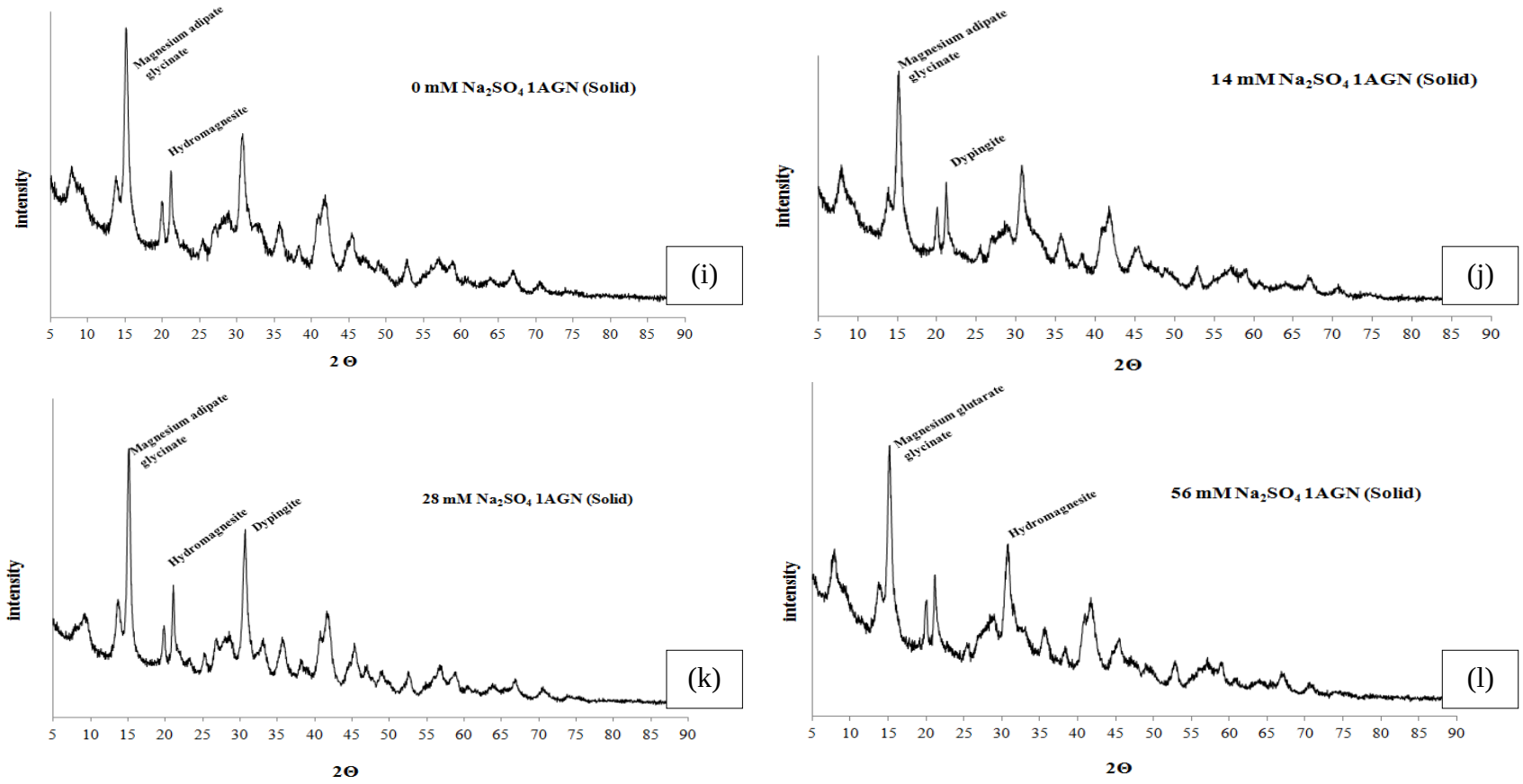

Figure D.3: XRD results of biominerals:(i) 0mM Na_2SO_4 containing solid media for 1AGN. (j) 14mM Na_2SO_4 containing solid media for 1AGN. (k) 28mM Na_2SO_4 containing solid media for 1AGN. (l) 56mM Na_2SO_4 containing solid media for 1AGN.

