ISOLATION OF HALOALKALIPHILIC MICROORGANISMS FROM LEATHER INDUSTRY

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ABSTRACT

Haloalkalophiles are extremophilic microorganisms that are adapted to saline and alkaline conditions. Different species of them have been isolated so far from soda lakes and soil samples. Haloalkalophilic microorganisms have significant adaptive mechanisms to avoid denaturing effect of salts and to balance their interior pH. Extracellular enzymes that are produced by these halophilic and alkalophilic microorganism are applicable for industrial purposes. Therefore isolation of these organisms from their habitats and study on genotypic characterization constitute initial steps for furter biotechnological studies.

In this study, processing steps of leather factories and their wastewater were chosen for sampling. In order to isolate target microorganisms Horikoshi-I medium including 12% NaCl was used. After isolation microorganisms were purified. Phenotypic tests were applied (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope, sensitivity to antibiotics and extracellular enzyme screenings). For genotypic characterization, genomic DNA was isolated and 16S-ITS rDNA region was amplified.

Differentiation was achieved according to Restriction Fragment Length Polymorphism (RFLP) method by using *Hae* III and *Taq* I endonucleases. Isolates, which represented the different RFLP patterns, were chosen for building up the haplotype groups.

As a result of the study seven different RFLP haplotypes were identified. Moreover, 16S ribosomal DNA partial sequencing was also performed on some of the strains in. These haloalkalophilic microorganisms and their enzymes could be used in different biotechnological studies in the future for various industrial applications.

ÖZ

Haloalkalofilik mikroorganizmalar yüksek alkali ve tuzlu koşullara adapte olmuş ekstremofilik canlılardır. Birçok farklı tür toprak ve soda gölü örneklerinden izole edilmiştir. Haloalkalofilik mikroorganizmalar tuzun zararlı etkilerin ve hücre içi pH değerlerini düzenleme yeteneğine sahiptirler. Halofilik ve alkalofilik olan bu organizmaların ürettiği ekstraselüler enzimler endüstride farklı amaçlar için kullanılanılabilir. Haloalkalofilik mikroorganizmaların yaşadıkları habitatlardan izole edilmeleri ve genotipik tanımlamalarının yapılması endüstri alanında kullanım amaçlı olarak genetik potansiyellerinin tanınması ve ileride yapılacak biyoteknolojik çalışmalar adına atılacak ilk adımı oluşturmaktadır.

Deri fabrikaları işlem basamakları ve atık suyu bu çalışma için örnekleme alanı olarak belirlenmiştir. Bu amaç için Horikoshi-I besi yerine %12 tuz ilave edilerek kullanılmıştır. İzolasyon aşamasının ardından organizmaların saflaştırma işlemi tamamlanmıştır. Fenotipik testler uygulanmıştır. Bunlar;Gram boyama, katalaz ve oksidaz testi, faz-kontrast mikroskobu ile spor gözlemi, ekstraselüler enzim taraması, farklı tuz konsantrasyonlarında (%0, %20, %25) gelişme, farklı sıcaklılarda (45-50 °C) gelişme, farklı pH (7-10-12) değerlerinde gelişme özellikleri). Genotipik karakterizasyon için genomik DNA izolasyonunun ardından 16S-ITS rDNA amplifikasyonu yapılmıştır. RFLP için iki farklı restriksiyon enzimi kullanıldı (*Hae* III and *Taq* I). Kesim ürünlerinin analizi yapılmıştır. Farklı jel profillerine sahip olanların oluşturduğu gruplar her iki enzim için de belirlendikten sonra grup temsilcileri kullanılarak dendogramlar oluşturulmuştur. Hae III restiriksiyon enzim kesimi sonucunda elde edilen grup temsilcilerinin 16S rDNA - PCR amplifikasyon ürünleri kısmi dizi analizleri için kullanıldı.

Haloalkalofilik mikroorganizmalar ve enzimleri daha ileride yapılacak biyoteknolojik çalışmalarda kullanılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES.. xi

LIST OF ABREVIATIONS xii

Chapter1 INTRODUCTION.. 1

1.1.Definition of Haloalkalophiles 1

1.2.Haloalkalophilic Microorganisms 1

1.2.1. Eubacterial Haloalkalophiliphiles 2

1.2.2. Haloalkalophilic Bacillus 2

1.2.3. Gram NegativeHaloalkalophilicSpecies 3

1.2.4. Archaeal Haloalkalophilic Species 3

1.2.5. Distribution and Isolation 5

1.2.6. Biotechnological and Industrial Applications........................ 5

1.2.7. Extracellular Enzymes and Their Industrial Application 6

1.2.8. Proteases from Haloalkalophilic Bacteria............................... 6

1.2.9. Lipases from Haloalkalophiles 8

1.3. Identification of the Haloalkaolphilic Organisms 9

1.3.1. Phenotypic Methods 9

1.3.2. Molecular Characterization Methods.................................... 10

1.3.2.1. Pulsed Field Gel Electrophoresis 10

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)............... 11

1.3.2.3. 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP 11

1.3.2.4. Plasmid Profiling 12

1.3.2.5. Ribotyping 12

1.3.2.6. DNA Sequencing 15

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir) 15
1.5.Thesis Objective 16

Chapter 2 MATERIAL METHODS 17
2.1. Materials 17
2.1.1. Chemicals 17
2.1.2. Samples used for isolation 17
2.2. Methods 17
2.2.1. Isolation and Preservation of the Isolates 17
2.2.1.2. Isolation of Haloalkalophilic Bacteria 17
2.2.1.3. Preservation and Activation of Isolates 18
2.2.2. Determination of Phenotypic Characteristics 18
2.2.2.1. Gram Staining 18
2.2.2.2. Examination of Endospores 19
2.2.2.3. Catalase Test 19
2.2.2.4. Oxidase Test 19
2.2.2.5. Growth at Different Temperatures 19
2.2.2.6. Growth at Different NaCl Concentrations 19
2.2.2.7. Growth at Different pH Ranges 20
2.2.3. Screening for Extracellular Enzymes 20
2.2.3.1. Screening for Protease Activity 20
2.2.3.2. Screening for Lipase Activity 20
2.2.4. Preparation of Genomic DNA 20
2.2.5. Genotypic Characterization 21
2.2.5.1. Identification of Isolates by 16SrDNA–ITS Region 21
2.2.5.1.1. Amplification of 16S rDNA – ITS Region 21
2.2.5.1.2. Electrophoresis of Amplified 16S-ITS Fragments 22
2.2.5.1.3. Chloroform Extraction of PCR Products 23
2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP) 23

2.2.5.1.5. Electrophoresis of Restriction Fragments........... 23
2.2.6. Sequencing... 24
2.2.6.1. Amplification of 16SrRNA Gene 24
2.2.6.2 Extraction of DNA Fragments from Agarose Gel 25
2.2.6.3 Sequencing Reactions 24
2.2.6.4. Purification of Sequencing Reactions.................................. 25
2.2.6.4.1. Removal of unincorporated dye terminators using ethanol presipitation 26
2.2.6.4.2. Assembling the SEQ Sequencer 26

Chapter 3 RESULTS AND DISCUSSION 28
3.1. Isolation of Haloalkalophilic Bacteria 28
3.2. Determination of Phenotypic Characteristics 28
3.2.1. Gram staining.. 28
3.2.2. Examination of Endospore... 28
3.2.3. Catalase and oxidase tests.. 28
3.2.4. Physiological Tests 29
3.3.Extracellular Enzyme Screening.. 32
3.3.1. Extracellular Enzyme Profiles of Isolates.. 33
3.4. Genotypic Characterization.. 36
3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon 36
3.4.2. 16S-ITS rDNA Based RFLP Profiling 37
3.4.3. Analysis of the Genotypic and Phenotypic Groups 40
3.4.4 Analysis of Sequencing Reactions.. 41

Chapter 4 CONCLUSION AND FUTURE PERSPECTIVE.. 42
REFERENCES 43
APENDICES AA1
APENDIX A. CHEMICHALS USED AA1
APENDIX B. RECIPIES MEDIA USED FOR EXPERIMENTS AB1
APPENDIX C. BUFFERS AND STOCK SOLUTIONS..AC1
APPENDIX D. STAINS AND INDICATORS AD1

APPENDIX E. PCR RECIPIES AE1
APPENDIX F. OLIGONUCLEOTIDE PRIMERS.. AF1
APPENDIX G. RECOGNITION SITES OF RESTRICTION ENZYMES AG1
APPENDIX H. ISOLATE NAMES AH1

LIST OF FIGURES

Figure 1.1. Archaea kingdom 4

Figure1.2. Rybotyping 14

Figure3.1. Apperance of extracellular protease 36

Figure 3.2. Apperance of extracellular lipase 36

Figure 3.3. 16S rDNA-ITS Amplification products of some isolates 36

Figure 3.4. *Hae* III and *Taq* I Digests of 16S-ITS rDNA Fragments of Representative Isolates 37

Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles of Isolates 38

Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles of Isolates 38

LIST OF TABLES

Table 1.1. Tolerance for pH, NaCl of selected species of the genus Bacillus ... 2

Table 1.2. "Natro group" of Halobacteria ... 5

Table 1.3. Detergent Proteases ... 7

Table.1.4. Lipolytic activity ... 9

Table 2.1. Samples used for isolation ... 17

Table 3.1. Results of the Physiologic test ... 29

Table.3.2. Tolerance for pH, NaCl of isolated strains ... 30

Table 3.3 Extracellular Enzyme Tests ... 33

Table 3.4 Extracellular Enzyme Profiles of isolates ... 34

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA ... 38

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups ... 39

Table 3.7. Analysis of the Genotypic and Phenotypic Groups ... 40

Table 3.8. Sequencing Results ... 41

ABBREVIATIONS

A	Adenine
bp	Base pair
C	Cytosine
CGT	Cyclodextrin Glucosyltransferase
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
G	Guanine
EDTA	Ethylenediamine Tetra Acetic Acid
ITS	Internal Transcribed Spacer
ISR	Internal Spacer Region
kb	Kilo base
PAGE	Polyacrylamide Gel Electrophoresis
P.E.	Pectinolytic Enzyme
PGA	Polygalacturonic Acid
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
T	Thymine
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA

CHAPTER 1

INTRODUCTION

1.1. Definition of Haloalkalophiles

Haloalkalophiles are one of the main physiological groups of microorganisms classified in alkaliphiles. Lake Magadii in Kenya, Wadi Natrun in Egypt have been defined as the most stable alkaline environments on earth. Therefore the first ecological studies related to haloalkalophilic microorganisms have been performed in these places. The conditions of high mineralization and/or alkalinity in such environments regulate their prokaryotic communities (Trotsenko and Khmelenia, 2001). Both alkalinity (pH 9) and salinity up to saturation (33% (wt/vol)) are needed for the survival of these microorganisms (Koki Horikoshi, 1999).

Alkaliphiles can be classified as obligately and facultatively alkaliphilic organisms. Obligately alkaliphilic microorganisms cannot grow below pH 9. Facultatively alkaliphilic organisms show optimal growth at pH 10.0 or above but they also have the ability to grow at neutral pH.

Halophilicity divided the organisms into three categories: slightly halophilic, moderately halophilic and extremely halophilic. Halophilic microorganisms require certain concentrations of NaCI for their growth, while the halotolerant ones have the ability to grow in the absence or presence of higher concentrations of NaCl (Mimura and Nagata, 2000).

1.2. Haloalkalophilic Microorganisms

Haloalkalophilic microorganisms inhabiting extremely alkaline and saline environments are found in both Archaea and Eubacteria. Most of the extremely salt tolerant haloalkaliphiles belong to *Halobacteriaceae*.

1.2.1. Eubacterial Haloalkalophilic

1.1.2.1 Haloalkalophilic *Bacillus*

Genus *Bacillus* can be characterized by unique physiological characteristics such as Gram staining, spore examination, catalase and oxidase tests. *Bacillus* is a Gram positive usually catalase and oxidase positive and spore producing organism that classified in Firmiculates. It is well known that *Bacillus* strains do not form endospores under many environmental conditions, and the ability to form spores is often difficult to demonstrate. This is very important for their taxonomy. There have been organisms described as non-spore-formers, which had to be reclassified after the detection of spores (Fritze and Claus, 1992). Recent taxonomic studies have separated some of the *Bacillus* members and grouped into new genera: *Alicyclobacillus, Aneurinibacillus, Brevibacillus, Gracilibacillus, Halobacillus, Paenibacillus, Virgibacillus* (Baumgart, 2003)

Table1.1. Tolerance for pH and NaCl of selected species of the genus *Bacillus.*

Species	Source	pH Tolerance	NaCl Tolerance
B. cohnii	Horse meadow soil	obligate alkalophilic	5%
B. horikoshii	Soil	obligate alkalophilic	17%
B. marismortui	Dead sea water	6.0-9.0, pH optimum 7.5	5-25%
B. agaradhaerens	Soil	obligate alkalophilic	16%
B. aclarkii	Soil	obligate alkalophilic	16%
B. horti	Soil, Japan	alkali tolerant	10%
B. vedderi	Bauxite waste	obligate alkalophilic	7.5%
B. alkalophilus	Soil and faeces	obligate alkalophilic	8%
B. clausii	Garden soil	alkali tolerant	10%
B. haloalkaliphilus	Brine/mud, Wadi Natrun	obligate alkalophilic	25%
B. halodurans	Soil	obligate alkalophilic	12%
B. pseudoalkaliphilus	Soil	obligate alkalophilic	10%
B. pseudofirmus	Lake bank soil	obligate alkalophilic	17%
Bacillus sp. DSM8714	River bank soil	alkalitolerant	10%
Bacillus sp. DSM8717	Horse and elephant manure	alkalitolerant	10%
Halobacillus halophilus	Salt marsh soil and solar salterns	7.0-9.0	15%

1.2.3. Gram Negative Haloalkalophilic Microorganisms

Halomonadaceae members are slightly and moderately halophilic bacteria with a broad pH tolerance. They are isolated from saline water, soil samples, leather processing, seawater and estuarine water. Species belong to the Halomonadaceae are Gram negative, rod shaped, non-spore forming, aerobic and also facultatively anaerobic in the presence of nitrate. (Dobzon and Franzman 1996; Franzman et al., 1988). By 16S rDNA sequence analysis, chemotaxonomic and physiological characteristics *Halomonas* have been defined as the only genus in Halomonadaceae (Dobson et al., 1996; Franzman and Tindall 1990). However, recently it has been proposed that Halomonadaceae has four different genera; *Halomonas*, *Alkanivorax, Carnivoras, Chromohalobacter* and *Zymobacte* (Bromguarte, 2003).

1.2.4. Haloalkalophilic Archea

Bacteria and archaea have been first recognized as two distinct groups of Prokarya by Woese et al. (1997). The original classification based on 16S ribosomal RNA gene sequence has been confirmed by extensive phylogenetic studies (Rosa et al., 2002). Extreme halophiles or Euryarchaeota require high salt concentrations, up to 20% NaCl for growth.

Archaea possess genes with recognizable counterparts in the Bacteria, showing that the two groups have functional similarities. Archaea also possess genes that also found in Eukarya (Rosa et al., 2002).

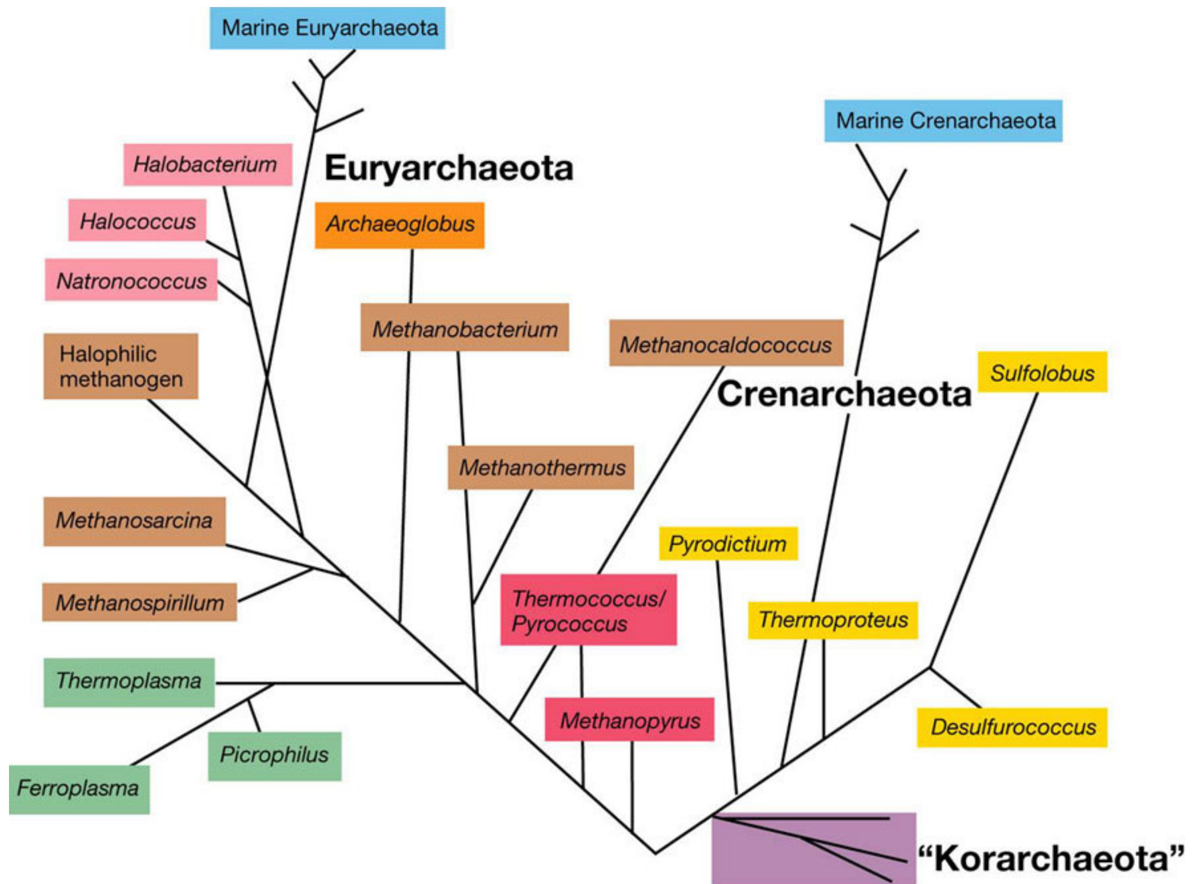


Figure1.1 Archaea kingdom

Euryarchaeaota (*Natronobacterium, Natronococcus, Natronomonas, Natrialba*, *Natronorubrum* genera are classified in "Natro group" of *Halobacteria*. These microorganism are extremely halophilic and alkaliphilic.

Table1.2. “Natro group” of Halobacteria.

Genus	Morphology	Number of species	Habitat
Natronobacterium	Rods	1	Highly saline soda lakes
Natrinema	Rods	2	Salted fish;hides
Natrialba	Rods	2	Soda lakes; beach sand
Natronomonas	Rods	1	Soda lakes
Natronococcus	Cocci	2	Soda lakes
Natronorubrum	Flattened cells	2	Soda lakes

1.2.5. Distribution and Isolation

Isolation of haloalkalophilic microorganisms is performed using different medium ingredients with respect to the group of target organism and the target environment. Actually the salt tolerance range plays an important role because the extremely halophilic organisms can not grow in low concentrations of salt although they have the ability to grow in minimal media. These microorganisms can live at high concentrations of different salts therefore they are populated in salt lakes. Limnological studies focus on the life conditions for extremely halophilic members during seasonal cycles. Anaerobic conditions are suitable in the presence of nitrate for facultatively anaerobes of eucaryotic members.

1.2.6. Biotechnological and Industrial Applications

Extremophilic microorganisms produce cell-associated products that can be used in different industrial applications. First of all their stability and activity in harsh conditions such as alkaline, acidic, high temperature, high concentration of salinity enables broad applications. Extracellular enzymes produced by these organisms are of great interest in biotechnology. Many *Bacillus* species are known to produce alkaline and saline stable proteases, amylases, cellulases, lipases, pectinases and xylanases (Martins et al., 2001)

Enzyme characterization studies with amylase from *Halobacillus* sp. strain MA-2, (Amoozegar et al., 2003) and *Halomonas mediteridiana* (J.Nieto et al., 1999) have

been known. Also the extracellular enzymes from archaeal members have unique features in their molecular structures.

Halophilic microorganisms also used for biological treatment (Kargı and Uygur, 1995).

Halophilic exopolysaccharides have been used in food manufacturing. Furthermore, there is a mereaning demand for non-toxic, biodegradable, environmentally friendly substances. *Halomonas maura* have been studied with novel composition exopolysaccharide (Arias et al., 2003).

1.2.7. Extracellular Enzymes and Their Industrial Application

Extracellular enzymes produced by haloalkalophilic microorganisms have unique properties. They have activity and stability at broad pH ranges and high concentrations of salts in that many other proteins are denaturated. Enzymes from archeal and eubacterial haloalkalophilic organisms have different characteristics. Archaeal enzymes need high concentrations of salt for their activity and stability (Mevarech et al., 2000). This is explained by the comparison of molecular structures from halophilic enzymes with those of nonhalophilic enzymes, this feature is related with adaptation mechanisms used for the protecting of cell from the denaturing effects of salt molecules (Ventosa et al., 1998)

1.2.8. Proteases from Haloalkalophilic Bacteria

Proteases are the enzymes that cleave protein molecules by hydrolysis. The first classification is made by using their cleavage mechanism. Exopeptidases work on the peptide bonds near the amino or carboxy-termini but endopeptidases are active on the peptide bonds distant from the termini of the substrate. The subclassification is based on their catalytic mechanism. Chemically different groups are found on active sites. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao et al., 1998). Subtilisins were grouped in serine type proteases. Catalytic triad of subtilisins consists of aspartic acid, histidine and serine. Although the size of subtilisins varies from 18 kDa to 90 kDa, all the subtilisins used in detergents have a size of

approximately 27 kDa. Most of the alkaline proteases were subtilisins. At present, less than 15 different enzyme molecules are used in detergents worldwide. These enzymes originate from *B. amyloliquefaciens, B. licheniformis, Bacillus clausii, Bacillus lentus, Bacillus alkaloophilus,* and *Bacillus halodurans*

The success of subtilisins is based on several factors, including their high stability and relatively low substrate specificity, features common in extracellular proteases. Their production as extracellular enzymes is of course an important factor in itself, as this greatly simplifies the separation of the enzyme from the biomass and facilitates other downstream processing steps. Another important point is the ability of *Bacillus* strains to secrete enzymes over a very short period of time into the fermentation broth.

Detergent industry prefers the enzymes, which are stable at high pH and compatible with chelating and oxidizing agents (Rao et al., 1998). Table represents the detergent proteases available (Maurer, 2004).

Table1.3. Detergent proteases (Maurer, 2004).

Trade mark	Origin	WT/PE	Production strain	Synonym
Genencor	*B. amyloliquefacies*	PE	*B. subtilis*	
Novozymes	*B. clausii*	WT	*B. clausii*	Subtilisin 309
Genencor	*B. lentus*	WT	*B. subtilis*	
Kao	*B. alkalophilus*	WT	*B. alkalophilus*	
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Novozymes	*B. halodurans*	WT	*B. halodurans*	Subtilisin 147
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. alkalophilus* PB92	PE	*B. alkaliphilus*	

PE, protein engineered; WT, wild type.

Since 1997, several gene-shuffling approaches have been performed with subtilisins. Interesting results from the shuffling of 26 protease genes have been described for properties such as activity in organic solvents, temperature stability, and activity at high or low pH (Minshull et al., 1999). Little, however, has been published on stain removal. Owing to the large number of variant molecules generated by

shuffling and other random techniques, screening methods with high-throughput and increased relevance have had to be developed. Unfortunately, these methods are still not entirely satisfactory, which might explain why no outstanding new subtilisin variant created by one of the gene shuffling technologies is yet present in detergents. Subtilisins for detergents are produced in *Bacillus*, because these species are able to secrete large a mounts of extracellular enzymes. The control mechanisms involved in the production of proteases in Bacillus are extremely complex and still not fully understood. An example is the two-component regulatory system that acts as a quorum sensing mechanism in *B. Subtilis* and which has been found to control the expression of the alkaline protease. This regulatory system is encoded on the chromosome and on endogenous plasmids. (Maurer, 2004)

Arhaea members have proteolytic activity in high salt concentrations (Eichler, 2001). Proteases from *Natronococcus occultus* and *Natrialba magadi* have been characterized (De Castro et al., 2001).

Leather industry uses proteases at dehairing and baiting steps (Kumar and Takagi, 1999). Stable proteases under alkaline and saline conditions would be most effective enzymes for such purposes.

It has been reported that Halophilic bacteria destroied brine-cured hides with their high proteoliytic activity (Bailey et al., 1998).

The studies about using proteases and gelatinases for the pretreatment of leather waste including cromium are important for the convertion of waste material to animal feed (Taylor, 2002).

Silver recovery has been achieved with different types of alkaline proteases at high alkaline conditions (Singh et al., 1999, Kumar and Takagi, 1999).

1.2.9. Lipases from Haloalkalophililes

Lipases, (triacylglycerol acylhydrolases, EC 3.1.1.3), are able to catalyse both the hydrolysis and the synthesis of esters bonds in lipid molecules. Lipid molecules are composed of glycerol and long-chain fatty acid molecules. Esterification and hydrolysis reactions that can occur in organic solvents are very important (Ishıkawa and Ogino,

2000). Most of the lipolytic enzymes used commercially are produced by alkalophilic microorganisms and they are used as detergent addiditives (Sharma et al. 2001).

Table.1.4. Lipolytic activity

Active site	Catalytic triad consisting of the amino acids serine, aspartic (or glutamic acid) and histidine; homologue to the triad of serine proteases.
Substrates	Oils (olive, soybean, mineral oils),Triglycerides (triolein, tributyrin), Synthetic surfactants (Tweens 20 and 40).
Other Activities	Phospholipase, lysophospholipase, cholesterol esterase, cutinase, amidase.

Lipases from *Halomonas* strains have been characterized (Mattiasson et al., 2004).

Alkaline lipases are mainly used in detergent industry. Approximately 1000 tons of lipases are added to the 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Alkaline lipases to be used in detergents should meet certain requirements. They should hydrolyze fats of various compositions, be stable in harsh washing conditions (pH 10-11, 30-60 °C) and in the presence of surfactants (linear alkyl benzene sulfonates (LAS) and proteases) (Sharma *et al.,* 2001).

1.3. Identification of the Haloalkalophilic Organisms

Identification of the haloalkalophilic microorganisms are performed by using the phenotypic and genotypic characteristics.

1.3.1. Phenotypic Methods

Morphological characteristics can be examined by direct observation, which give simple information on the organism for example colony formation that includes colony shape, diameter and color. Furthermore, microscopic observations after different chemical treatments give detailed information on the cell morphology. These are cell shape, Gram behavior and sporulation type. Phase contrast microscopy gives

information on the cell shape and sporulation type without any chemical treatment. Some tests can provide information about the adaptive mechanisms and relation between interior of the cell and the medium. These tests conditions are: growth at different pH ranges, temperatures, pressure and salt concentrations. Bergey's Manual of Systematic Bacteriology a major taxonomic compilation of prokaryotes gives information in all recognized species of prokaryotes and contains number of keys to evaluate the properties given above (Madigan *et al.,* 1997).

Phenotypic methods also include biotyping, antibiogram, phage typing, serotyping, PAGE/immunoblotting and multilocus enzyme electrophoresis (Busch and Nitschko, 1999).

Although molecular characterization methods provide clear differentiation and precise identification, using of phenotypic characterization methods combined with molecular techniques are required for taxonomic studies and functional purposes.

1.3.2. Molecular Characterization Methods

1.3.2.1. Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is used for the analysis of microbial genomes and molecular characterization of the microorganisms below species level. It enables to the seperation of large DNA molecules and therefore whole genomic DNA can be analysed. Analysis of chromosomal macrorestriction patterns by PFGE is a powerful method to produce fingerprints of closely related strains (Farber, 1996). Application of the method can differ for different type of organism. Different organism can require different cell disruption treatments for the releasing the genomic DNA. Also the size and content of genomic DNA can differ among the organisms. Thus, optimization parameters such as lysis of cells, electrophoresis conditions (pulse time, voltage) are the main points for obtaining fine discriminative PFGE results.

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)

DNA fragments are amplified by the polymerase chain reaction (PCR) using short (generally 10 bp) synthetic primers of random sequence. These oligonucleotides serve as both forward and reverse primer at the same time, and are usually able to amplify fragments at 3-10 genomic sites simultaneously. Amplified fragments (within the 0.5-5 kb range) are separated by gel-electrophoresis and polymorphisms are detected as the presence or absence of bands of particular size. These polymorphisms are considered to be primarily due to variation in the primer annealing sites. Some advantages of RAPD are as follows: low quantities of template DNA are required (5-50 ng per reaction), no sequence data for primer construction are required, easy and quick to assay, low costs involved, generation of multiple bands per reaction, and it suitable for automation.

1.3.2.3 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP (Restriction Fragment Length Polymorphism)

In most prokaryotes the ribosomal RNA genes reside sequentially in the chromosome (16S-23S-5S) and they are transcribed as a single polycistronic RNA molecule (Luz et al., 1998). These ordered genes have long been served as a genetic marker to study microbial diversity. The 16S rRNA gene sequence has been used for assessing bacterial phylogenies (Abd-El-Haleem et al., 2002). However, it has only provided information useful for identifications at genus level (Shaver et al., 2002).

Below the genus level, additional rDNA sequences have been necessary. The use of 23S rDNA has also been limited by its larger size and insufficient sequence data (Abd- El-Haleem et al., 2002). The intergenic spacer region (ISR) or also known as intergenic/internal transcribed spacer (ITS) (Abd-El-Haleem et al., 2002; Daffonchio et al., 2000; Shaver et al., 2002; Fisher and Triplett, 1999; Toth et al., 2001) between 16S and 23S rDNA genes, includes both conserved and highly variable sequence motifs, such as tRNA genes, and anti-terminators (boxA) (Abd-El Haleem et al., 2002). ISRs are generally found in multiple copies in most bacteria genomes. Since they are

hypervariable with respect to adjacent genes, they can differentiate between multiple operons in the cell (Daffonchio et al., 1998). The size of the spacer has been found to differ considerably among different species, and even among the different operons within a single cell (Garcia-Martinez et al., 1999).

Figure 1.8 Schematic representation of a 16S-23S spacer and organization of ITS functional regions (Garcia-Martinez et al., 1999).

The method is based on the amplification of 16S rDNA and ISR regions as singleamplicon. The resulting amplicon is then digested with frequent cutting enzymes. Finally restriction fragments obtained are separated by agarose gel electrophoresis and restriction patterns are then compared. The method could discriminate different groups of bacteria at species level.

1.3.2.4. Plasmid Profiling

Plasmids are the genetic materials of bacteria carried as extracromosomal elements. They are self-replicative and they can be transferred naturally between the microorganisms. Genes coded in plasmid DNA structures give different characteristics to microorganisms.

Plasmids isolated from the microorganisms give variable profiles with respect to the organism type and the habitats. Therefore the gel profiles of plasmids for closely related organism would be useful in taxonomic studies (Farber, 1996).

1.3.2.5. Ribotyping

Nucleic acid probes that recognize ribosomal genes are used for ribotying. Ribosomal RNA (rRNA) genes are composed of 23S, 16S and 5S regions. They are known to be highly conserved. Whereas most of the bacterial genes present in one copy, rRNA operons can be present in anywhere more than one copy (2-11 copies per bacterial cell). Thus the more copies of rRNA operon enables more discriminatory riboyping. In practice, first bacterial DNA is isolated and digested by restriction endonucleases. After agarose gel electrophoresing, DNA is transferred onto nylon or nitrocellulose membrane by capillary system or electrophoresis. The nylon membranes

are used as a solid support for probing. Probes specific to 23S, 16S, 5S rRNA genes are labeled using radioactive or non radioactive labeling techniques (non isotopic cold-labelling systems). Hence fingerprint patterns of bacterial DNA containing ribosomal genes are created. These patterns are varied from 1 to 15 bands and compared among the isolates. For another ribotying technique called as chemiluminescence ribotyping, digoxigenin-labeled cDNA (DNA which has been made by reverse transcription of rRNA) can be seen in figure 2. One of the major advantages of ribotyping is that because of the similarity of ribosomal genes, universal probes can be used. Reproducibility is also another advantage of this genotypic method.

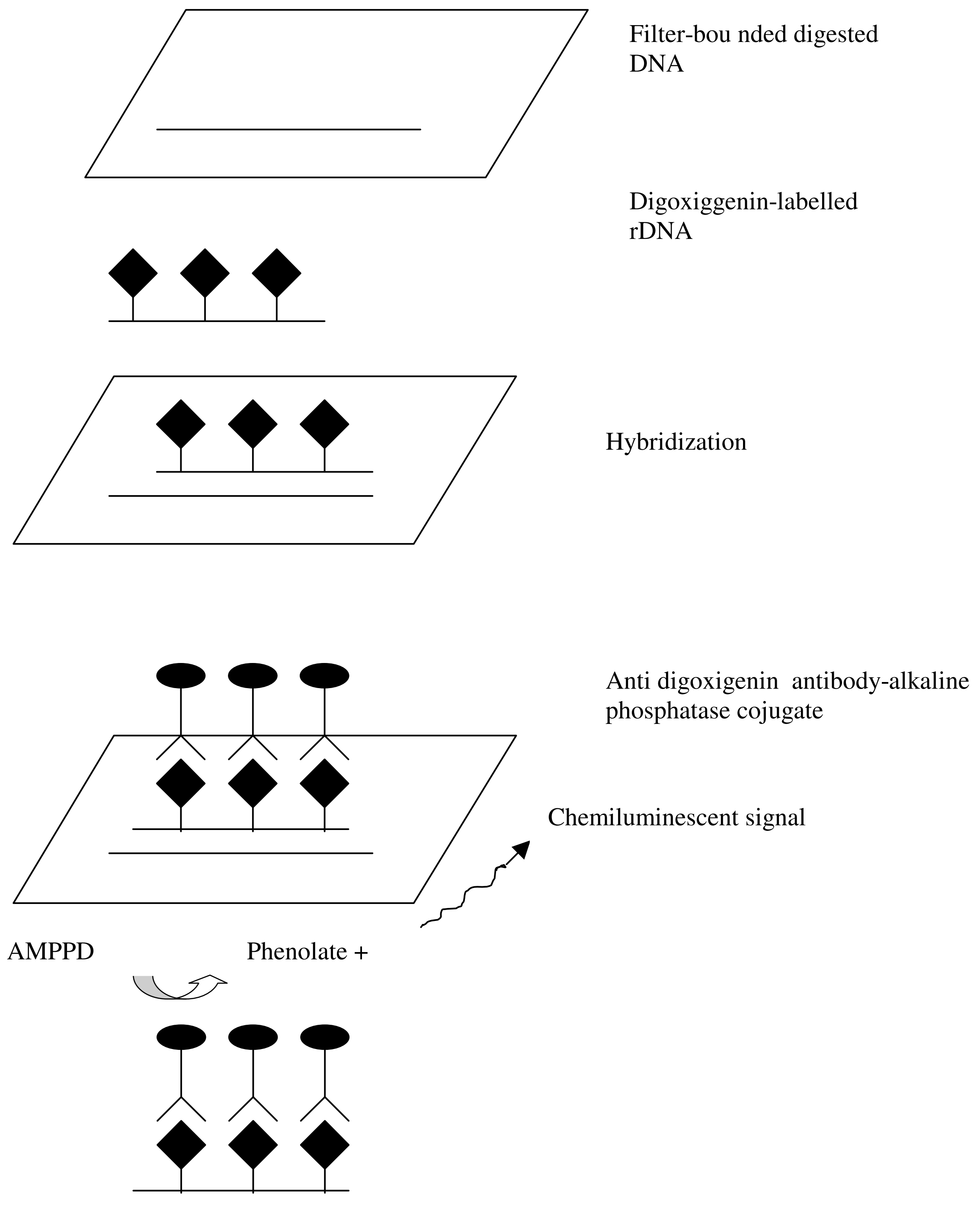


Figure1.2. Schematic representation of chemiluminescence ribotyping. AMPPD, adamantly-1, 2-dioxetane phosphate (Bingen et al., 1994)

1.3.2.6. DNA Sequencing

DNA sequencing is used for investigation of the phylogenetic relationship of organisms. This tecnique is based on the exact order of nucleotides in a region of the DNA molecule Automated DNA sequencing generally includes PCR amplification of a DNA region of interest, computer-aided sequence analysis and interpretation. For DNA sequencing the region of the chromosome to be analysed must be short consisting of a variable sequence flanked by highly conserved region. The variability within the selected sequence must be sufficient to differentiate microorganisms. And also the selected sequence should not be horizontally transmissible to other strains of species (Olive and Bean, 1999).

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir)

Leather factories of Ege Region are populated in Menemen – İzmir. twenty different companies have leather factories in Menemen region. It is known that industrial activities bring the most important pollution problems. For this reason leather factories were placed far from the centre of the city. Leather processing steps are as fallows:

- Curing
- Beamhouse operations which wash and soak the hides or skins and (at most tanneries) remove the attached hair.
- Tanyard processes in which the tanning agent (primarily chromium) reacts with and stabilizes the proteinaceous matter in the hides or skins.
- Finishing or post-tanning processes.

Waste Water of the leather factories are removed from the other factories at Menemen region and a simple waste water treatment is applied. The classical scheme is given below (UNEP/IEO, 1994).

- Pre-treatment: mechanical screening to remove coarse material.

- Primary treatment: sulphide removal from beamhouse effluents; chrome removal from tanning effluents; flow equalization; physical-chemical treatment for

-BOD removal and neutralization.

- Secondary treatment, usually biological.

- Tertiary treatment, including nitrification and denitrification.

- Sedimentation and sludge handling

1.5Thesis Objectives

The purpose of this study was,

- Isolation of haloalkalophilic strains
- Screening for their extracellular enzymes: protease and lipase
- Characterization of these isolates by phenotypic and genotypic methods.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used were listed in Appendix A.

2.1.2. Samples used for isolation

Table 2.1. Samples used for isolation

Sample Type	Location
Polluted Soil	Leather Factories waste (İzmir – Menemen)
Leather processing	Leather Factories (Izmir-Menemen)

2.2. Methods

2.2.1. Isolation and Preservation of the Isolates

2.2.1.2. Isolation of Haloalkalophilic Bacteria

Dilution plate method was used for the isolation of haloalkalophilic bacteria (Mora et al., 1998). Ten g soil, leather or 10 ml of water samples were transferred in 90 ml of 0.85% saline water. In order to homogenize the samples 6h incubation was performed in orbital shaker at room temperature. The samples were pasteurized for 10 m. at 80°C in a water bath in order to kill most of the vegetative cells and eliminate non-

spore forming bacteria (Mora et al., 1998). After the heat treatment, for dilution, 1ml aliquots from each sample was transferred in 9 ml of 0.85 % saline water and 6 fold dilutions were prepared. One ml of the dilutions was plated on appropriate solid media (Appendix B.1) and incubated for 5 days at 37 °C. Single colonies were picked and they were purified using streak plate method.

2.2.1.3. Preservation and Activation of Isolates

Cultures were grown in Horikoshi-I (Apendix B.1) agar plates containing 12% NaCl and incubated for 2 days at 37°C they were then transferred into the isolation broth. After turbidity formation in isolation broth, 0.5ml of each culture were transferred into cryotubes and 0.5ml of isolation broth containing 20% glycerol was added. These steps were all performed on ice. Then tubes were mixed gently by pipetting. Cultures were stored at -20°C for an hour they were then stored at -80°C. For the phenotypic test culture activation was applied from 20% glycerol stocks. For phenotypic and genotypic identification of the isolates, cultures were prepared from these stocks.

2.2.2. Determination of Phenotypic Characteristics

2.2.2.1. Gram Staining

Isolates were stained by modified Gram method for halophilic bacteria. Eighty µl of sterile 0.85% saline water were pipetted onto slides the organisms were then suspended and mixed thoroughly. Overnight culture was spread onto the microscope slides until a thin film formed by using tooth stick. After being air dried, the slides were fixed and desalted simultaneously by immersing in 2% acetic acid for 5 min. Then they were dried again. The smears were covered with 0.25% aqueous solution of crystal violet (Apendix D.2.1) for 1 min. After rinsing under the tap water, the slides were transferred into iodine (Apendix D.2.2) solution and were kept for 1 min. Following this step, slides were washed in alcohol for 6 s. They were then stained with safranin (Apendix D.2.3) for 30 s. After staining, the slides were dried on paper towels and the

cells were examined under light microscope. Gram (+) cells assumed purple color while Gram (-) cells appeared pink or red.

2.2.2.2. Examination of Endospores

Isolates were grown on Horikoshi-I (Apendix B.1) agar plates and 0.0005% $MnCl_2$ (Travers, 1987) were added in to the medium. Observations for spore formation were monitored at 3-4- 5-6th day.