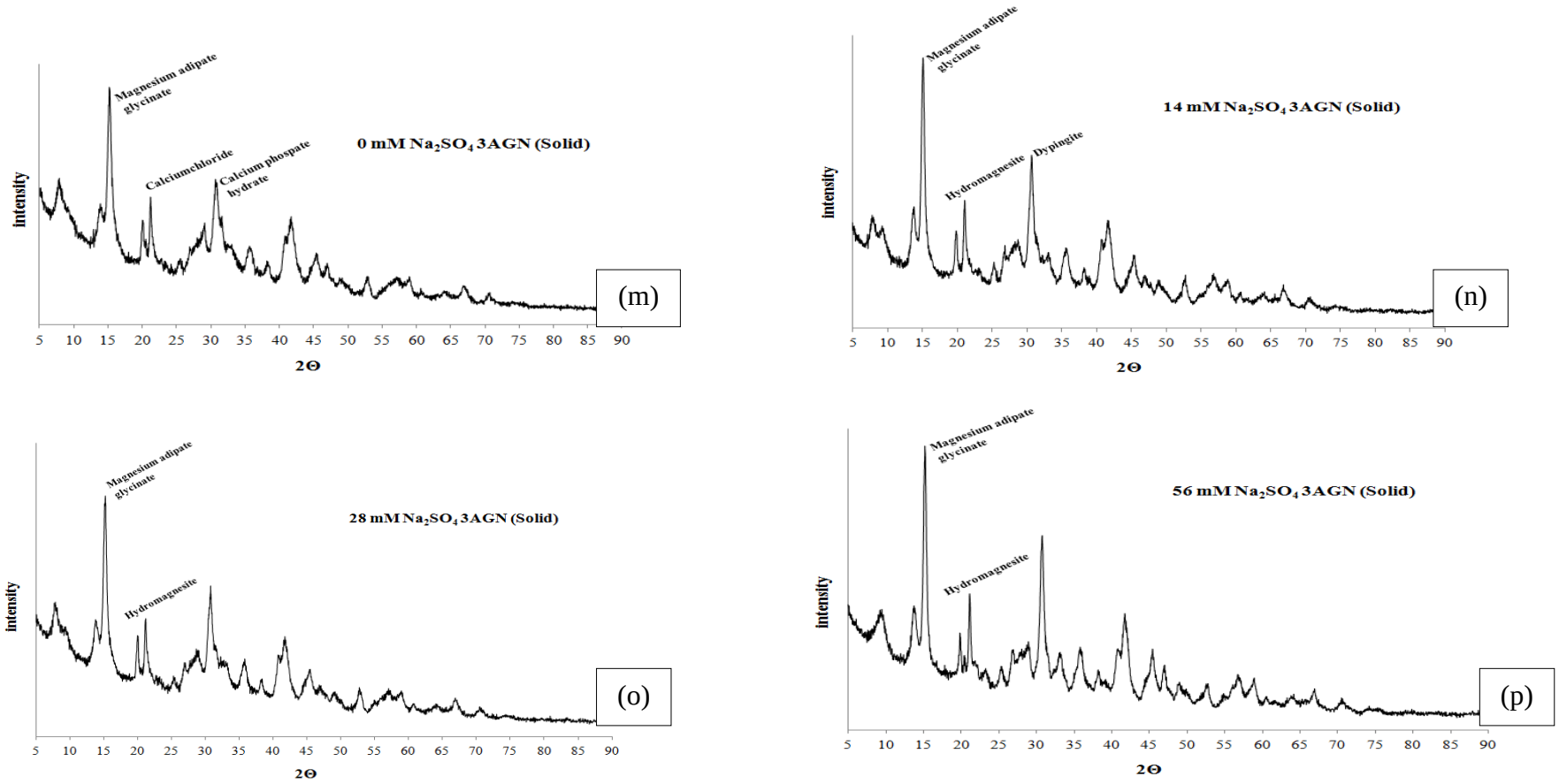

Figure D.4: XRD results of biominerals: (m) 0mM Na_2SO_4 containing solid media for 3AGN. (n) 14mM Na_2SO_4 containing solid media for 3AGN. (o) 28mM Na_2SO_4 containing solid media for 3AGN. (p) 56mM Na_2SO_4 containing solid media for 3AGN.