2.2.2.3. Catalase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. Catalase activity was observed after dropping 3% hydrogen peroxide solution onto the target colony. Formation of the air bubbles indicated the presence of catalase activity.

2.2.2.4. Oxidase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. 1% solution of tetramethyl-p-phenylenediamine was poured onto a filter paper placed in a petri dish. Then the target colony was taken from the agar plate and spotted onto wet filter paper. Observation of the blue-purple color indicated the oxsidase activity.

2.2.2.5. Growth at Different Temperatures

The isolates were grown on nutrient agar (Apendix B.2) 45°C and 50°C. Observations for growth were noted during the 5-day incubation.

2.2.2.6. Growth at Different NaCl Concentrations

Cultures were tested for their salt tolerance on Horikoshi-I (Apendix B.1) agar plates containing 0%, 20%, 25 % NaCl during the 5-day incubation.

2.2.2.7. Growth at Different pH Ranges

Cultures were tested for pH tolerance in nutrient agar plates at different pH points (7, 10, and 12). Growth behaviors were observed during the 5-day incubation

2.2.3. Screening for Extracellular Enzymes

2.2.3.1. Screening for Protease Activity

Proteteolytic activity of the isolates observed by adding casein into Horikoshi–I (Apendix B.1) medium adding sikimmed milk. Different concentrations of NaCl (12% and 20%) were added. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete proteases extracellularly, give clear zones around the colony (Horikoshi, 1999).

2.2.3.2. Screening for Lipase Activity

Lipolytic activity of the isolates was observed by using the medium described in Appendix B.5. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete lipase extracellularly, gave opaque zones around the colony (Haba et al., 2000).

2.2.4. Preparation of Genomic DNA

Cultures were grown on solid Horikoshi – I (Apendix B.1) medium. Cells were suspended in 1.5 ml of sterile water in petri dishes. Then cell suspension was transferred into eppendorf tubes. In order to prepare cell pellets, tubes were centrifugated for 5 min at 5000 rpm. Pellets were resuspended in 567 µl 1Xte (Apendix C.4). Thirty µl 10% SDS and 3µl proteinase K solution (20 mg/ml) were added and the solution was mixed thoroughly. Cell lysis was performed with 1h incubation at 37°C. Afterwards 0.1 ml of 5 M NaCl were added and mixed well. Then 80µl of CTAB/NaCl (Appendix C.8) were added, the tubes were then mixed and incubated for 10 min at 65°C. Following the incubation, 0.7 ml of chloroform/isoamyl alcohol (Appendix C.2) were added, mixed thoroughly and centrifuged for 5 min at 10000 rpm. Aqueous, viscous supernatant was

transferred into a fresh eppendorf tube. An equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged for 5 min. The supernatant was again transferred into a fresh eppendorf tube and 0.6 volume of isopropanol were added to precipitate the nucleic acids. The tube was then shaken back and forth until a white DNA precipitate became visible. DNA wool were taken with a pipet tip and placed into a new tube, contain 300µl 70% ethanol. The tubes were stored at -20°C for 1h. Afterwards tubes were centrifuged for 15 min at 6000 rpm. Then the supernatant was discarded and DNA pellet was washed with 500 µl 70% ethanol. It was centrifuged for 10 min at 8000 rpm. After this, ethanol was discarded. The pellet was dried and dissolved in 100 µl 1xTE containing 100 µg/ml RNAase. Then tubes were incubated for 1h at 37°C. After the incubation the sample volume was adjusted to 400 µl with 1xTE. DNA was dissolved by alternating cold-heat shocks (for 10 min at 80°C and 20 min at -20°C, repeated twice). To further purify DNA an equal volume of phenol was added and mixed well. An equal volume of chloroform was added and mixed well. Then the tubes were centrifuged for 5 min at 6000 rpm. The aqueous phase was transferred into a fresh eppendorf tube. Equal volume of chloroform /isoamyl alcohol was added and mixed well. Samples were centrifuged for 5 min at 6000 rpm. Chloroform /isoamyl alcohol step was repeated once again and DNA was precipitated by adding 1/10 sample volume of 5 M NaCl and 2 volumes of 99% ethanol. Pellet was washed in 500 µl 70% ethanol by centrifugation for 5 min at 6000 rpm. After that, ethanol was removed and samples were dried for 10 min at 37°C. DNA was dissolved in 20µl, 50µl, 100µl or 200µl 1xTE according to the pellet size. Then the samples were preserved at -20 °C.

2.2.5. Genotypic Characterization

2.2.5.1. Identification of isolates by 16S rDNA - ITS rDNA - RFLP

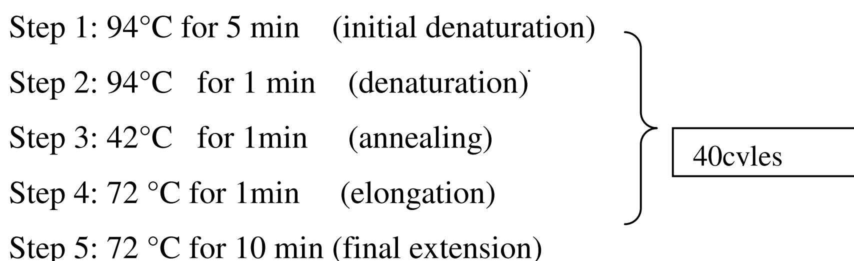
2.2.5.1.1. Amplification of 16S ITS rDNA

PCR amplification of 16S-ITS rDNA of the isolates was performed by using two DNA primers (Apendix F). The forward primer was complementary to the upstream of 16S rRNA gene and the reverse was complementary to the upstream of 23S rRNA gene.

Forward: 5. -AGAGTTTGATCCTGGCTCAG-3 (Mora et al., 1998).

Reverse: 5. -CAAGGCATCCACCGT-3 (Jensen et al., 1993).

Amplifications were performed in a Mini Cycler System. PCR conditions were as follows:



The final reaction volume was 50µl. PCR mix (Appendix E.1) was prepared after 2 µl -4µl of genomic DNA (approx. 500 µg) was placed into 1.5ml eppendorf tubes. During the preperation of the mix, tubes were kept on icebox. Taq DNA polymerase (1.25U) was added and mixed gently and then tubes were centrifuged for 2-3 s. The mix were added to each of the tube. Then 60 µl of mineral oil was added into the tubes while they were on ice. Tubes were centrifuged for 2-3 s then they were placed into the wells of Mini Cycler System (MJ Research INC, USA).

2.2.5.1.2. Electrophoresis of Amplified 16S-ITS rDNA Fragments

Amplified fragments were separated in 0.8 % agarose gel. First, 0.96 g of agarose was dissolved in 120 ml of 1×TAE buffer (Appendix C.4) by boiling (15 µl of ethidium bromide solution (10 mg/ml) were added after cooling the solution to 40°C). The gel was then poured into the gel-casting stand and the combs were placed. 1×TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. Five µl of PCR products were taken and mixed with 1 µl, 6 × gel-loading buffers. The samples were then loaded into the wells of agarose gel starting from the second well. Finally, 5 µl of DNA molecular weight marker were loaded into the first well and they

were electrophoresed at 40 mA until bromophenol blue have reached 2/3 of the gel. DNA fragments were visualized in a photodocumentation system (Vilber-Lourmat, France).

2.2.5.1.3. Chloroform Extraction of PCR Products

Final volumes of the PCR products were adjusted to 100 µl with 1×TE buffer. Then two volume of chloroform were added. After centrifugation for 2 min at 6000 rpm, organic phase was discarded and chloroform extraction was repeated. Then the tubes were centrifuged for 2 min at 6000 rpm. The aqueous phase was transferred into new eppendorf tubes containing 10 µl of 3 M sodium acetate (pH 5.2) and mixed well. After that, 450 µl of 99% ethanol were added and mixed. The samples were centrifuged for 15 min at 7000 rpm. Pellet was washed with 500 µl 70% ethanol. After centrifugation for 5 min at 6000 rpm, alcohol was removed. Pellet was dried for 10 min at 37°C, and they were dissolved in 25 µl 1xTE, and stored at 20°C.

2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP)

Purified PCR products were separated in 1% agarose gel in order to estimate DNA concentrations for each sample. Then 12.5µl of PCR products were aliquoted into new tubes for digestion. *Taq* I and *Hae* III were the enzymes chosen for this purpose. Reaction mix (Appendix F2) was added on PCR products while the tubes were stored on ice. 5 U of enzyme was used for each reaction. The final volume of the reaction was adjusted to 50 µl. Incubation period was 5 h for both of the enzymes. But the optimum reaction temperatures were 65°C for *Taq I* and 37°C for *Hae* III. Mineral oil was added over the reaction mixtures to avoid evaporations at 65°C. After the incubation DNA was extracted as described in section 2.2.6.1.3. and dissolved in 15 µl of 1xTE.

2.2.5.1.5. Electrophoresis of Restriction Fragments

Restriction fragments were first separated in 2 % agarose gel containing 20 µl of ethidium bromide solution (10mg/ml). Electrophoresis was carried out at 60 mA for the

first 20 min, then the current was adjusted to 80 mA and the electrophoresis was continued further for 2.5 h. Some isolates produced similar restriction fragment patterns. These were contained within distinct groups. One digested sample from each group was picked and re-electrophoresed in order to produce representative restriction patterns for further restriction profile analyses.

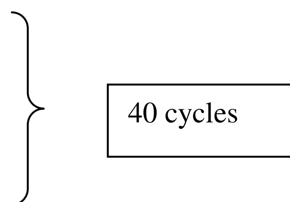
2.2.6 Sequencing

2.2.6.1. Amplification of 16SrRNA Gene

16SrDNA region of rRNA genes of the isolates were amplified using the following DNA oligoprimers for sequencing reactions
EGE 1. 5. - AGAGTTTGATCCTGGCTCAG –3 (Jensen *et al.,* 1993)
EGE 2. 5. - CTACGGCTACCTTGTTACGA –3 (Jensen *et al.,* 1993)

Two µl of genomic DNA (approx. 500 µg) were transferred into 0.5 ml eppendorf tubes, as the template. PCR mix was prepared and after the addition of *Taq* DNA polymerase (1.25 U), it was mixed gently and centrifuged for 2-3 s. Forty-eight ml of the mix were distributed into the tubes. All the manipulations were performed on ice. The tubes were then overlaid with 60µl mineral oil, centrifuged for 2-3 s at 6000 rpm. Finally, they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) using the following program:

Step1: 95°C for 5 min
Step2: 95°C for 1 min
Step3: 56°C for 1 min
Step4: 72°C for 1 min
Step4: 72°C for 10 min



2.2.6.2 Extraction of DNA Fragments from Agarose Gel

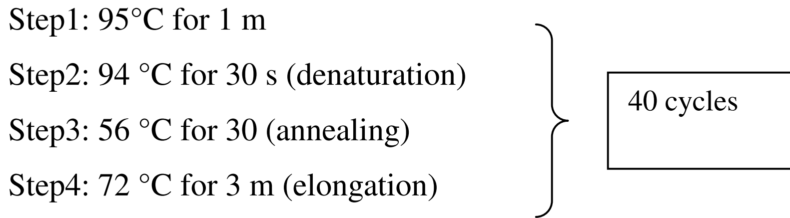
DNA isolation kit (Applichem A3421) was used for extraction of amplification products from agarose gel.

DNA bands (approximately 1600 bp) were excised from the agarose gel by a rasor blade. Three gel slice volume of 6 M NaI (for TAE gels) was added. The samples were incubated for 5 min at 55°C, mixed and continued for incubation for 2 min. Ten µl of well mixed glass powder suspension was then added, mixed well and incubated for 5 min at 55°C by occasional mixing. The samples were then centrifuged for 10 s at 10.000 rpm and the supernatant was discharged. The glass pellet was rinsed with 500 µl wash buffer. And resusupended by pipetting or gently flicking the tube. The samples were then centrifuged for 10 s at 10,000 rpm. The washing step was repeated twice. After the last wash, the supernatant was removed as much as possible. The pellet was suspended in 25 µl of 1xTE buffer. The samples were eluted for 5 min at 55°C with occasional mixing. Centrifugation was performed for 45 s at 10,000 rpm. The supernatant was finally transferred carefully into a new eppendorf tube. The eluted DNA samples were stored at -20°C until use.

2.2.6.3 Sequencing Reactions

The sequencing reactions were performed by using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit.

Four eppendorf tubes were firstly labeled as "A", "C", "G", and "T" for respective termination reactions. Two µl of corresdonding Cy5.5 ddNTP termination mix were then dispensed into the appropriately labelled tubes. The tubes were then capped to prevent evaporation. The master mix (Appendix I) was prepared in an eppendorf tube for each template to be sequenced. The content of master mix was mixed thoroughly and 7µl of the mix was dispensed into each tube labelled "A", "C", "G", and "T". Each sequencing reaction was mixed thoroughly and overlaid with one drop of mineral oil. Each tube was capped and placed into the thermal cycler. The sequencing condition reastions were:



40 amplification cycles of Step 2, 3 and 4 were performed

The primer used was ;

EGE 1; 5. - AGAGTTTGATCCTGGCTCAG -3. (Jensen *et al.,* 1993)

2.2.6.4 Purification of Sequencing Reactions

2.2.6.4.1 Removal of unincorporated dye terminators using ethanol precipitation

The volume of the sample was adjusted to 50 µl by adding 1xTE. The samples under the mineral oil were then transferred into fresh eppendorf tubes. Twenty µl of 7.5 M ammonium acetate and 200 µl (3 times the reaction volume) of chilled 100% ethanol were added to each tube. The tubes were then mixed thoroughly by vortexing and placed on ice for 20 min to precipitate the DNA. Centrifugation was then performed at 12, 000 rpm for 30 min at 4°C. Supernatants were removed carefully and the pellets were washed with 200 µl of chilled 70% ethanol. After the centrifugation for 5 min, the supernatants were removed and the pellets were dried. Each pellet was then resuspended in 6 µl of formamide loading dye and vortexed vigorously (2-3 min) to ensure complete resuspension. Just prior to loading the samples onto gel, each sample was heated at 70 °C for 2-3 min for denaturation and then placed on ice. One and a half or 2µl of each sample were then loaded into the lanes of sequencing gel.

2.2.6.4.2 Assembling the SEQ Sequencer

The sequencing reactions were performed with SEQ personal sequencing system (Amercham Pharmacia Biotech). The system consists of SEQ personal sequencer, SEQ

Rapidset gel polymerizer, RapidGel QuickFill cassettes, RapidGel-XL-6% cartridge and filling gun and filling fixture.

Preparing a gel involves attaching the RapidGel-XL cartridge to the casting gun, filling the RapidGel cassettes, polymerizing the acyrlamide in the gel polymerizer. After the polymerization the comb was removed from the cassette. After cleaning the cassette, it was placed properly into the buffer chamber. At this step pre-run was performed to measure the backgraound redding. Once the gel cassette was aligned and the pre-run was complete, 1xTE buffer was loaded into the upper and lower buffer chambers until it reached the fill lines. The prepared samples (1-2 μl) were then loaded into the gel with a micropipette. Then the analysis was started. After the run was finished, data were analysed by SEQ software.

The sequences obteined were evaluated in and submitted to GenBank.

Chapter 3

RESULTS AND DISCUSSION

3.1. Isolation of Haloalkalophilic Bacteria

Haloalkalophilic bacteria were isolated from Menemen Region. Target places were leather factories and the wastes of this industry. Therefore the samples were taken from both the processing steps and waste.

3.2. Determination of Phenotypic Characteristics

3.2.1. Gram staining

The modified Gram staining method specific for halophilic bacteria gave desired results with these isolates. All the isolates were gram positive, rod shaped except one cocci (C1).

3.2.2. Examination of Endospore

Isolates were grown on agar plates (Appendix) for 5 days. Isolates were non-sporforming.

3.2.3. Catalase and oxidase tests

All the isolates were catalase and oxsidase positive.

3.2.4. Physiological Tests

Growth at pH 7 was observed to determine whether the isolates were obligate alkalophilic (Table 3.2). Four isolates could not grow at this pH value. Twenty-five of the isolates were alkali tolerant and could not grow at pH 12. Isolates were not thermotolerant since they could not grow at 45ºC and 50ºC on alkaline nutrient agar plates.

Table 3.1. Results of the Physiological Test

Physiologic test	Isolate number
No Growth at 20%NaCl	D2, D10, D20. D26, E4, E16, E24, F22
No Growth at PH 7	D1, D20, F9, F21
No Growth at 0%NaCl pH 10	D2, D6, D18, D23, E4, E43, F5, F7, F29
No Growth at pH 12	D1, D6, D10, D18, D20, D23, D24, D30, D32, E2, E4, E10, E12, E14, E16, E23, E24, E36, F7, F10, F13, F16, F20, F22, F29,

Growth of the isolates was also observed at 0% and 20% NaCl concentrations. Nine of the isolates were oblitely halophilic. Eight of the isolates were not extremely halophilic.

Table3.2. Differentiation of Isolates according to growth at different NaCl concentrations and pH ranges.

İsolate Name	20% NaCl	PH 7	0%NaCl pH 10	pH 12
D1	+	-	+	-
D2	-	+	-	+
D5	+	+	+	+
D6	+	+	-	-
D7	+	+	+	+
D10	-	+	+	-
D11	+	+	+	+
D12	+	+	+	+
D14	+	+	+	+
D18	+	+	-	-
D20	-	-	+	-
D23	+	+	-	-
D24	+	+	+	-
D25	+	+	+	+
D26	-	+	+	+
D29	+	+	+	+
D30	+	+	+	-
D31	+	+	+	+
D32	+	+	+	-
E1	+	+	+	+
E2	+	+	+	-
E3	+	+	+	+
E4	-	+	-	-
E6	+	+	+	+
E7	+	+	+	+
E9	+	+	+	+
E10	+	+	+	-
E12	+	+	+	-
E13	+	+	+	+
E14	+	+	+	-
E15	+	+	+	+

E16	-	+	+	-
E17	+	+	+	+
E20	+	+	+	+
E23	+	+	+	-
E24	-	+	+	-
E25	+	+	+	+
E26	+	+	+	+
E27	+	+	+	+
E28	+	+	+	+
E29	+	+	+	+
E30	+	+	+	+
E33	+	+	+	+
E34	+	+	+	+
E35	+	+	+	+
E36	+	+	+	-
E39	+	+	+	+
E42	+	+	+	+
E43	+	+	-	+
E47	+	+	+	+
F1	+	+	+	+
F2	+	+	+	+
F3	+	+	+	+
F4	+	+	+	+
F5	+	+	-	+
F7	+	+	-	-
F8	+	+	+	+
F9	+	-	+	+
F10	+	+	+	-
F11	+	+	+	+
F12	+	+	+	+
F13	+	+	+	-
F14	+	+	+	+
F15	+	+	+	+
F16	+	+	+	-
F17	+	+	+	+
F18	+	+	+	+

F19	+	+	+	+
F20	+	+	+	-
F21	+	-	+	+
F22	-	+	+	-
F23	+	+	+	+
F24	+	+	+	+
F25	+	+	+	+
F26	+	+	+	+
F27	+	+	+	+
F28	+	+	+	+
F29	+	+	-	-
F30	+	+	+	+
F32	+	+	+	+
F34	+	+	+	+
F35	+	+	+	+

3.3. Extracellular Enzyme Screening

Extracellular enzyme screening was performed on solid agar plates by using different substrates for each enzyme activity. Protease screening based on the observation of clear zones around the colonies (Figure). Lipase activity gave opaque zones around the colonies (Figure). Different NaCI concentratios were used for lipase screening. The enzyme screening studies were repeated performed twice.

3.3.1 Extracellular Enzyme Profiles of the Isolates

Table 3.3 Extracellular Enzyme Tests

Extracellular Enzyme Type	Isolate Name
Did not produce lipase	D5, D10, D20, D26 D31, E23, E43, F11, F29 F33
Utilized Tween80 at 0% NaCl	D1, D2, D30, F25, F15
Utilized Tween 20 at 12% NaCl	D23, D24, D25, D30, E1, E2, E3, E42, F5, F12, F13, F14, F15, F16, F18, F23, F25
Did not Utilized Tween 20	D7, E7, E9, E10, E13, E14, E15, E16, E23, E24, E25, E26, E27, E28, E29, E30, E39, E47, F26, F27, F31
Did not utilase Tween80	D12, E12, E34, F1, F20, F30, F32,
Did not produce lipase without NaCI	D12, D14, E12, E34, F1, F30, F32
Utilase casein	F17, D5, F29, D32, E34, F1, F15, E36, D24, F10, F13, F12, E20, F32, E1, E47, E33, E14, E25, E42, F22

Table 3.4 Extracellular Enzyme Profiles of isolates

Isolate Name	Protease	0%NaCl Tween20	12% NaCl Tween20	20% NaCl Tween20	0% NaCl Tween80	12% NaCl Tween80	20% NaCl Tween 80
D1	-	-	-	-	+	+	-
D2	-	-	-	+	+	-	-
D5	+	-	-	-	-	-	-
D6	-	+	-	-	-	+	-
D7	-	-	-	-	-	+	-
D10	-	-	-	-	-	-	-
D11	-	+	-	+	-	+	+
D12	-	+	-	-	-	-	-
D14	-	+	-	-	-	-	-
D18	-	+	-	+	-	-	+
D20	-	-	-	-	-	-	-
D23	-	+	+	-	-	+	+
D24	+	+	+	-	-	+	+
D25	-	+	+	-	-	-	+
D26	-	-	-	-	-	-	-
D29	-	+	-	-	-	+	+
D30	-	+	+	+	+	+	+
D31	-	-	-	-	-	-	-
D32	+	-	-	-	-	+	+
E1	+	+	+	-	-	+	+
E2	-	+	+	-	-	+	
E3	-	+	+	-	-	+	+
E4	-	+	-	-	-	+	-
E6	-	+	-	-	-	+	+
E7	-	-	-	-	-	+	-
E9	-	-	-	-	-	+	-
E10	-	-	-	-	-	-	+
E12	-	+	-	-	-	-	-
E13	-	-	-	-	-	+	-
E14	+	-	-	-	-	+	-
E15	-	-	-	-	-	+	-
E16	-	-	-	-	-	+	-
E17	-	-	-	-	-	+	-
E20	+	+	-	-	-	+	+
E23	-	-	-	-	-	+	-
E24	-	-	-	-	-	+	-
E25	+	-	-	-	-	+	-
E26	-	-	-	-	-	+	-
E27	-	-	-	-	-	+	-
E28	-	-	-	-	-	+	-
E29	-	-	-	-	-	+	-
E30	-	-	-	-	-	-	+
E33	+	-	-	+	-	+	+
E34	+	+	-	-	-	-	-
E35	-	-	-	+	-	-	+

E36	+	+	-	-	-	+	+
E39	-	-	-	-	-	+	-
E42	+	+	+	-	-	-	+
E43	-	-	-	-	-	-	-
E47	+	-	-	-	-	-	+
F1	+	+	-	-	-	-	-
F2	-	+	-	+	-	+	+
F3	-	+	-	+	-	+	-
F4	-	+	-	-	-	+	+
F5	-	+	+	+	-	+	+
F7	-	+	-	+	-	+	+
F8	-	+	-	-	-	+	+
F9	-	+	-	-	-	-	+
F10	+	+	-	+	-	+	+
F11	-	-	-	-	-	-	-
F12	+	+	+	+	-	+	+
F13	+	+	+	-	-	+	+
F14	-	-	+	-	-	+	+
F15	+	+	+	+	+	-	+
F16	-	+	+	-	-	+	+
F17	+	+	-	-	-	+	+
F18	-	-	+	-	-	+	+
F19	-	+	-	-	-	+	+
F20	-	+	-	+	-	-	-
F21	-	+	-	-	-	+	-
F22	+	+	-	-	-	+	-
F23	-	+	+	+	-	+	+
F24	-	-	-	-	-	-	+
F25	-	+	+	+	+	+	+
F26	-	-	-	-	-	+	+
F27	-	-	-	-	-	-	+
F28	-	+	-	-	-	-	+
F29	+	-	-	-	-	-	-
F30	-	+	-	-	-	-	-
F32	+	+	-	-	-		-
F35	-	-	-	-	-	-	-
F34	-		-	-	-	-	-

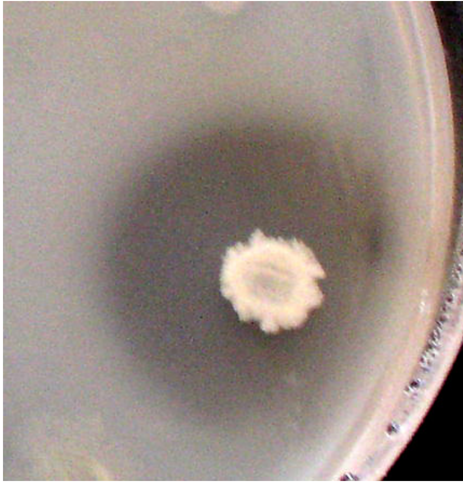


Figure 3.1. Apperance of extracellular protease

Figure 3.2. Apperance of extracellular lipase

3.4. Genotypic Characterization

3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon

The amplification product size differed between 1800 and 2000 bp (Figure)

.

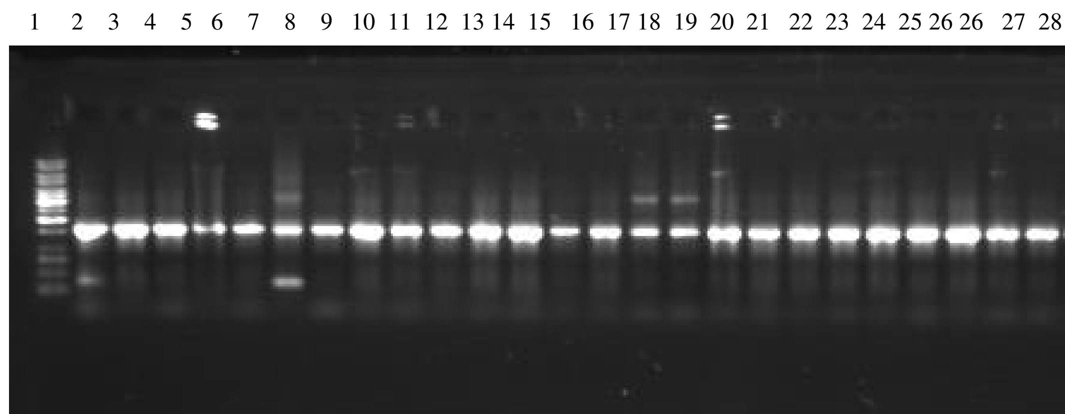


Figure 3.3. 16S rDNA-ITS Amplification products of some isolates. Lanes 1. 1 kb DNA Lac D1, 3. Isolate F2, 4. Isolate F1 5. Isolate D8, 6. Isolate D10, 7. Isolate 12,8. Is Isolate E15, 10. Isolate E17, 11. Isolate E19, 12. Isolate D23 lane 13. Isolate D24

3.4.2. 16S-ITS rDNA - RFLP Profiling

In order to identify the isolates amplification products of 16S-ITS rDNA region was digested with restriction endonucleases. *Taq* I and *Hae* III endonucleases were chosen for digestion. Digestion products were first run in 2% agarose gel to see all different profiles and to make groups within the digestion products. For a specific RFLP group, one representative digestion product was chosen and electrophoresed together in 2.5% agarose gel (Figure 3.3).

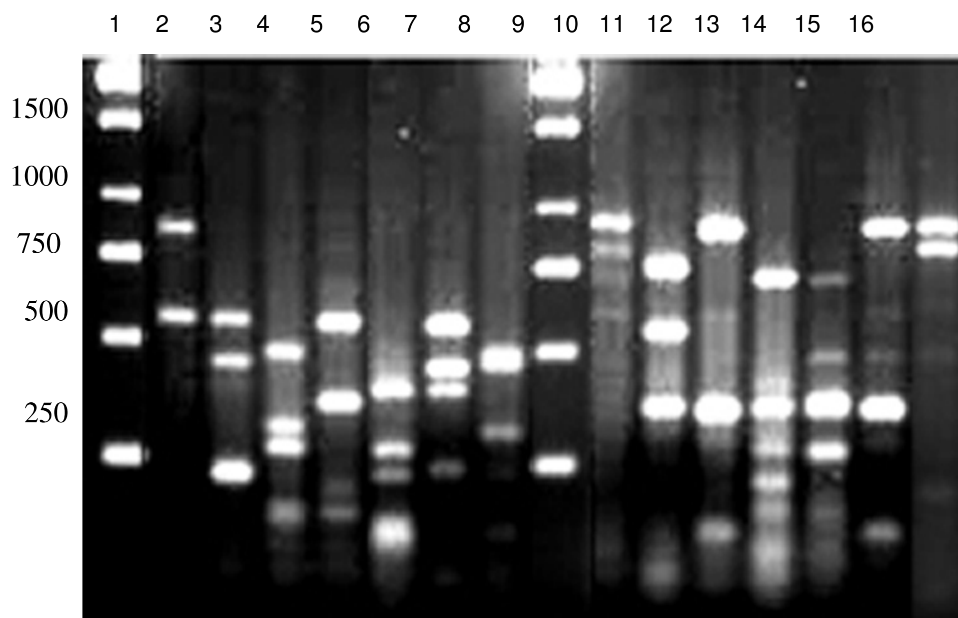


Figure 3.4 16S-ITS rDNA RFLP profiles of *Hae* III groups. Lanes 1. 1 kb DNA ladder, 2. Isolate D5, 3. Isolate D7, 4. Isolate D30, 5. Isolate D12, 6. Isolate D23, 7. Isolate F28, 8. Isolate, F21 16S-ITS rDNA RFLP profiles of *Taq*I groups. Lanes 9. 1 kb DNA ladder,10. Isolate D2, 11. Isolate D18, 12. Isolate D12, 13. Isolate F6, 14. Isolate F20, 15. Isolate F28, 16. IsolateE30,

Resulting gel profiles were analyzed using the program BIO-1D++. Dendrogram of representative strains were carried out using 15% homology coefficient. (Figure 3.3 and 3.4). Fragment sizes were given in Table 3.6.

Hae III and *Taq* I restriction analysis of 16S-ITS rDNA fragments of strains revealed 7 distinct groups (Table 3.3.).

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2000	854	546	465	546	384	546	465	2000	886	673	808	661	361	838	870
1500	555	440	309	354	257	440	432	1473		509	338	354	338	338	778
1000		219	272	125	211	377	294	934		354	78	265	257	78	
750			125		94	219		685				195			
500					70			465				133			
250								227				31			

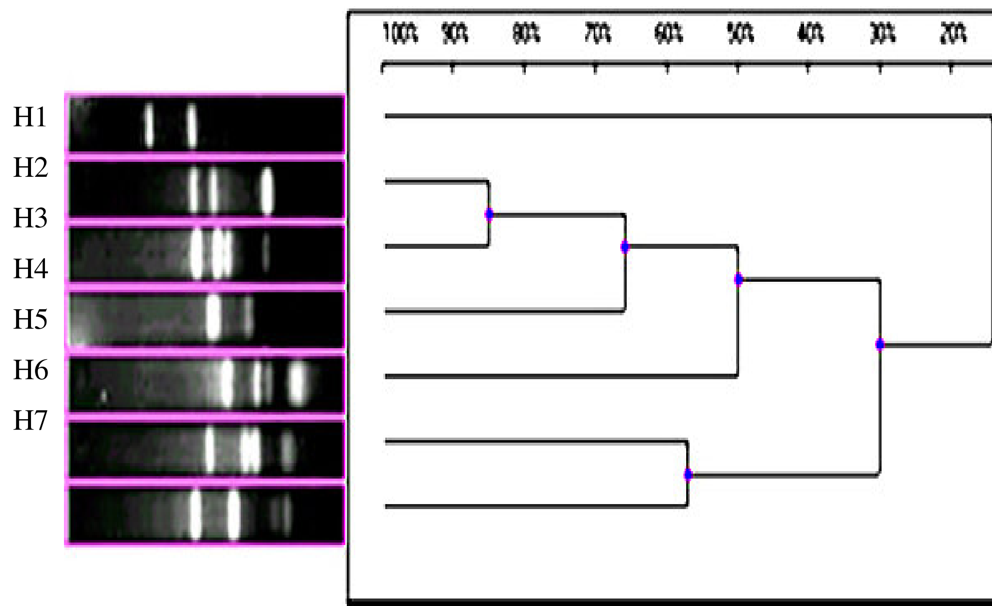


Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles.

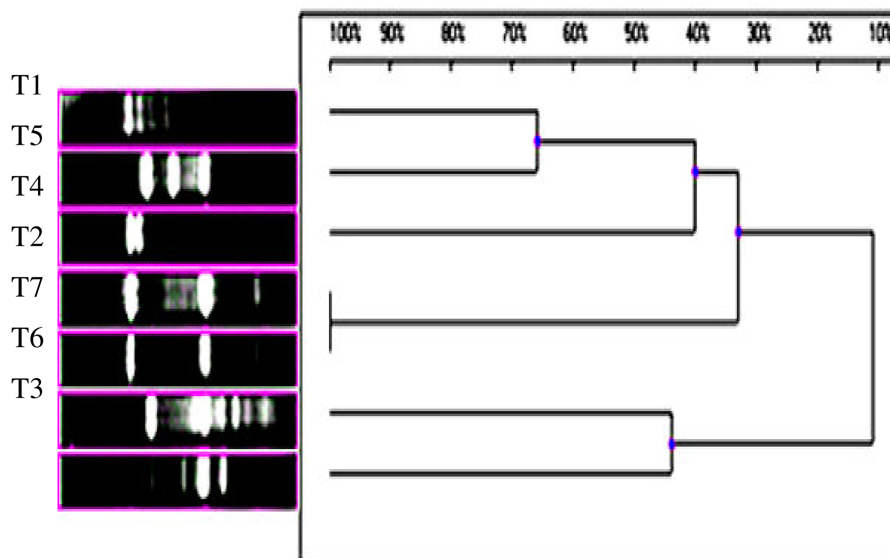


Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles.

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups.

Genotypic groups	Name of the Isolates
HT1	D1, D2, D5, E43,E47,F1
HT2	D6, D7, D10, D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E30, E34, E35
HT3	D12, F32
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E20, E33, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F19, F22, F23, F24, F25, F26, F27
HT5	D30
HT6	F20, F21
HT7	F28

The use of two restriction enzymes enabled the revelation of diversity among the isolates. When the results of both digestion were combined, 7 distinct genotypes were obtained (Table 3.10). The results indicated that isolate group members were differentiated by each restriction enzyme, were the same.

In this study, 16S ITS and rDNA regions were used as a single amplicon for RFLP analysis. No report was found in literature on genotypic characterization of haloalkalophiles using this method. Similar studies have been performed in this institute for genotypic characterization of alkalophilic bacilli (Akbalik, 2004), thermophilic bacilli, (Yavuz, 2004), lactic acid bacteria (Yavuz, 2003, Bulut, 2003.

3.4.3. Analysis of the Genotypic and Phenotypic Groups

Table 3.7. Analysis of the Genotypic and Phenotypic Groups

	Isolate number	20%NaCI	pH 7	0%NaCl	pH 12	Protease	0%NaCl Tween 20	12%NaCl Tween 20	20%NaCl Tween 20	0%NaCl Tween 80
HT1	D1,D2,D5,E34, E43, E47, F1	%80	80%	70%	80%	40%	0%20	0%	20%	%30
HT2	D6,D7,D10,D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E39, F29, F30, F34, F35,	100%	100%	100%	100%	10%	10%	0%	0%	0%
HT3	D12, F32	100%	100%	100%	100%	50%	100%	0%	0%	0%
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E30, E33, E35, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18, F19, F22, F23, F24, F25, F26, F27	90%	100%	90%	70%	30%	90%	40%	30%	20%
HT5	D30	100%	100%	100%	0%	0%	100%	100%	100%	100%
HT6	F20, F21	100%	50%	100%	50%	0%	100%	0%	50%	0%
HT7	F28	100 %	100%	100%	100%	0%	100%	0%	0%	0%

Phenotypic characterization of the isolates indicated that strains of different species had different phenotypic features. When the genotypic results were compared with the results of enzyme screening and phsyical tests, a correlation between a few groups were observed. Most of the isolates produced lipase (Tween 20 and Tween 80) at all NaCI concentrations used grouped in HT4. All members of HT2, HT3, HT5, HT6, HT7 were also able to grow in the presence of 20% NaCI. Most of the isolates that could not grow at pH7 were placed in HT6. Isolates that could not grow at pH12 were placed in HT1, HT5, and HT6.

3.4.4 Analysis of Sequencing Reactions

16S partial sequencing results of six representative isolates are as listed below.

Table 3.8. Sequencing results

Genotypic Group	Isolate Name-Accession Number	Length of bp.-Similarity	Accession Number - Organism	Name of the Family
HT2	D7-AY604872	-89%	*AY345480.1-Bacterium K-29*	Unclassified *Bacillaceae*
HT3	D12-AY601901	716-93%	AF237976.1-*Salinococcus roseus*	Unclassified *Bacillaceae*
HT4	D23-AY601902	323-92%	AJ640133.1-*Halomonas* sp.18bAG	*Halomonadaceae*
HT5	D30-AY601903	-92%	AY383044.1-*Halomonas meridiana*	*Halomonadaceae*
HT6	F20 (C1)-AY604871	-89%	AJ29397-*Nestenkonia* sp.EL-30	*Micrococcaceae*
HT7	F28 (15B)-AY601904	-91%	AY3091336-Bacterium K-2	Unclassified *Bacillaceae*

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, haloalkalophilic bacteria were isolated from samples of waste soil and leather processing. They were first characterized phenotypically (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope and extracellular enzyme screening, physiological tests such as growth at pH 7 and pH 12 , different NaCl concentrations and at different temperatures). Extracellular enzyme screening was performed on solid agar media. Molecular characterization was performed by using 16S-ITS rDNA RFLP.

All the isolated strains were Gram positive but they were not sporeforming. All the strains were catalase positive. Most of the isolates contained oxidase. Four strains could not grow at pH7. They were thus obligately alkalophilic. Twentyfive isolated strain were alkalitolerant because they could not grow at pH 12. Nine isolated strain could not grow with in 0% NaCl they were thus obligately halophilic. Eight of the isolates could not grow at 20% NaCl concentration.

Extracellular enzyme screening resulted in 21 protease and 72 lipase activity. Isolates produced protease were grouped in HT1, HT2, HT3, HT4.Different concentrations of NaCl were used for lipase screening. Seventeen of the isolated strains which could utilize Tween 20 in the presence of 12% NaCI were all grouped in HT4. Isolated strains, which could not utilize T20, were grouped in HT2 except E47, F26, and F27. Seven of the isolated strains were not produce lipase in the presence of NaCl were grouped in HT1, HT2 and HT3.

For restriction analysis of 16S-ITS rDNA fragments of isolated strains, two restriction enzymes, *Taq* I and Hae III, were used. Both enzymes produced similar RFLP groups. Representative group members were subjected to 16S partial sequencing. They had high similarity with *Bacillaceae*, *Halomonadaceae* and *Micrococcaceae* families.

Characterization of the extracellular enzymes from different isolated strains is the essential work for these Haloalkalophilic bacteria because of their unique futures.

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APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Experiments

NO	CHEMICAL	CODE
1	Agar Merck	1.01613
2	Bacteriological pepton Oxoid	LP037
3	D-Glucose AppliChem	A3666
4	Yeast Extract Merck	1.03753
5	Skimmed milk LabM	MC27
6	Glycerol AppliChem	A2926
7	NaCI AppliChem	A2942
8	K2HPO4 AppliChem	A2945
9	MgSO4.7H2O Merck	1.05886
10	Tween80 Applichem	A1390
11	Ammonium sulfate Applichem	A3485
12	Nutrient broth Merck	1.05443
13	Sodium carbonate Merck 1.06392	
14	KH2PO4 Merck	1.04871
15	Disodium hydrogen phosphate Applichem	A2943
16	Immersion oil Applichem	A0699
17	Calcium chloride Applichem	A3652
18	Crystal violet Sigma	C3886
19	Safranin Merck	1.15948
20	N,N,N.,N.- Tetramethyl-p-phenylenediamine Sigma	T3134
21	Congo Red Sigma	C6767
22	Potassium Iodide Sigma	P8256
23	Tris Base Sigma	T6066
24	EDTA AppliChem	A2937
24	Isopropanol AppliChem	A3928
25	Proteinase K AppliChem	A3830
24	Ethidium bromide AppliChem	A1151
25	Ethanol AppliChem	A3678
26	Taq DNA polymerase Promega	M1865
27	Primers: Ege 1 and L1 Promega	
28	dNTP set MBI, Fermentas,	R0181
29	Standard agarose (low electroendoosmosis) AppliChem	A2114
30	Chloroform AppliChem	A3633
31	Isoamyl alcohol AppliChem	A2610
32	Bromophenol blue Merck	1.08122
33	Boric acid AppliChem	A2940

APPENDIX B

RECIPIES FOR MEDIA USED IN EXPERIMENTS

B.1 HORIKOSHI BROTH AND AGAR

HORIKOSHI-I BROTH

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water. Medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

HORIKOSHI-I AGAR (pH=10.2)

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

B.2 NUTRIENT AGAR (pH:10.3) USED FOR GROWTH TESTS AT DIFFERENT TEMPERATURES AND SODIUM CHLORIDE CONCENTRATIONS

Nutrient Broth	8
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 was sterilized separately and added into medium.