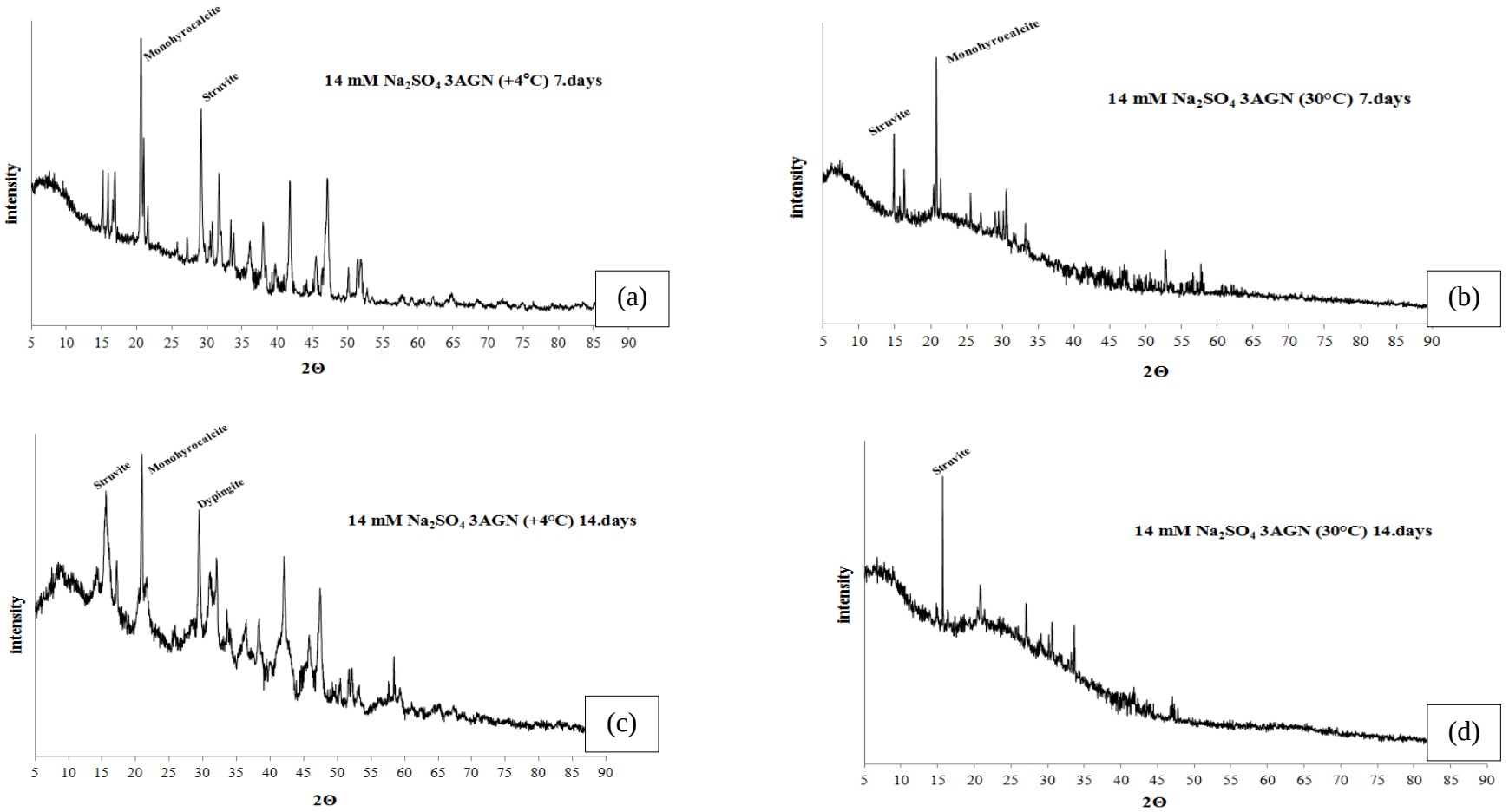

Figure D.5: XRD results of biominerals versus time and different temperature: (a) 14mM Na_2SO_4 containing media for 3AGN at 4°C, 7.days. (b) 14mM Na_2SO_4 containing media for 3AGN at 30°C, 7.days. (c) 14mM Na_2SO_4 containing media for 3AGN at 4°C, 14.days. (d) 14mM Na_2SO_4 containing media for 3AGN at 30°C, 14.days.