B.5 MEDIUM USED FOR LIPASE SCREENING (pH: 10.3)

	g/l
Nutrient Broth	8
$CaCl_2.H_2O$	0.1
Agar	13
Tween20/Tween80	10ml
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 and Tween20/80 was sterilized separately and added into medium.

APPENDIX C

BUFFERS AND STOCK SOLUTIONS

C.1 ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

Ethidium bromide (0.5 g) was dissolved in 50 ml of deionized water.

C.2 CHLOROFORM-ISOAMYL ALCOHOL SOLUTION (24:1)

Forty eight milliliters of chloroform was mixed with 2 ml of isoamyl alcohol.

C.3 50 X TAE

Two hundred and fifty-two grams Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

C.4 1XTAE

Twenty milliliters of 50X TAE buffer was taken and the volume was adjusted to1000 ml with deionized water to obtain 1 liter 1X TAE buffer.

C.5 1X TE BUFFER

10 mM Tris pH 8.0
1 mM EDTA pH 8.0

C.6 10 X TBE

One hundred and eight grams Tris Base and 55 g boric acid were weighed. They

were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was added. The volume was brought to 1000 ml with deionized water.

C.7 1X TBE

Hundred milliliters, 10X TBE was taken and the volume was brought to 1000 ml with deionized water to obtain 1X TBE buffer.

C.8 CTAB/ NaCl (10% CTAB in 0,7 M NaCl)

NaCl (4.1 g) was dissolved in 80 ml water and CTAB was added slowly while heating and stirring. Final volume was adjusted to 100ml.

APPENDIX D

STAINS AND INDICATORS

D.1. BROMTYMOL BLUE SOLUTION

Bromtymol blue	1 g
N/10 NaOH	25 ml
Deionized water	475 ml

D.2. SOLUTIONS FOR GRAM STAINING

D.2.1. CRYSTAL VIOLET STAINING REAGENT

Solution A

Crystal violet	2g
Ethanol (95%)	20ml 83

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

D.2.2. IODINE SOLUTION

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Iodine and potassium iodide were grinded in a mortar, water was added slowly with continuous grinding until the iodine was dissolved. The solution was stored in amber bottle.

D.2.3. SAFRANIN SOLUTION

Safranin (2.5% in 95% alcohol)	10ml
Distilled water	100ml

APPENDIX E

PCR RECIPIES

E.1. PCR MIX

Mg free Taq DNA polymerase buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Sterile deionized water	32 µl
Oligo forward (10 picomole/µl)	1 µl
Oligo reverse (10 picomole/ µl)	1 µl
dNTP (2mM each) 10X	5 µl

E.2. 6X GEL LOADING BUFFER (20 ML)

10x TBE	2 ml
Glycerol	6 ml
Deionized water	12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

E.3. dNTP (10X)

Ten microliters of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at .20 °C.

E.4. RESTRICTION ENZYME MIX

Restriction enzyme buffer	5 µl
DNA	8 µl
Restriction enzyme (10U/ µl)	1.5 µl
Sterile deionized water	35.5 µl

APPENDIX F

OLIGONUCLEOTIDE PRIMERS

L1: 5.- CAAGGCATCCACCGT -3.

Three hundred and fifty micrograms primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliters of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

EGE 1 5.- AGAGTTTGATCCTGGCTCAG -3.

Five hundred and ninty micrograms primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain final concentration of 2 µg / µl stock solutions. Five microliters of stock solution were then taken and mixed with 95 µl sterile deionized water. Therefore, 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

APPENDIX G

RECOGNITION SITES OF RESTRICTION ENZYMES

G1. ***Taq*** **I**

5.- T▼CG A -3.
5.- A GC ▲T -3.

G2. ***Hae*** **III**

5.-GG▼CC-3.
5.-CC▲GG-3.

APPENDIX H

ISOLATE NAMES

DI- C12a	F26- Ü4z
D2- Ş6a	F27- Ü6a
D5- E15	F28- 15b
D6-C15a	F29- F1
D7- 20a	F30- 24a
D10-19a	F31- 27b
D11- B5c	F32- B4c
D12- B1a	F33- T1b
D14- 23a	F34- E6z
D18- C4	F35- B8a
D20- B8b0	E42- C8
D22- H2	E43- C14a
D23- H1a	E47- C19y
D24- P5z	F1- E11x
D25- C17	F2- F2x
D26- 2b	F3- F2y
D29- D3c	F4- F2z
D30- B9	F5- G5
D31- A9	F6- G8
D32- D2b	F7- H3x
E1- A2	F8- H3y
E2- P2y	F9- P1x
E3- C6	F10- P4a
E4- C2a	F11- P5y
E6- G9a	F12- S1
E7- 3a	F13- S2
E9-24a	F14- Ş1

E10- 13b	F15- V3
E12- 29c	F16- A1
E13- 22c	F17- A4
E14- 12c	F18- A6
E15- 27c	F19- A7
E16- 1a	F20- B10
E17- 2d	F21- C1
E20- B2	F22- C2
E23- B8c	F23- C3
E24- 18b	F24- C24
E25- 11a	F25- C10

ISOLATION OF HALOALKALIPHILIC MICROORGANISMS FROM LEATHER INDUSTRY

Hatice Sevgi ÇOBAN

October, 2004

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By

Hatice Sevgi ÇOBAN

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Graduate School in Partial Fulfilment of the
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ABSTRACT

Haloalkalophiles are extremophilic microorganisms that are adapted to saline and alkaline conditions. Different species of them have been isolated so far from soda lakes and soil samples. Haloalkalophilic microorganisms have significant adaptive mechanisms to avoid denaturing effect of salts and to balance their interior pH. Extracellular enzymes that are produced by these halophilic and alkalophilic microorganism are applicable for industrial purposes. Therefore isolation of these organisms from their habitats and study on genotypic characterization constitute initial steps for furter biotechnological studies.

In this study, processing steps of leather factories and their wastewater were chosen for sampling. In order to isolate target microorganisms Horikoshi-I medium including 12% NaCl was used. After isolation microorganisms were purified. Phenotypic tests were applied (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope, sensitivity to antibiotics and extracellular enzyme screenings). For genotypic characterization, genomic DNA was isolated and 16S-ITS rDNA region was amplified.

Differentiation was achieved according to Restriction Fragment Length Polymorphism (RFLP) method by using *Hae* III and *Taq* I endonucleases. Isolates, which represented the different RFLP patterns, were chosen for building up the haplotype groups.

As a result of the study seven different RFLP haplotypes were identified. Moreover, 16S ribosomal DNA partial sequencing was also performed on some of the strains in. These haloalkalophilic microorganisms and their enzymes could be used in different biotechnological studies in the future for various industrial applications.

ÖZ

Haloalkalofilik mikroorganizmalar yüksek alkali ve tuzlu koşullara adapte olmuş ekstremofilik canlılardır. Birçok farklı tür toprak ve soda gölü örneklerinden izole edilmiştir. Haloalkalofilik mikroorganizmalar tuzun zararlı etkilerin ve hücre içi pH değerlerini düzenleme yeteneğine sahiptirler. Halofilik ve alkalofilik olan bu organizmaların ürettiği ekstraselüler enzimler endüstride farklı amaçlar için kullanılanılabilir. Haloalkalofilik mikroorganizmaların yaşadıkları habitatlardan izole edilmeleri ve genotipik tanımlamalarının yapılması endüstri alanında kullanım amaçlı olarak genetik potansiyellerinin tanınması ve ileride yapılacak biyoteknolojik çalışmalar adına atılacak ilk adımı oluşturmaktadır.

Deri fabrikaları işlem basamakları ve atık suyu bu çalışma için örnekleme alanı olarak belirlenmiştir. Bu amaç için Horikoshi-I besi yerine %12 tuz ilave edilerek kullanılmıştır. İzolasyon aşamasının ardından organizmaların saflaştırma işlemi tamamlanmıştır. Fenotipik testler uygulanmıştır. Bunlar;Gram boyama, katalaz ve oksidaz testi, faz-kontrast mikroskobu ile spor gözlemi, ekstraselüler enzim taraması, farklı tuz konsantrasyonlarında (%0, %20, %25) gelişme, farklı sıcaklılarda (45-50 °C) gelişme, farklı pH (7-10-12) değerlerinde gelişme özellikleri). Genotipik karakterizasyon için genomik DNA izolasyonunun ardından 16S-ITS rDNA amplifikasyonu yapılmıştır. RFLP için iki farklı restriksiyon enzimi kullanıldı (*Hae* III and *Taq* I). Kesim ürünlerinin analizi yapılmıştır. Farklı jel profillerine sahip olanların oluşturduğu gruplar her iki enzim için de belirlendikten sonra grup temsilcileri kullanılarak dendogramlar oluşturulmuştur. Hae III restiriksiyon enzim kesimi sonucunda elde edilen grup temsilcilerinin 16S rDNA - PCR amplifikasyon ürünleri kısmi dizi analizleri için kullanıldı.

Haloalkalofilik mikroorganizmalar ve enzimleri daha ileride yapılacak biyoteknolojik çalışmalarda kullanılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES.. xi

LIST OF ABREVIATIONS xii

Chapter1 INTRODUCTION.. 1

1.1.Definition of Haloalkalophiles 1

1.2.Haloalkalophilic Microorganisms 1

1.2.1. Eubacterial Haloalkalophiliphiles 2

1.2.2. Haloalkalophilic Bacillus 2

1.2.3. Gram NegativeHaloalkalophilicSpecies 3

1.2.4. Archaeal Haloalkalophilic Species 3

1.2.5. Distribution and Isolation 5

1.2.6. Biotechnological and Industrial Applications.. 5

1.2.7. Extracellular Enzymes and Their Industrial Application 6

1.2.8. Proteases from Haloalkalophilic Bacteria.. 6

1.2.9. Lipases from Haloalkalophiles 8

1.3. Identification of the Haloalkaolphilic Organisms 9

1.3.1. Phenotypic Methods 9

1.3.2. Molecular Characterization Methods... 10

1.3.2.1. Pulsed Field Gel Electrophoresis 10

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)............... 11

1.3.2.3. 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP 11

1.3.2.4. Plasmid Profiling 12

1.3.2.5. Ribotyping 12

1.3.2.6. DNA Sequencing 15

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir) 15
1.5.Thesis Objective 16

Chapter 2 MATERIAL METHODS 17
2.1. Materials 17
2.1.1. Chemicals 17
2.1.2. Samples used for isolation 17
2.2. Methods 17
2.2.1. Isolation and Preservation of the Isolates 17
2.2.1.2. Isolation of Haloalkalophilic Bacteria 17
2.2.1.3. Preservation and Activation of Isolates 18
2.2.2. Determination of Phenotypic Characteristics 18
2.2.2.1. Gram Staining 18
2.2.2.2. Examination of Endospores 19
2.2.2.3. Catalase Test 19
2.2.2.4. Oxidase Test 19
2.2.2.5. Growth at Different Temperatures 19
2.2.2.6. Growth at Different NaCl Concentrations 19
2.2.2.7. Growth at Different pH Ranges 20
2.2.3. Screening for Extracellular Enzymes 20
2.2.3.1. Screening for Protease Activity 20
2.2.3.2. Screening for Lipase Activity 20
2.2.4. Preparation of Genomic DNA 20
2.2.5. Genotypic Characterization 21
2.2.5.1. Identification of Isolates by 16SrDNA–ITS Region 21
2.2.5.1.1. Amplification of 16S rDNA – ITS Region 21
2.2.5.1.2. Electrophoresis of Amplified 16S-ITS Fragments 22
2.2.5.1.3. Chloroform Extraction of PCR Products 23
2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP) 23

2.2.5.1.5. Electrophoresis of Restriction Fragments 23
2.2.6. Sequencing 24
2.2.6.1. Amplification of 16SrRNA Gene 24
2.2.6.2 Extraction of DNA Fragments from Agarose Gel 25
2.2.6.3 Sequencing Reactions 24
2.2.6.4. Purification of Sequencing Reactions 25
2.2.6.4.1. Removal of unincorporated dye terminators using ethanol presipitation 26
2.2.6.4.2. Assembling the SEQ Sequencer 26

Chapter 3 RESULTS AND DISCUSSION 28
3.1. Isolation of Haloalkalophilic Bacteria 28
3.2. Determination of Phenotypic Characteristics 28
3.2.1. Gram staining 28
3.2.2. Examination of Endospore 28
3.2.3. Catalase and oxidase tests 28
3.2.4. Physiological Tests 29
3.3.Extracellular Enzyme Screening 32
3.3.1. Extracellular Enzyme Profiles of Isolates 33
3.4. Genotypic Characterization 36
3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon 36
3.4.2. 16S-ITS rDNA Based RFLP Profiling 37
3.4.3. Analysis of the Genotypic and Phenotypic Groups 40
3.4.4 Analysis of Sequencing Reactions 41

Chapter 4 CONCLUSION AND FUTURE PERSPECTIVE 42
REFERENCES 43
APENDICES AA1
APENDIX A. CHEMICHALS USED AA1
APENDIX B. RECIPIES MEDIA USED FOR EXPERIMENTS AB1
APPENDIX C. BUFFERS AND STOCK SOLUTIONS AC1
APPENDIX D. STAINS AND INDICATORS AD1

APPENDIX E. PCR RECIPIES AE1
APPENDIX F. OLIGONUCLEOTIDE PRIMERS.. AF1
APPENDIX G. RECOGNITION SITES OF RESTRICTION ENZYMES................AG1
APPENDIX H. ISOLATE NAMES AH1

LIST OF FIGURES

Figure 1.1. Archaea kingdom 4

Figure1.2. Rybotyping 14

Figure3.1. Apperance of extracellular protease 36

Figure 3.2. Apperance of extracellular lipase 36

Figure 3.3. 16S rDNA-ITS Amplification products of some isolates 36

Figure 3.4. *Hae* III and *Taq* I Digests of 16S-ITS rDNA Fragments of Representative Isolates 37

Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles of Isolates 38

Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles of Isolates 38

LIST OF TABLES

Table 1.1. Tolerance for pH, NaCl of selected species of the genus Bacillus 2

Table 1.2. "Natro group" of Halobacteria 5

Table 1.3. Detergent Proteases 7

Table.1.4. Lipolytic activity 9

Table 2.1. Samples used for isolation 17

Table 3.1. Results of the Physiologic test 29

Table.3.2. Tolerance for pH, NaCl of isolated strains 30

Table 3.3 Extracellular Enzyme Tests 33

Table 3.4 Extracellular Enzyme Profiles of isolates 34

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA 38

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups 39

Table 3.7. Analysis of the Genotypic and Phenotypic Groups 40

Table 3.8. Sequencing Results 41

ABBREVIATIONS

A	Adenine
bp	Base pair
C	Cytosine
CGT	Cyclodextrin Glucosyltransferase
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
G	Guanine
EDTA	Ethylenediamine Tetra Acetic Acid
ITS	Internal Transcribed Spacer
ISR	Internal Spacer Region
kb	Kilo base
PAGE	Polyacrylamide Gel Electrophoresis
P.E.	Pectinolytic Enzyme
PGA	Polygalacturonic Acid
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
T	Thymine
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA

CHAPTER 1

INTRODUCTION

1.1. Definition of Haloalkalophiles

Haloalkalophiles are one of the main physiological groups of microorganisms classified in alkaliphiles. Lake Magadii in Kenya, Wadi Natrun in Egypt have been defined as the most stable alkaline environments on earth. Therefore the first ecological studies related to haloalkalophilic microorganisms have been performed in these places. The conditions of high mineralization and/or alkalinity in such environments regulate their prokaryotic communities (Trotsenko and Khmelenia, 2001). Both alkalinity (pH 9) and salinity up to saturation (33% (wt/vol)) are needed for the survival of these microorganisms (Koki Horikoshi, 1999).

Alkaliphiles can be classified as obligately and facultatively alkaliphilic organisms. Obligately alkaliphilic microorganisms cannot grow below pH 9. Facultatively alkaliphilic organisms show optimal growth at pH 10.0 or above but they also have the ability to grow at neutral pH.

Halophilicity divided the organisms into three categories: slightly halophilic, moderately halophilic and extremely halophilic. Halophilic microorganisms require certain concentrations of NaCI for their growth, while the halotolerant ones have the ability to grow in the absence or presence of higher concentrations of NaCl (Mimura and Nagata, 2000).

1.2. Haloalkalophilic Microorganisms

Haloalkalophilic microorganisms inhabiting extremely alkaline and saline environments are found in both Archaea and Eubacteria. Most of the extremely salt tolerant haloalkaliphiles belong to *Halobacteriaceae*.

1.2.1. Eubacterial Haloalkalophilic

1.1.2.1 Haloalkalophilic *Bacillus*

Genus *Bacillus* can be characterized by unique physiological characteristics such as Gram staining, spore examination, catalase and oxidase tests. *Bacillus* is a Gram positive usually catalase and oxidase positive and spore producing organism that classified in Firmiculates. It is well known that *Bacillus* strains do not form endospores under many environmental conditions, and the ability to form spores is often difficult to demonstrate. This is very important for their taxonomy. There have been organisms described as non-spore-formers, which had to be reclassified after the detection of spores (Fritze and Claus, 1992). Recent taxonomic studies have separated some of the *Bacillus* members and grouped into new genera: *Alicyclobacillus, Aneurinibacillus, Brevibacillus, Gracilibacillus, Halobacillus, Paenibacillus, Virgibacillus* (Baumgart, 2003)

Table1.1. Tolerance for pH and NaCl of selected species of the genus *Bacillus.*

Species	Source	pH Tolerance	NaCl Tolerance
B. cohnii	Horse meadow soil	obligate alkalophilic	5%
B. horikoshii	Soil	obligate alkalophilic	17%
B. marismortui	Dead sea water	6.0-9.0, pH optimum 7.5	5-25%
B. agaradhaerens	Soil	obligate alkalophilic	16%
B. aclarkii	Soil	obligate alkalophilic	16%
B. horti	Soil, Japan	alkali tolerant	10%
B. vedderi	Bauxite waste	obligate alkalophilic	7.5%
B. alkalophilus	Soil and faeces	obligate alkalophilic	8%
B. clausii	Garden soil	alkali tolerant	10%
B. haloalkaliphilus	Brine/mud, Wadi Natrun	obligate alkalophilic	25%
B. halodurans	Soil	obligate alkalophilic	12%
B. pseudoalkaliphilus	Soil	obligate alkalophilic	10%
B. pseudofirmus	Lake bank soil	obligate alkalophilic	17%
Bacillus sp. DSM8714	River bank soil	alkalitolerant	10%
Bacillus sp. DSM8717	Horse and elephant manure	alkalitolerant	10%
Halobacillus halophilus	Salt marsh soil and solar salterns	7.0-9.0	15%

1.2.3. Gram Negative Haloalkalophilic Microorganisms

Halomonadaceae members are slightly and moderately halophilic bacteria with a broad pH tolerance. They are isolated from saline water, soil samples, leather processing, seawater and estuarine water. Species belong to the Halomonadaceae are Gram negative, rod shaped, non-spore forming, aerobic and also facultatively anaerobic in the presence of nitrate. (Dobzon and Franzman 1996; Franzman et al., 1988). By 16S rDNA sequence analysis, chemotaxonomic and physiological characteristics *Halomonas* have been defined as the only genus in Halomonadaceae (Dobson et al., 1996; Franzman and Tindall 1990). However, recently it has been proposed that Halomonadaceae has four different genera; *Halomonas*, *Alkanivorax, Carnivoras, Chromohalobacter* and *Zymobacte* (Bromguarte, 2003).

1.2.4. Haloalkalophilic Archea

Bacteria and archaea have been first recognized as two distinct groups of Prokarya by Woese et al. (1997). The original classification based on 16S ribosomal RNA gene sequence has been confirmed by extensive phylogenetic studies (Rosa et al., 2002). Extreme halophiles or Euryarchaeota require high salt concentrations, up to 20% NaCl for growth.

Archaea possess genes with recognizable counterparts in the Bacteria, showing that the two groups have functional similarities. Archaea also possess genes that also found in Eukarya (Rosa et al., 2002).

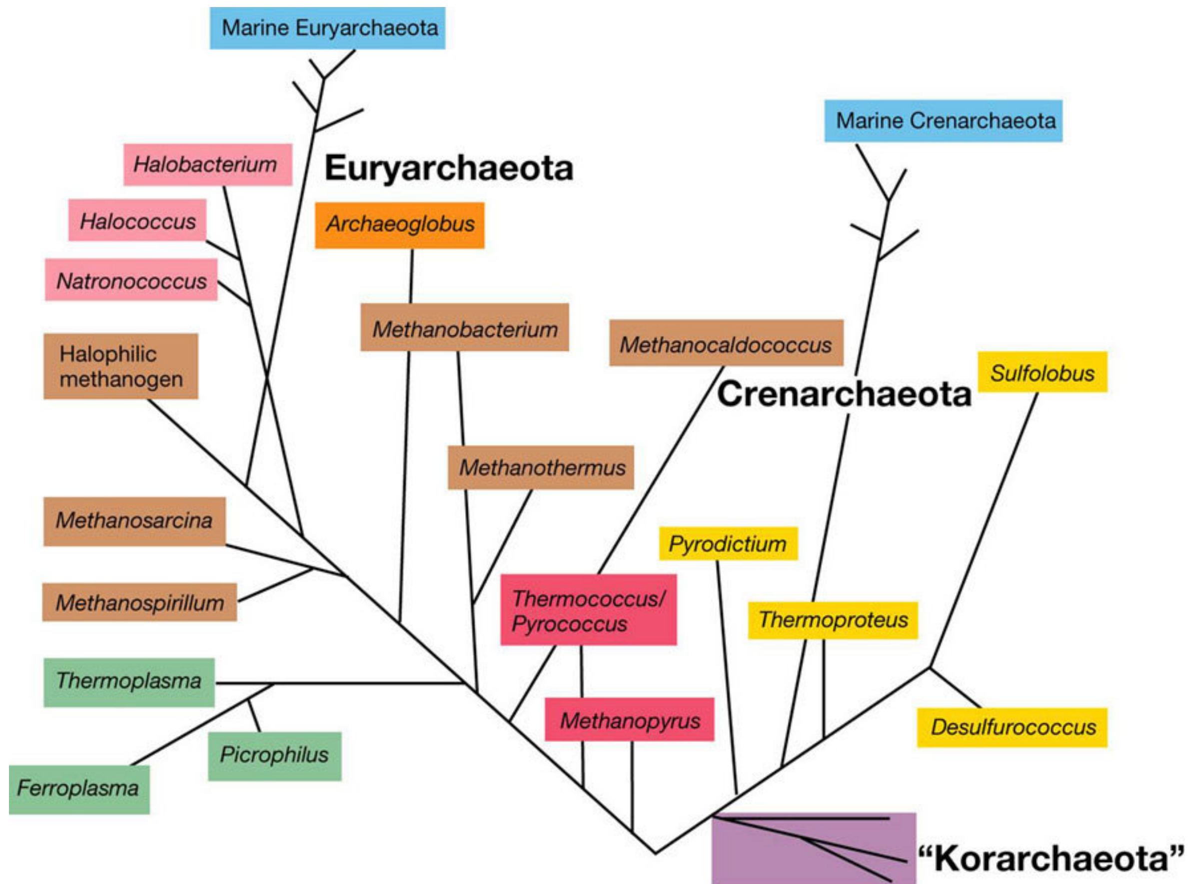


Figure1.1 Archaea kingdom

Euryarchaeaota (*Natronobacterium, Natronococcus, Natronomonas, Natrialba*, *Natronorubrum* genera are classified in "Natro group" of *Halobacteria*. These microorganism are extremely halophilic and alkaliphilic.

Table1.2. “Natro group” of Halobacteria.

Genus	Morphology	Number of species	Habitat
Natronobacterium	Rods	1	Highly saline soda lakes
Natrinema	Rods	2	Salted fish;hides
Natrialba	Rods	2	Soda lakes; beach sand
Natronomonas	Rods	1	Soda lakes
Natronococcus	Cocci	2	Soda lakes
Natronorubrum	Flattened cells	2	Soda lakes

1.2.5. Distribution and Isolation

Isolation of haloalkalophilic microorganisms is performed using different medium ingredients with respect to the group of target organism and the target environment. Actually the salt tolerance range plays an important role because the extremely halophilic organisms can not grow in low concentrations of salt although they have the ability to grow in minimal media. These microorganisms can live at high concentrations of different salts therefore they are populated in salt lakes. Limnological studies focus on the life conditions for extremely halophilic members during seasonal cycles. Anaerobic conditions are suitable in the presence of nitrate for facultatively anaerobes of eucaryotic members.

1.2.6. Biotechnological and Industrial Applications

Extremophilic microorganisms produce cell-associated products that can be used in different industrial applications. First of all their stability and activity in harsh conditions such as alkaline, acidic, high temperature, high concentration of salinity enables broad applications. Extracellular enzymes produced by these organisms are of great interest in biotechnology. Many *Bacillus* species are known to produce alkaline and saline stable proteases, amylases, cellulases, lipases, pectinases and xylanases (Martins et al., 2001)

Enzyme characterization studies with amylase from *Halobacillus* sp. strain MA-2, (Amoozegar et al., 2003) and *Halomonas mediteridiana* (J.Nieto et al., 1999) have

been known. Also the extracellular enzymes from archaeal members have unique features in their molecular structures.

Halophilic microorganisms also used for biological treatment (Kargı and Uygur, 1995).

Halophilic exopolysaccharides have been used in food manufacturing. Furthermore, there is a mereaning demand for non-toxic, biodegradable, environmentally friendly substances. *Halomonas maura* have been studied with novel composition exopolysaccharide (Arias et al., 2003).

1.2.7. Extracellular Enzymes and Their Industrial Application

Extracellular enzymes produced by haloalkalophilic microorganisms have unique properties. They have activity and stability at broad pH ranges and high concentrations of salts in that many other proteins are denaturated. Enzymes from archeal and eubacterial haloalkalophilic organisms have different characteristics. Archaeal enzymes need high concentrations of salt for their activity and stability (Mevarech et al., 2000). This is explained by the comparison of molecular structures from halophilic enzymes with those of nonhalophilic enzymes, this feature is related with adaptation mechanisms used for the protecting of cell from the denaturing effects of salt molecules (Ventosa et al., 1998)

1.2.8. Proteases from Haloalkalophilic Bacteria

Proteases are the enzymes that cleave protein molecules by hydrolysis. The first classification is made by using their cleavage mechanism. Exopeptidases work on the peptide bonds near the amino or carboxy-termini but endopeptidases are active on the peptide bonds distant from the termini of the substrate. The subclassification is based on their catalytic mechanism. Chemically different groups are found on active sites. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao et al., 1998). Subtilisins were grouped in serine type proteases. Catalytic triad of subtilisins consists of aspartic acid, histidine and serine. Although the size of subtilisins varies from 18 kDa to 90 kDa, all the subtilisins used in detergents have a size of

approximately 27 kDa. Most of the alkaline proteases were subtilisins. At present, less than 15 different enzyme molecules are used in detergents worldwide. These enzymes originate from *B. amyloliquefaciens, B. licheniformis, Bacillus clausii, Bacillus lentus, Bacillus alkaloophilus,* and *Bacillus halodurans*

The success of subtilisins is based on several factors, including their high stability and relatively low substrate specificity, features common in extracellular proteases. Their production as extracellular enzymes is of course an important factor in itself, as this greatly simplifies the separation of the enzyme from the biomass and facilitates other downstream processing steps. Another important point is the ability of *Bacillus* strains to secrete enzymes over a very short period of time into the fermentation broth.

Detergent industry prefers the enzymes, which are stable at high pH and compatible with chelating and oxidizing agents (Rao et al., 1998). Table represents the detergent proteases available (Maurer, 2004).

Table1.3. Detergent proteases (Maurer, 2004).

Trade mark	Origin	WT/PE	Production strain	Synonym
Genencor	*B. amyloliquefacies*	PE	*B. subtilis*	
Novozymes	*B. clausii*	WT	*B. clausii*	Subtilisin 309
Genencor	*B. lentus*	WT	*B. subtilis*	
Kao	*B. alkalophilus*	WT	*B. alkalophilus*	
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Novozymes	*B. halodurans*	WT	*B. halodurans*	Subtilisin 147
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. alkalophilus* PB92	PE	*B. alkaliphilus*	

PE, protein engineered; WT, wild type.

Since 1997, several gene-shuffling approaches have been performed with subtilisins. Interesting results from the shuffling of 26 protease genes have been described for properties such as activity in organic solvents, temperature stability, and activity at high or low pH (Minshull et al., 1999). Little, however, has been published on stain removal. Owing to the large number of variant molecules generated by

shuffling and other random techniques, screening methods with high-throughput and increased relevance have had to be developed. Unfortunately, these methods are still not entirely satisfactory, which might explain why no outstanding new subtilisin variant created by one of the gene shuffling technologies is yet present in detergents. Subtilisins for detergents are produced in *Bacillus*, because these species are able to secrete large a mounts of extracellular enzymes. The control mechanisms involved in the production of proteases in Bacillus are extremely complex and still not fully understood. An example is the two-component regulatory system that acts as a quorum sensing mechanism in *B. Subtilis* and which has been found to control the expression of the alkaline protease. This regulatory system is encoded on the chromosome and on endogenous plasmids. (Maurer, 2004)

Arhaea members have proteolytic activity in high salt concentrations (Eichler, 2001). Proteases from *Natronococcus occultus* and *Natrialba magadi* have been characterized (De Castro et al., 2001).

Leather industry uses proteases at dehairing and baiting steps (Kumar and Takagi, 1999). Stable proteases under alkaline and saline conditions would be most effective enzymes for such purposes.

It has been reported that Halophilic bacteria destroied brine-cured hides with their high proteoliytic activity (Bailey et al., 1998).

The studies about using proteases and gelatinases for the pretreatment of leather waste including cromium are important for the convertion of waste material to animal feed (Taylor, 2002).

Silver recovery has been achieved with different types of alkaline proteases at high alkaline conditions (Singh et al., 1999, Kumar and Takagi, 1999).

1.2.9. Lipases from Haloalkalophililes

Lipases, (triacylglycerol acylhydrolases, EC 3.1.1.3), are able to catalyse both the hydrolysis and the synthesis of esters bonds in lipid molecules. Lipid molecules are composed of glycerol and long-chain fatty acid molecules. Esterification and hydrolysis reactions that can occur in organic solvents are very important (Ishıkawa and Ogino,

2000). Most of the lipolytic enzymes used commercially are produced by alkalophilic microorganisms and they are used as detergent addiditives (Sharma et al. 2001).

Table.1.4. Lipolytic activity

Active site	Catalytic triad consisting of the amino acids serine, aspartic (or glutamic acid) and histidine; homologue to the triad of serine proteases.
Substrates	Oils (olive, soybean, mineral oils),Triglycerides (triolein, tributyrin), Synthetic surfactants (Tweens 20 and 40).
Other Activities	Phospholipase, lysophospholipase, cholesterol esterase, cutinase, amidase.

Lipases from *Halomonas* strains have been characterized (Mattiasson et al., 2004).

Alkaline lipases are mainly used in detergent industry. Approximately 1000 tons of lipases are added to the 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Alkaline lipases to be used in detergents should meet certain requirements. They should hydrolyze fats of various compositions, be stable in harsh washing conditions (pH 10-11, 30-60 °C) and in the presence of surfactants (linear alkyl benzene sulfonates (LAS) and proteases) (Sharma *et al.,* 2001).

1.3. Identification of the Haloalkalophilic Organisms

Identification of the haloalkalophilic microorganisms are performed by using the phenotypic and genotypic characteristics.

1.3.1. Phenotypic Methods

Morphological characteristics can be examined by direct observation, which give simple information on the organism for example colony formation that includes colony shape, diameter and color. Furthermore, microscopic observations after different chemical treatments give detailed information on the cell morphology. These are cell shape, Gram behavior and sporulation type. Phase contrast microscopy gives

information on the cell shape and sporulation type without any chemical treatment. Some tests can provide information about the adaptive mechanisms and relation between interior of the cell and the medium. These tests conditions are: growth at different pH ranges, temperatures, pressure and salt concentrations. Bergey's Manual of Systematic Bacteriology a major taxonomic compilation of prokaryotes gives information in all recognized species of prokaryotes and contains number of keys to evaluate the properties given above (Madigan *et al.,* 1997).

Phenotypic methods also include biotyping, antibiogram, phage typing, serotyping, PAGE/immunoblotting and multilocus enzyme electrophoresis (Busch and Nitschko, 1999).

Although molecular characterization methods provide clear differentiation and precise identification, using of phenotypic characterization methods combined with molecular techniques are required for taxonomic studies and functional purposes.

1.3.2. Molecular Characterization Methods

1.3.2.1. Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is used for the analysis of microbial genomes and molecular characterization of the microorganisms below species level. It enables to the seperation of large DNA molecules and therefore whole genomic DNA can be analysed. Analysis of chromosomal macrorestriction patterns by PFGE is a powerful method to produce fingerprints of closely related strains (Farber, 1996). Application of the method can differ for different type of organism. Different organism can require different cell disruption treatments for the releasing the genomic DNA. Also the size and content of genomic DNA can differ among the organisms. Thus, optimization parameters such as lysis of cells, electrophoresis conditions (pulse time, voltage) are the main points for obtaining fine discriminative PFGE results.

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)

DNA fragments are amplified by the polymerase chain reaction (PCR) using short (generally 10 bp) synthetic primers of random sequence. These oligonucleotides serve as both forward and reverse primer at the same time, and are usually able to amplify fragments at 3-10 genomic sites simultaneously. Amplified fragments (within the 0.5-5 kb range) are separated by gel-electrophoresis and polymorphisms are detected as the presence or absence of bands of particular size. These polymorphisms are considered to be primarily due to variation in the primer annealing sites. Some advantages of RAPD are as follows: low quantities of template DNA are required (5-50 ng per reaction), no sequence data for primer construction are required, easy and quick to assay, low costs involved, generation of multiple bands per reaction, and it suitable for automation.

1.3.2.3 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP (Restriction Fragment Length Polymorphism)

In most prokaryotes the ribosomal RNA genes reside sequentially in the chromosome (16S-23S-5S) and they are transcribed as a single polycistronic RNA molecule (Luz et al., 1998). These ordered genes have long been served as a genetic marker to study microbial diversity. The 16S rRNA gene sequence has been used for assessing bacterial phylogenies (Abd-El-Haleem et al., 2002). However, it has only provided information useful for identifications at genus level (Shaver et al., 2002).

Below the genus level, additional rDNA sequences have been necessary. The use of 23S rDNA has also been limited by its larger size and insufficient sequence data (Abd- El-Haleem et al., 2002). The intergenic spacer region (ISR) or also known as intergenic/internal transcribed spacer (ITS) (Abd-El-Haleem et al., 2002; Daffonchio et al., 2000; Shaver et al., 2002; Fisher and Triplett, 1999; Toth et al., 2001) between 16S and 23S rDNA genes, includes both conserved and highly variable sequence motifs, such as tRNA genes, and anti-terminators (boxA) (Abd-El Haleem et al., 2002). ISRs are generally found in multiple copies in most bacteria genomes. Since they are

hypervariable with respect to adjacent genes, they can differentiate between multiple operons in the cell (Daffonchio et al., 1998). The size of the spacer has been found to differ considerably among different species, and even among the different operons within a single cell (Garcia-Martinez et al., 1999).

Figure 1.8 Schematic representation of a 16S-23S spacer and organization of ITS functional regions (Garcia-Martinez et al., 1999).

The method is based on the amplification of 16S rDNA and ISR regions as singleamplicon. The resulting amplicon is then digested with frequent cutting enzymes. Finally restriction fragments obtained are separated by agarose gel electrophoresis and restriction patterns are then compared. The method could discriminate different groups of bacteria at species level.

1.3.2.4. Plasmid Profiling

Plasmids are the genetic materials of bacteria carried as extracromosomal elements. They are self-replicative and they can be transferred naturally between the microorganisms. Genes coded in plasmid DNA structures give different characteristics to microorganisms.

Plasmids isolated from the microorganisms give variable profiles with respect to the organism type and the habitats. Therefore the gel profiles of plasmids for closely related organism would be useful in taxonomic studies (Farber, 1996).

1.3.2.5. Ribotyping

Nucleic acid probes that recognize ribosomal genes are used for ribotying. Ribosomal RNA (rRNA) genes are composed of 23S, 16S and 5S regions. They are known to be highly conserved. Whereas most of the bacterial genes present in one copy, rRNA operons can be present in anywhere more than one copy (2-11 copies per bacterial cell). Thus the more copies of rRNA operon enables more discriminatory riboyping. In practice, first bacterial DNA is isolated and digested by restriction endonucleases. After agarose gel electrophoresing, DNA is transferred onto nylon or nitrocellulose membrane by capillary system or electrophoresis. The nylon membranes

are used as a solid support for probing. Probes specific to 23S, 16S, 5S rRNA genes are labeled using radioactive or non radioactive labeling techniques (non isotopic cold-labelling systems). Hence fingerprint patterns of bacterial DNA containing ribosomal genes are created. These patterns are varied from 1 to 15 bands and compared among the isolates. For another ribotying technique called as chemiluminescence ribotyping, digoxigenin-labeled cDNA (DNA which has been made by reverse transcription of rRNA) can be seen in figure 2. One of the major advantages of ribotyping is that because of the similarity of ribosomal genes, universal probes can be used. Reproducibility is also another advantage of this genotypic method.

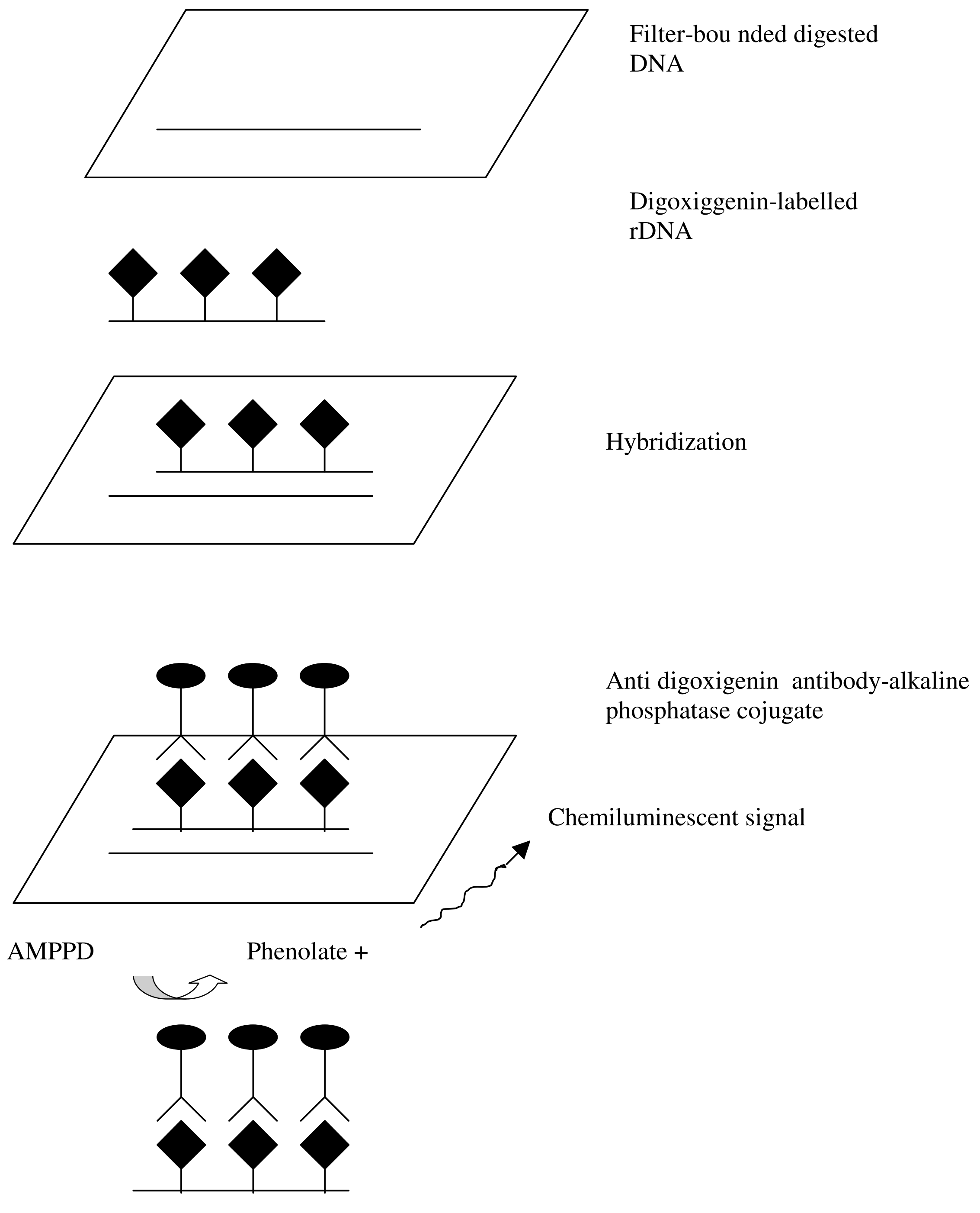


Figure1.2. Schematic representation of chemiluminescence ribotyping. AMPPD, adamantly-1, 2-dioxetane phosphate (Bingen et al., 1994)

1.3.2.6. DNA Sequencing

DNA sequencing is used for investigation of the phylogenetic relationship of organisms. This tecnique is based on the exact order of nucleotides in a region of the DNA molecule Automated DNA sequencing generally includes PCR amplification of a DNA region of interest, computer-aided sequence analysis and interpretation. For DNA sequencing the region of the chromosome to be analysed must be short consisting of a variable sequence flanked by highly conserved region. The variability within the selected sequence must be sufficient to differentiate microorganisms. And also the selected sequence should not be horizontally transmissible to other strains of species (Olive and Bean, 1999).