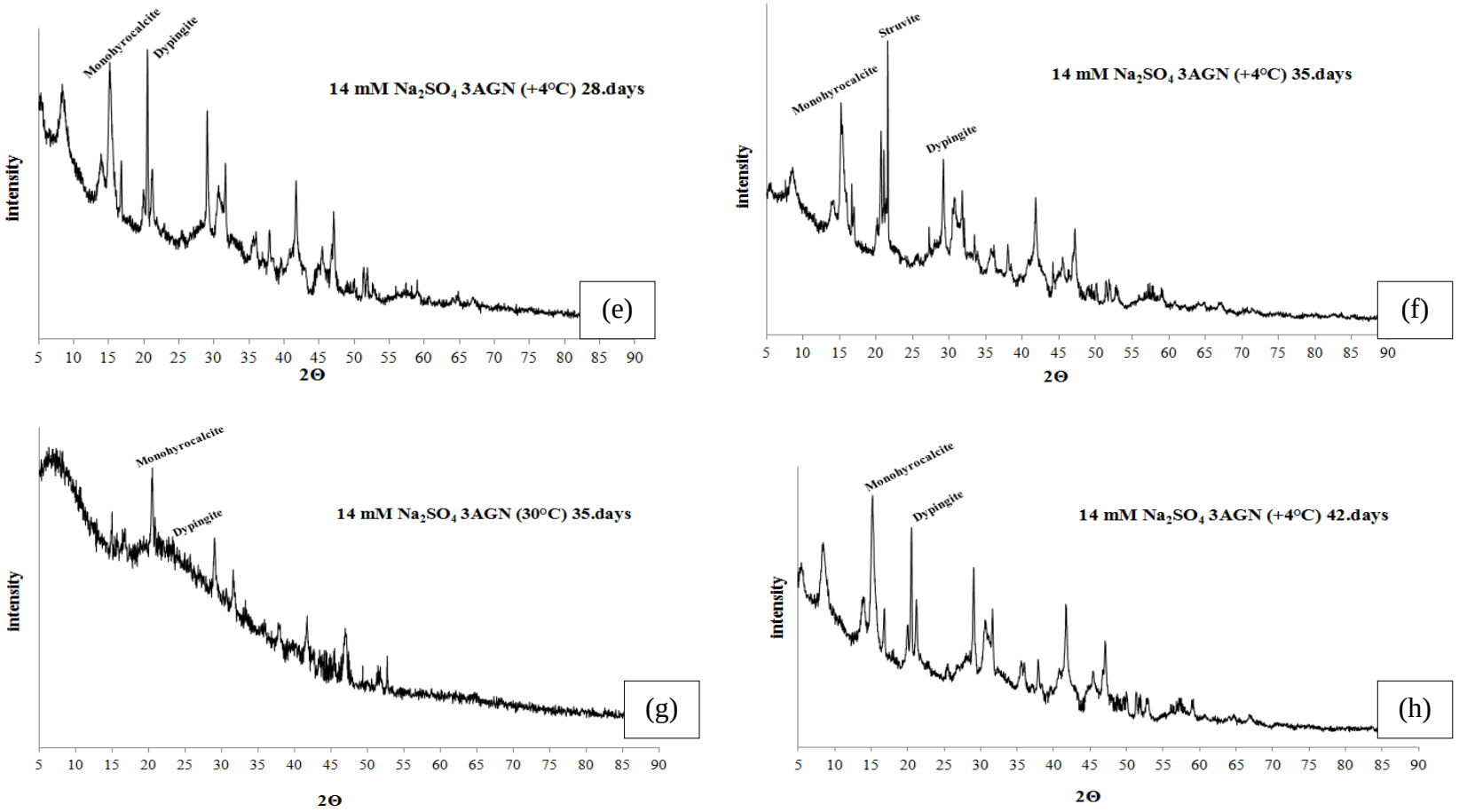

Figure D.6: XRD results of biominerals versus time and different temperature: (e) 14mM Na_2SO_4 containing media for 3AGN at 4°C,28.days. (f) 14mM Na_2SO_4 containing media for 3AGN at 4°C, 35.days. (g) 14mM Na_2SO_4 containing media for 3AGN at 30°C, 35.days. (h) 14mM Na_2SO_4 containing media for 3AGN at 4°C, 42.days