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir)

Leather factories of Ege Region are populated in Menemen – İzmir. twenty different companies have leather factories in Menemen region. It is known that industrial activities bring the most important pollution problems. For this reason leather factories were placed far from the centre of the city. Leather processing steps are as fallows:

- Curing
- Beamhouse operations which wash and soak the hides or skins and (at most tanneries) remove the attached hair.
- Tanyard processes in which the tanning agent (primarily chromium) reacts with and stabilizes the proteinaceous matter in the hides or skins.
- Finishing or post-tanning processes.

Waste Water of the leather factories are removed from the other factories at Menemen region and a simple waste water treatment is applied. The classical scheme is given below (UNEP/IEO, 1994).

- Pre-treatment: mechanical screening to remove coarse material.

- Primary treatment: sulphide removal from beamhouse effluents; chrome removal from tanning effluents; flow equalization; physical-chemical treatment for

-BOD removal and neutralization.

- Secondary treatment, usually biological.

- Tertiary treatment, including nitrification and denitrification.

- Sedimentation and sludge handling

1.5Thesis Objectives

The purpose of this study was,

- Isolation of haloalkalophilic strains
- Screening for their extracellular enzymes: protease and lipase
- Characterization of these isolates by phenotypic and genotypic methods.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used were listed in Appendix A.

2.1.2. Samples used for isolation

Table 2.1. Samples used for isolation

Sample Type	Location
Polluted Soil	Leather Factories waste (İzmir – Menemen)
Leather processing	Leather Factories (Izmir-Menemen)

2.2. Methods

2.2.1. Isolation and Preservation of the Isolates

2.2.1.2. Isolation of Haloalkalophilic Bacteria

Dilution plate method was used for the isolation of haloalkalophilic bacteria (Mora et al., 1998). Ten g soil, leather or 10 ml of water samples were transferred in 90 ml of 0.85% saline water. In order to homogenize the samples 6h incubation was performed in orbital shaker at room temperature. The samples were pasteurized for 10 m. at 80°C in a water bath in order to kill most of the vegetative cells and eliminate non-

spore forming bacteria (Mora et al., 1998). After the heat treatment, for dilution, 1ml aliquots from each sample was transferred in 9 ml of 0.85 % saline water and 6 fold dilutions were prepared. One ml of the dilutions was plated on appropriate solid media (Appendix B.1) and incubated for 5 days at 37 °C. Single colonies were picked and they were purified using streak plate method.

2.2.1.3. Preservation and Activation of Isolates

Cultures were grown in Horikoshi-I (Apendix B.1) agar plates containing 12% NaCl and incubated for 2 days at 37°C they were then transferred into the isolation broth. After turbidity formation in isolation broth, 0.5ml of each culture were transferred into cryotubes and 0.5ml of isolation broth containing 20% glycerol was added. These steps were all performed on ice. Then tubes were mixed gently by pipetting. Cultures were stored at -20°C for an hour they were then stored at -80°C. For the phenotypic test culture activation was applied from 20% glycerol stocks. For phenotypic and genotypic identification of the isolates, cultures were prepared from these stocks.

2.2.2. Determination of Phenotypic Characteristics

2.2.2.1. Gram Staining

Isolates were stained by modified Gram method for halophilic bacteria. Eighty µl of sterile 0.85% saline water were pipetted onto slides the organisms were then suspended and mixed thoroughly. Overnight culture was spread onto the microscope slides until a thin film formed by using tooth stick. After being air dried, the slides were fixed and desalted simultaneously by immersing in 2% acetic acid for 5 min. Then they were dried again. The smears were covered with 0.25% aqueous solution of crystal violet (Apendix D.2.1) for 1 min. After rinsing under the tap water, the slides were transferred into iodine (Apendix D.2.2) solution and were kept for 1 min. Following this step, slides were washed in alcohol for 6 s. They were then stained with safranin (Apendix D.2.3) for 30 s. After staining, the slides were dried on paper towels and the

cells were examined under light microscope. Gram (+) cells assumed purple color while Gram (-) cells appeared pink or red.

2.2.2.2. Examination of Endospores

Isolates were grown on Horikoshi-I (Apendix B.1) agar plates and 0.0005% $MnCl_2$ (Travers, 1987) were added in to the medium. Observations for spore formation were monitored at 3-4- 5-6th day.

2.2.2.3. Catalase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. Catalase activity was observed after dropping 3% hydrogen peroxide solution onto the target colony. Formation of the air bubbles indicated the presence of catalase activity.

2.2.2.4. Oxidase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. 1% solution of tetramethyl-p-phenylenediamine was poured onto a filter paper placed in a petri dish. Then the target colony was taken from the agar plate and spotted onto wet filter paper. Observation of the blue-purple color indicated the oxsidase activity.

2.2.2.5. Growth at Different Temperatures

The isolates were grown on nutrient agar (Apendix B.2) 45°C and 50°C. Observations for growth were noted during the 5-day incubation.

2.2.2.6. Growth at Different NaCl Concentrations

Cultures were tested for their salt tolerance on Horikoshi-I (Apendix B.1) agar plates containing 0%, 20%, 25 % NaCl during the 5-day incubation.

2.2.2.7. Growth at Different pH Ranges

Cultures were tested for pH tolerance in nutrient agar plates at different pH points (7, 10, and 12). Growth behaviors were observed during the 5-day incubation

2.2.3. Screening for Extracellular Enzymes

2.2.3.1. Screening for Protease Activity

Proteteolytic activity of the isolates observed by adding casein into Horikoshi–I (Apendix B.1) medium adding sikimmed milk. Different concentrations of NaCl (12% and 20%) were added. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete proteases extracellularly, give clear zones around the colony (Horikoshi, 1999).

2.2.3.2. Screening for Lipase Activity

Lipolytic activity of the isolates was observed by using the medium described in Appendix B.5. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete lipase extracellularly, gave opaque zones around the colony (Haba et al., 2000).

2.2.4. Preparation of Genomic DNA

Cultures were grown on solid Horikoshi – I (Apendix B.1) medium. Cells were suspended in 1.5 ml of sterile water in petri dishes. Then cell suspension was transferred into eppendorf tubes. In order to prepare cell pellets, tubes were centrifugated for 5 min at 5000 rpm. Pellets were resuspended in 567 µl 1Xte (Apendix C.4). Thirty µl 10% SDS and 3µl proteinase K solution (20 mg/ml) were added and the solution was mixed thoroughly. Cell lysis was performed with 1h incubation at 37°C. Afterwards 0.1 ml of 5 M NaCl were added and mixed well. Then 80µl of CTAB/NaCl (Appendix C.8) were added, the tubes were then mixed and incubated for 10 min at 65°C. Following the incubation, 0.7 ml of chloroform/isoamyl alcohol (Appendix C.2) were added, mixed thoroughly and centrifuged for 5 min at 10000 rpm. Aqueous, viscous supernatant was

transferred into a fresh eppendorf tube. An equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged for 5 min. The supernatant was again transferred into a fresh eppendorf tube and 0.6 volume of isopropanol were added to precipitate the nucleic acids. The tube was then shaken back and forth until a white DNA precipitate became visible. DNA wool were taken with a pipet tip and placed into a new tube, contain 300µl 70% ethanol. The tubes were stored at -20°C for 1h. Afterwards tubes were centrifuged for 15 min at 6000 rpm. Then the supernatant was discarded and DNA pellet was washed with 500 µl 70% ethanol. It was centrifuged for 10 min at 8000 rpm. After this, ethanol was discarded. The pellet was dried and dissolved in 100 µl 1xTE containing 100 µg/ml RNAase. Then tubes were incubated for 1h at 37°C. After the incubation the sample volume was adjusted to 400 µl with 1xTE. DNA was dissolved by alternating cold-heat shocks (for 10 min at 80°C and 20 min at -20°C, repeated twice). To further purify DNA an equal volume of phenol was added and mixed well. An equal volume of chloroform was added and mixed well. Then the tubes were centrifuged for 5 min at 6000 rpm. The aqueous phase was transferred into a fresh eppendorf tube. Equal volume of chloroform /isoamyl alcohol was added and mixed well. Samples were centrifuged for 5 min at 6000 rpm. Chloroform /isoamyl alcohol step was repeated once again and DNA was precipitated by adding 1/10 sample volume of 5 M NaCl and 2 volumes of 99% ethanol. Pellet was washed in 500 µl 70% ethanol by centrifugation for 5 min at 6000 rpm. After that, ethanol was removed and samples were dried for 10 min at 37°C. DNA was dissolved in 20µl, 50µl, 100µl or 200µl 1xTE according to the pellet size. Then the samples were preserved at -20 °C.

2.2.5. Genotypic Characterization

2.2.5.1. Identification of isolates by 16S rDNA - ITS rDNA - RFLP

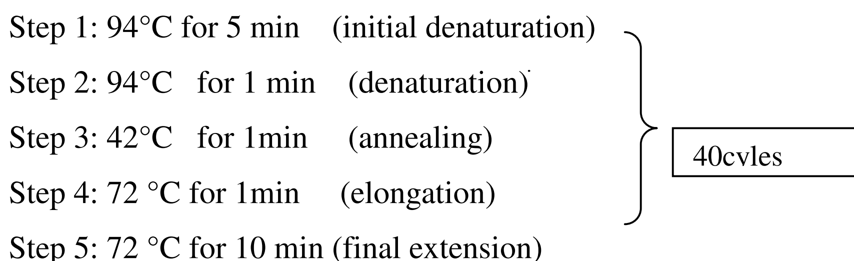
2.2.5.1.1. Amplification of 16S ITS rDNA

PCR amplification of 16S-ITS rDNA of the isolates was performed by using two DNA primers (Apendix F). The forward primer was complementary to the upstream of 16S rRNA gene and the reverse was complementary to the upstream of 23S rRNA gene.

Forward: 5. -AGAGTTTGATCCTGGCTCAG-3 (Mora et al., 1998).

Reverse: 5. -CAAGGCATCCACCGT-3 (Jensen et al., 1993).

Amplifications were performed in a Mini Cycler System. PCR conditions were as follows:



The final reaction volume was 50µl. PCR mix (Appendix E.1) was prepared after 2 µl -4µl of genomic DNA (approx. 500 µg) was placed into 1.5ml eppendorf tubes. During the preperation of the mix, tubes were kept on icebox. Taq DNA polymerase (1.25U) was added and mixed gently and then tubes were centrifuged for 2-3 s. The mix were added to each of the tube. Then 60 µl of mineral oil was added into the tubes while they were on ice. Tubes were centrifuged for 2-3 s then they were placed into the wells of Mini Cycler System (MJ Research INC, USA).

2.2.5.1.2. Electrophoresis of Amplified 16S-ITS rDNA Fragments

Amplified fragments were separated in 0.8 % agarose gel. First, 0.96 g of agarose was dissolved in 120 ml of 1×TAE buffer (Appendix C.4) by boiling (15 µl of ethidium bromide solution (10 mg/ml) were added after cooling the solution to 40°C). The gel was then poured into the gel-casting stand and the combs were placed. 1×TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. Five µl of PCR products were taken and mixed with 1 µl, 6 × gel-loading buffers. The samples were then loaded into the wells of agarose gel starting from the second well. Finally, 5 µl of DNA molecular weight marker were loaded into the first well and they

were electrophoresed at 40 mA until bromophenol blue have reached 2/3 of the gel. DNA fragments were visualized in a photodocumentation system (Vilber-Lourmat, France).

2.2.5.1.3. Chloroform Extraction of PCR Products

Final volumes of the PCR products were adjusted to 100 µl with 1×TE buffer. Then two volume of chloroform were added. After centrifugation for 2 min at 6000 rpm, organic phase was discarded and chloroform extraction was repeated. Then the tubes were centrifuged for 2 min at 6000 rpm. The aqueous phase was transferred into new eppendorf tubes containing 10 µl of 3 M sodium acetate (pH 5.2) and mixed well. After that, 450 µl of 99% ethanol were added and mixed. The samples were centrifuged for 15 min at 7000 rpm. Pellet was washed with 500 µl 70% ethanol. After centrifugation for 5 min at 6000 rpm, alcohol was removed. Pellet was dried for 10 min at 37°C, and they were dissolved in 25 µl 1xTE, and stored at 20°C.

2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP)

Purified PCR products were separated in 1% agarose gel in order to estimate DNA concentrations for each sample. Then 12.5µl of PCR products were aliquoted into new tubes for digestion. *Taq* I and *Hae* III were the enzymes chosen for this purpose. Reaction mix (Appendix F2) was added on PCR products while the tubes were stored on ice. 5 U of enzyme was used for each reaction. The final volume of the reaction was adjusted to 50 µl. Incubation period was 5 h for both of the enzymes. But the optimum reaction temperatures were 65°C for *Taq I* and 37°C for *Hae* III. Mineral oil was added over the reaction mixtures to avoid evaporations at 65°C. After the incubation DNA was extracted as described in section 2.2.6.1.3. and dissolved in 15 µl of 1xTE.

2.2.5.1.5. Electrophoresis of Restriction Fragments

Restriction fragments were first separated in 2 % agarose gel containing 20 µl of ethidium bromide solution (10mg/ml). Electrophoresis was carried out at 60 mA for the

first 20 min, then the current was adjusted to 80 mA and the electrophoresis was continued further for 2.5 h. Some isolates produced similar restriction fragment patterns. These were contained within distinct groups. One digested sample from each group was picked and re-electrophoresed in order to produce representative restriction patterns for further restriction profile analyses.

2.2.6 Sequencing

2.2.6.1. Amplification of 16SrRNA Gene

16SrDNA region of rRNA genes of the isolates were amplified using the following DNA oligoprimers for sequencing reactions

EGE 1. 5. - AGAGTTTGATCCTGGCTCAG –3 (Jensen *et al.,* 1993)

EGE 2. 5. - CTACGGCTACCTTGTTACGA –3 (Jensen *et al.,* 1993)

Two µl of genomic DNA (approx. 500 µg) were transferred into 0.5 ml eppendorf tubes, as the template. PCR mix was prepared and after the addition of *Taq* DNA polymerase (1.25 U), it was mixed gently and centrifuged for 2-3 s. Forty-eight ml of the mix were distributed into the tubes. All the manipulations were performed on ice. The tubes were then overlaid with 60µl mineral oil, centrifuged for 2-3 s at 6000 rpm. Finally, they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) using the following program:

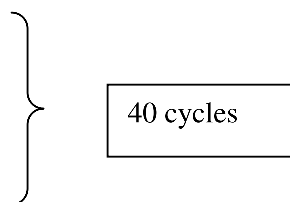
Step1: 95°C for 5 min

Step2: 95°C for 1 min

Step3: 56°C for 1 min

Step4: 72°C for 1 min

Step4: 72°C for 10 min



2.2.6.2 Extraction of DNA Fragments from Agarose Gel

DNA isolation kit (Applichem A3421) was used for extraction of amplification products from agarose gel.

DNA bands (approximately 1600 bp) were excised from the agarose gel by a rasor blade. Three gel slice volume of 6 M NaI (for TAE gels) was added. The samples were incubated for 5 min at 55°C, mixed and continued for incubation for 2 min. Ten µl of well mixed glass powder suspension was then added, mixed well and incubated for 5 min at 55°C by occasional mixing. The samples were then centrifuged for 10 s at 10.000 rpm and the supernatant was discharged. The glass pellet was rinsed with 500 µl wash buffer. And resusupended by pipetting or gently flicking the tube. The samples were then centrifuged for 10 s at 10,000 rpm. The washing step was repeated twice. After the last wash, the supernatant was removed as much as possible. The pellet was suspended in 25 µl of 1xTE buffer. The samples were eluted for 5 min at 55°C with occasional mixing. Centrifugation was performed for 45 s at 10,000 rpm. The supernatant was finally transferred carefully into a new eppendorf tube. The eluted DNA samples were stored at -20°C until use.

2.2.6.3 Sequencing Reactions

The sequencing reactions were performed by using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit.

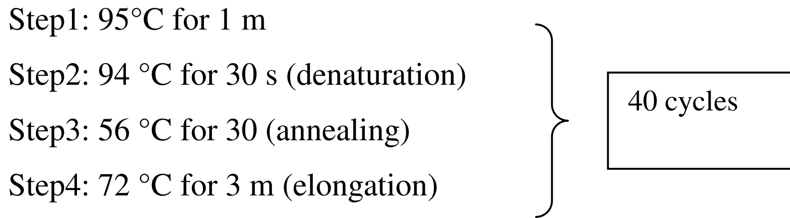
Four eppendorf tubes were firstly labeled as “A”, “C”, “G”, and “T” for respective termination reactions. Two µl of corresdonding Cy5.5 ddNTP termination mix were then dispensed into the appropriately labelled tubes. The tubes were then capped to prevent evaporation. The master mix (Appendix I) was prepared in an eppendorf tube for each template to be sequenced. The content of master mix was mixed thoroughly and 7µl of the mix was dispensed into each tube labelled “A”, “C”, “G”, and “T”. Each sequencing reaction was mixed thoroughly and overlaid with one drop of mineral oil. Each tube was capped and placed into the thermal cycler. The sequencing condition reastions were:

Step1: 95°C for 1 m

Step2: 94 °C for 30 s (denaturation)

Step3: 56 °C for 30 (annealing)

Step4: 72 °C for 3 m (elongation)



40 amplification cycles of Step 2, 3 and 4 were performed

The primer used was ;

EGE 1; 5. - AGAGTTTGATCCTGGCTCAG -3. (Jensen *et al.,* 1993)

2.2.6.4 Purification of Sequencing Reactions

2.2.6.4.1 Removal of unincorporated dye terminators using ethanol precipitation

The volume of the sample was adjusted to 50 µl by adding 1xTE. The samples under the mineral oil were then transferred into fresh eppendorf tubes. Twenty µl of 7.5 M ammonium acetate and 200 µl (3 times the reaction volume) of chilled 100% ethanol were added to each tube. The tubes were then mixed thoroughly by vortexing and placed on ice for 20 min to precipitate the DNA. Centrifugation was then performed at 12, 000 rpm for 30 min at 4°C. Supernatants were removed carefully and the pellets were washed with 200 µl of chilled 70% ethanol. After the centrifugation for 5 min, the supernatants were removed and the pellets were dried. Each pellet was then resuspended in 6 µl of formamide loading dye and vortexed vigorously (2-3 min) to ensure complete resuspension. Just prior to loading the samples onto gel, each sample was heated at 70 °C for 2-3 min for denaturation and then placed on ice. One and a half or 2µl of each sample were then loaded into the lanes of sequencing gel.

2.2.6.4.2 Assembling the SEQ Sequencer

The sequencing reactions were performed with SEQ personal sequencing system (Amercham Pharmacia Biotech). The system consists of SEQ personal sequencer, SEQ

Rapidset gel polymerizer, RapidGel QuickFill cassettes, RapidGel-XL-6% cartridge and filling gun and filling fixture.

Preparing a gel involves attaching the RapidGel-XL cartridge to the casting gun, filling the RapidGel cassettes, polymerizing the acyrlamide in the gel polymerizer. After the polymerization the comb was removed from the cassette. After cleaning the cassette, it was placed properly into the buffer chamber. At this step pre-run was performed to measure the backgraound redding. Once the gel cassette was aligned and the pre-run was complete, 1xTE buffer was loaded into the upper and lower buffer chambers until it reached the fill lines. The prepared samples (1-2 µl) were then loaded into the gel with a micropipette. Then the analysis was started. After the run was finished, data were analysed by SEQ software.

The sequences obteined were evaluated in and submitted to GenBank.

Chapter 3

RESULTS AND DISCUSSION

3.1. Isolation of Haloalkalophilic Bacteria

Haloalkalophilic bacteria were isolated from Menemen Region. Target places were leather factories and the wastes of this industry. Therefore the samples were taken from both the processing steps and waste.