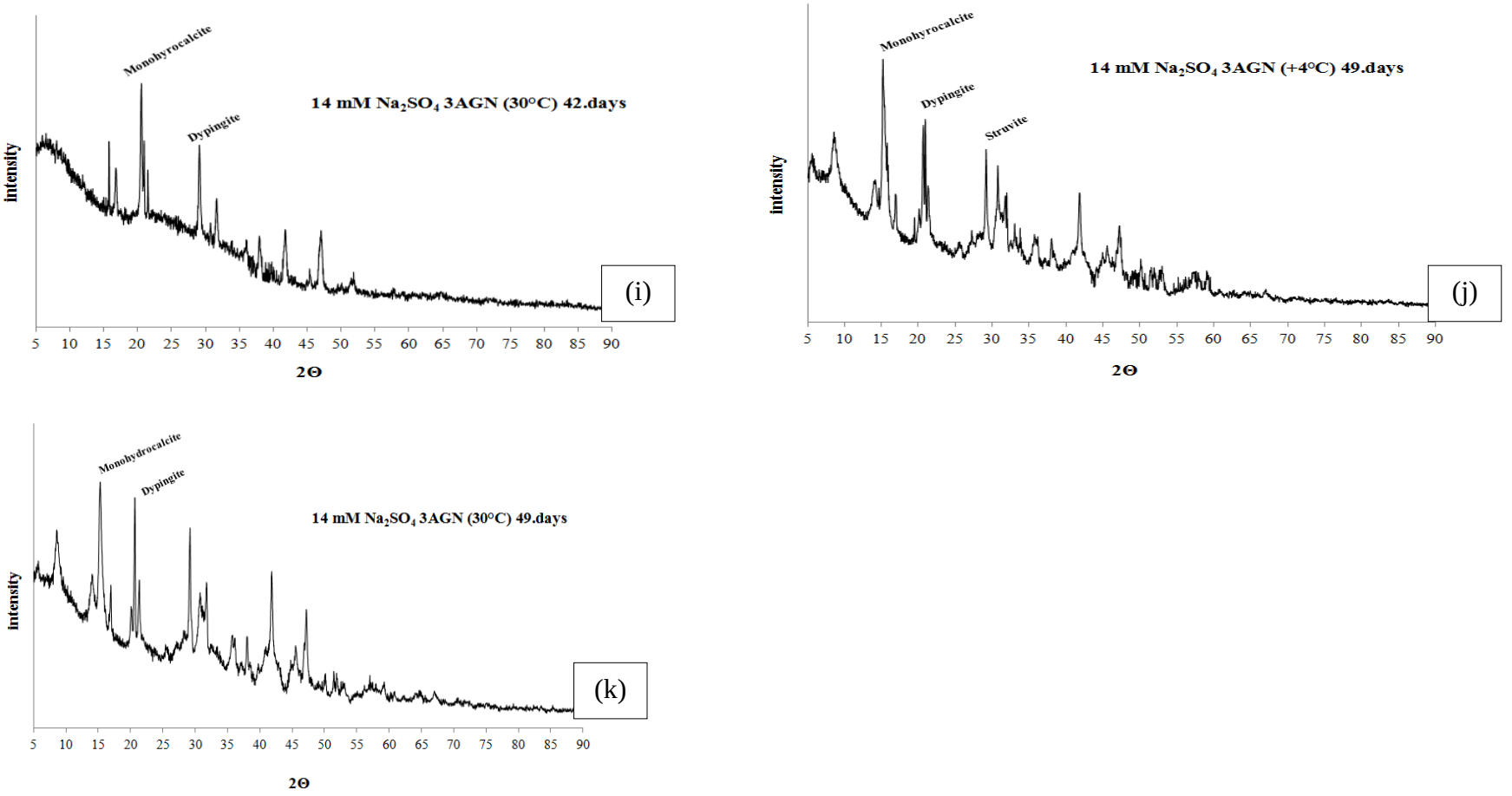

Figure D.7: XRD results of biominerals versus time and different temperature: (i) 14mM Na_2SO_4 containing media for 3AGN at 30˚C,42.days. (j) 14mM Na_2SO_4 containing media for 3AGN at 4˚C, 49.days. (k) 14mM Na_2SO_4 containing media for 3AGN at 30˚C, 49.days.

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