3.2. Determination of Phenotypic Characteristics

3.2.1. Gram staining

The modified Gram staining method specific for halophilic bacteria gave desired results with these isolates. All the isolates were gram positive, rod shaped except one cocci (C1).

3.2.2. Examination of Endospore

Isolates were grown on agar plates (Appendix) for 5 days. Isolates were non-sporforming.

3.2.3. Catalase and oxidase tests

All the isolates were catalase and oxsidase positive.

3.2.4. Physiological Tests

Growth at pH 7 was observed to determine whether the isolates were obligate alkalophilic (Table 3.2). Four isolates could not grow at this pH value. Twenty-five of the isolates were alkali tolerant and could not grow at pH 12. Isolates were not thermotolerant since they could not grow at 45ºC and 50ºC on alkaline nutrient agar plates.

Table 3.1. Results of the Physiological Test

Physiologic test	Isolate number
No Growth at 20%NaCl	D2, D10, D20. D26, E4, E16, E24, F22
No Growth at PH 7	D1, D20, F9, F21
No Growth at 0%NaCl pH 10	D2, D6, D18, D23, E4, E43, F5, F7, F29
No Growth at pH 12	D1, D6, D10, D18, D20, D23, D24, D30, D32, E2, E4, E10, E12, E14, E16, E23, E24, E36, F7, F10, F13, F16, F20, F22, F29,

Growth of the isolates was also observed at 0% and 20% NaCl concentrations. Nine of the isolates were oblitely halophilic. Eight of the isolates were not extremely halophilic.

Table3.2. Differentiation of Isolates according to growth at different NaCl concentrations and pH ranges.

İsolate Name	20% NaCl	PH 7	0%NaCl pH 10	pH 12
D1	+	-	+	-
D2	-	+	-	+
D5	+	+	+	+
D6	+	+	-	-
D7	+	+	+	+
D10	-	+	+	-
D11	+	+	+	+
D12	+	+	+	+
D14	+	+	+	+
D18	+	+	-	-
D20	-	-	+	-
D23	+	+	-	-
D24	+	+	+	-
D25	+	+	+	+
D26	-	+	+	+
D29	+	+	+	+
D30	+	+	+	-
D31	+	+	+	+
D32	+	+	+	-
E1	+	+	+	+
E2	+	+	+	-
E3	+	+	+	+
E4	-	+	-	-
E6	+	+	+	+
E7	+	+	+	+
E9	+	+	+	+
E10	+	+	+	-
E12	+	+	+	-
E13	+	+	+	+
E14	+	+	+	-
E15	+	+	+	+

E16	-	+	+	-
E17	+	+	+	+
E20	+	+	+	+
E23	+	+	+	-
E24	-	+	+	-
E25	+	+	+	+
E26	+	+	+	+
E27	+	+	+	+
E28	+	+	+	+
E29	+	+	+	+
E30	+	+	+	+
E33	+	+	+	+
E34	+	+	+	+
E35	+	+	+	+
E36	+	+	+	-
E39	+	+	+	+
E42	+	+	+	+
E43	+	+	-	+
E47	+	+	+	+
F1	+	+	+	+
F2	+	+	+	+
F3	+	+	+	+
F4	+	+	+	+
F5	+	+	-	+
F7	+	+	-	-
F8	+	+	+	+
F9	+	-	+	+
F10	+	+	+	-
F11	+	+	+	+
F12	+	+	+	+
F13	+	+	+	-
F14	+	+	+	+
F15	+	+	+	+
F16	+	+	+	-
F17	+	+	+	+
F18	+	+	+	+

F19	+	+	+	+
F20	+	+	+	-
F21	+	-	+	+
F22	-	+	+	-
F23	+	+	+	+
F24	+	+	+	+
F25	+	+	+	+
F26	+	+	+	+
F27	+	+	+	+
F28	+	+	+	+
F29	+	+	-	-
F30	+	+	+	+
F32	+	+	+	+
F34	+	+	+	+
F35	+	+	+	+

3.3. Extracellular Enzyme Screening

Extracellular enzyme screening was performed on solid agar plates by using different substrates for each enzyme activity. Protease screening based on the observation of clear zones around the colonies (Figure). Lipase activity gave opaque zones around the colonies (Figure). Different NaCI concentratios were used for lipase screening. The enzyme screening studies were repeated performed twice.

3.3.1 Extracellular Enzyme Profiles of the Isolates

Table 3.3 Extracellular Enzyme Tests

Extracellular Enzyme Type	Isolate Name
Did not produce lipase	D5, D10, D20, D26 D31, E23, E43, F11, F29 F33
Utilized Tween80 at 0% NaCl	D1, D2, D30, F25, F15
Utilized Tween 20 at 12% NaCl	D23, D24, D25, D30, E1, E2, E3, E42, F5, F12, F13, F14, F15, F16, F18, F23, F25
Did not Utilized Tween 20	D7, E7, E9, E10, E13, E14, E15, E16, E23, E24, E25, E26, E27, E28, E29, E30, E39, E47, F26, F27, F31
Did not utilase Tween80	D12, E12, E34, F1, F20, F30, F32,
Did not produce lipase without NaCI	D12, D14, E12, E34, F1, F30, F32
Utilase casein	F17, D5, F29, D32, E34, F1, F15, E36, D24, F10, F13, F12, E20, F32, E1, E47, E33, E14, E25, E42, F22

Table 3.4 Extracellular Enzyme Profiles of isolates

Isolate Name	Protease	0%NaCl Tween20	12% NaCl Tween20	20% NaCl Tween20	0% NaCl Tween80	12% NaCl Tween80	20% NaCl Tween 80
D1	-	-	-	-	+	+	-
D2	-	-	-	+	+	-	-
D5	+	-	-	-	-	-	-
D6	-	+	-	-	-	+	-
D7	-	-	-	-	-	+	-
D10	-	-	-	-	-	-	-
D11	-	+	-	+	-	+	+
D12	-	+	-	-	-	-	-
D14	-	+	-	-	-	-	-
D18	-	+	-	+	-	-	+
D20	-	-	-	-	-	-	-
D23	-	+	+	-	-	+	+
D24	+	+	+	-	-	+	+
D25	-	+	+	-	-	-	+
D26	-	-	-	-	-	-	-
D29	-	+	-	-	-	+	+
D30	-	+	+	+	+	+	+
D31	-	-	-	-	-	-	-
D32	+	-	-	-	-	+	+
E1	+	+	+	-	-	+	+
E2	-	+	+	-	-	+	
E3	-	+	+	-	-	+	+
E4	-	+	-	-	-	+	-
E6	-	+	-	-	-	+	+
E7	-	-	-	-	-	+	-
E9	-	-	-	-	-	+	-
E10	-	-	-	-	-	-	+
E12	-	+	-	-	-	-	-
E13	-	-	-	-	-	+	-
E14	+	-	-	-	-	+	-
E15	-	-	-	-	-	+	-
E16	-	-	-	-	-	+	-
E17	-	-	-	-	-	+	-
E20	+	+	-	-	-	+	+
E23	-	-	-	-	-	+	-
E24	-	-	-	-	-	+	-
E25	+	-	-	-	-	+	-
E26	-	-	-	-	-	+	-
E27	-	-	-	-	-	+	-
E28	-	-	-	-	-	+	-
E29	-	-	-	-	-	+	-
E30	-	-	-	-	-	-	+
E33	+	-	-	+	-	+	+
E34	+	+	-	-	-	-	-
E35	-	-	-	+	-	-	+

E36	+	+	-	-	-	+	+
E39	-	-	-	-	-	+	-
E42	+	+	+	-	-	-	+
E43	-	-	-	-	-	-	-
E47	+	-	-	-	-	-	+
F1	+	+	-	-	-	-	-
F2	-	+	-	+	-	+	+
F3	-	+	-	+	-	+	-
F4	-	+	-	-	-	+	+
F5	-	+	+	+	-	+	+
F7	-	+	-	+	-	+	+
F8	-	+	-	-	-	+	+
F9	-	+	-	-	-	-	+
F10	+	+	-	+	-	+	+
F11	-	-	-	-	-	-	-
F12	+	+	+	+	-	+	+
F13	+	+	+	-	-	+	+
F14	-	-	+	-	-	+	+
F15	+	+	+	+	+	-	+
F16	-	+	+	-	-	+	+
F17	+	+	-	-	-	+	+
F18	-	-	+	-	-	+	+
F19	-	+	-	-	-	+	+
F20	-	+	-	+	-	-	-
F21	-	+	-	-	-	+	-
F22	+	+	-	-	-	+	-
F23	-	+	+	+	-	+	+
F24	-	-	-	-	-	-	+
F25	-	+	+	+	+	+	+
F26	-	-	-	-	-	+	+
F27	-	-	-	-	-	-	+
F28	-	+	-	-	-	-	+
F29	+	-	-	-	-	-	-
F30	-	+	-	-	-	-	-
F32	+	+	-	-	-		-
F35	-	-	-	-	-	-	-
F34	-		-	-	-	-	-

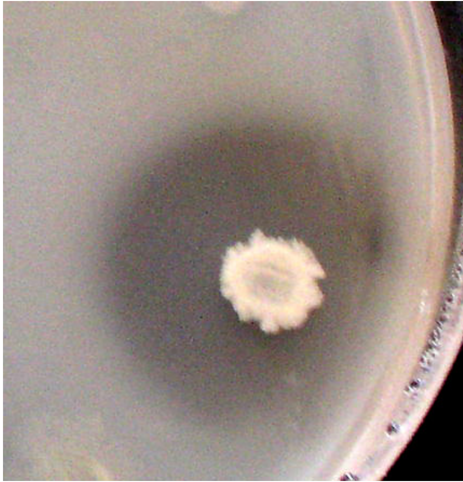


Figure 3.1. Apperance of extracellular protease

Figure 3.2. Apperance of extracellular lipase

3.4. Genotypic Characterization

3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon

The amplification product size differed between 1800 and 2000 bp (Figure)

.

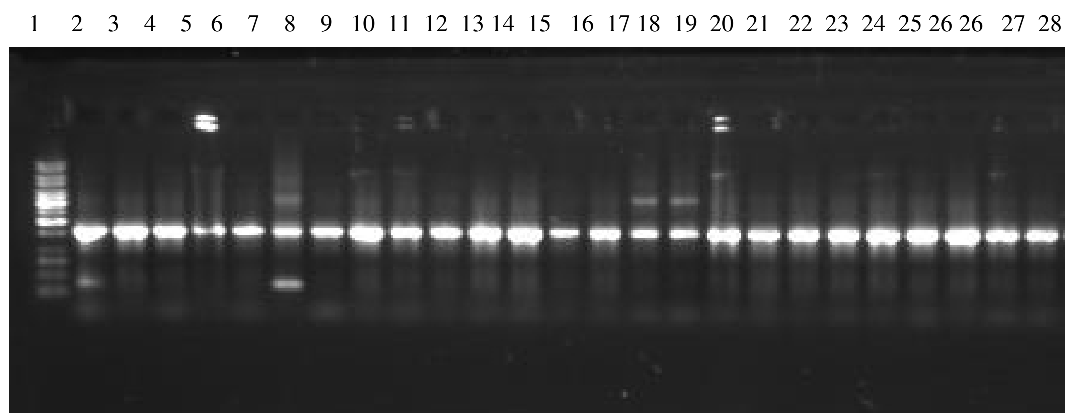


Figure 3.3. 16S rDNA-ITS Amplification products of some isolates. Lanes 1. 1 kb DNA Lac D1, 3. Isolate F2, 4. Isolate F1 5. Isolate D8, 6. Isolate D10, 7. Isolate 12,8. Is Isolate E15, 10. Isolate E17, 11. Isolate E19, 12. Isolate D23 lane 13. Isolate D24

3.4.2. 16S-ITS rDNA - RFLP Profiling

In order to identify the isolates amplification products of 16S-ITS rDNA region was digested with restriction endonucleases. *Taq* I and *Hae* III endonucleases were chosen for digestion. Digestion products were first run in 2% agarose gel to see all different profiles and to make groups within the digestion products. For a specific RFLP group, one representative digestion product was chosen and electrophoresed together in 2.5% agarose gel (Figure 3.3).

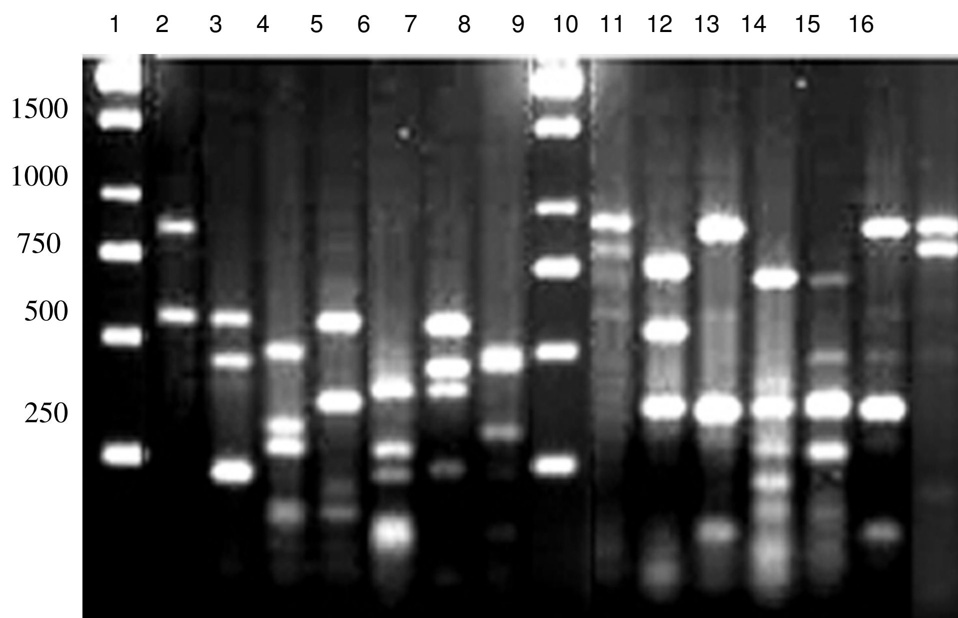


Figure 3.4 16S-ITS rDNA RFLP profiles of *Hae* III groups. Lanes 1. 1 kb DNA ladder, 2. Isolate D5, 3. Isolate D7, 4. Isolate D30, 5. Isolate D12, 6. Isolate D23, 7. Isolate F28, 8. Isolate, F21 16S-ITS rDNA RFLP profiles of *Taq*I groups. Lanes 9. 1 kb DNA ladder,10. Isolate D2, 11. Isolate D18, 12. Isolate D12, 13. Isolate F6, 14. Isolate F20, 15. Isolate F28, 16. IsolateE30,

Resulting gel profiles were analyzed using the program BIO-1D++. Dendrogram of representative strains were carried out using 15% homology coefficient. (Figure 3.3 and 3.4). Fragment sizes were given in Table 3.6.

Hae III and *Taq* I restriction analysis of 16S-ITS rDNA fragments of strains revealed 7 distinct groups (Table 3.3.).

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2000	854	546	465	546	384	546	465	2000	886	673	808	661	361	838	870
1500	555	440	309	354	257	440	432	1473		509	338	354	338	338	778
1000		219	272	125	211	377	294	934		354	78	265	257	78	
750			125		94	219		685				195			
500					70			465				133			
250								227				31			

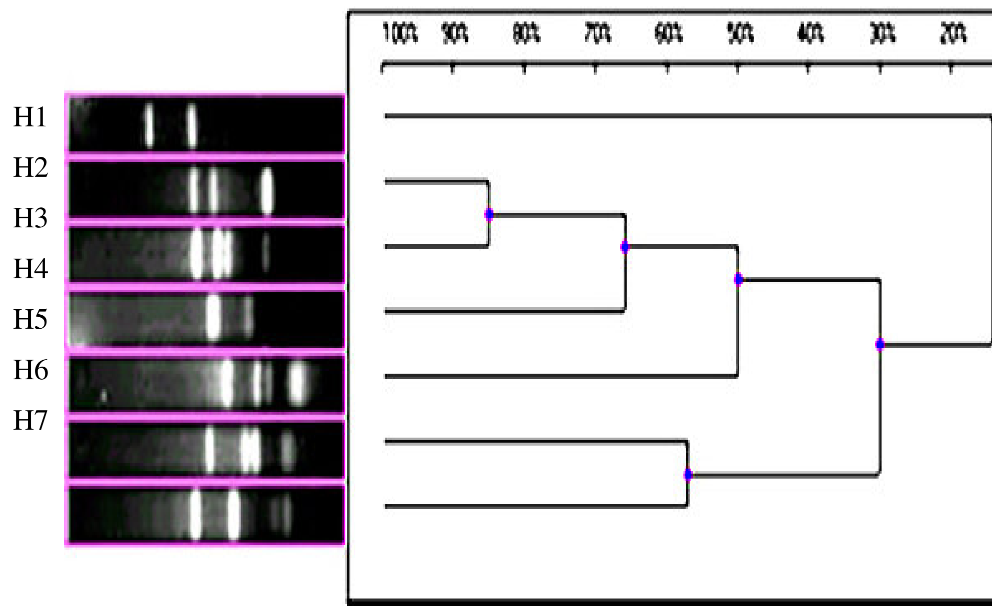


Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles.

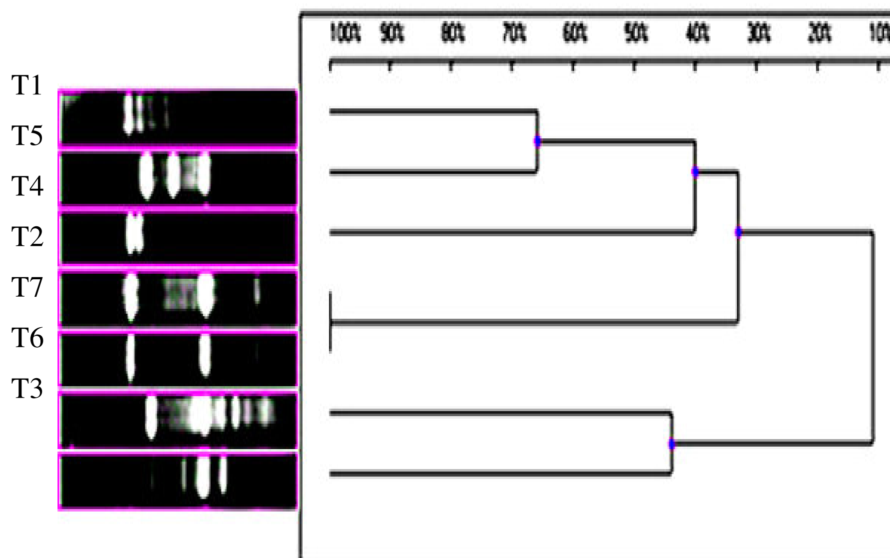


Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles.

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups.

Genotypic groups	Name of the Isolates
HT1	D1, D2, D5, E43,E47,F1
HT2	D6, D7, D10, D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E30, E34, E35
HT3	D12, F32
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E20, E33, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F19, F22, F23, F24, F25, F26, F27
HT5	D30
HT6	F20, F21
HT7	F28

The use of two restriction enzymes enabled the revelation of diversity among the isolates. When the results of both digestion were combined, 7 distinct genotypes were obtained (Table 3.10). The results indicated that isolate group members were differentiated by each restriction enzyme, were the same.

In this study, 16S ITS and rDNA regions were used as a single amplicon for RFLP analysis. No report was found in literature on genotypic characterization of haloalkalophiles using this method. Similar studies have been performed in this institute for genotypic characterization of alkalophilic bacilli (Akbalik, 2004), thermophilic bacilli, (Yavuz, 2004), lactic acid bacteria (Yavuz, 2003, Bulut, 2003.

3.4.3. Analysis of the Genotypic and Phenotypic Groups

Table 3.7. Analysis of the Genotypic and Phenotypic Groups

	Isolate number	20%NaCI	pH 7	0%NaCl	pH 12	Protease	0%NaCl Tween 20	12%NaCl Tween 20	20%NaCl Tween 20	0%NaCl Tween 80
HT1	D1,D2,D5,E34, E43, E47, F1	%80	80%	70%	80%	40%	0%20	0%	20%	%30
HT2	D6,D7,D10,D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E39, F29, F30, F34, F35,	100%	100%	100%	100%	10%	10%	0%	0%	0%
HT3	D12, F32	100%	100%	100%	100%	50%	100%	0%	0%	0%
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E30, E33, E35, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18, F19, F22, F23, F24, F25, F26, F27	90%	100%	90%	70%	30%	90%	40%	30%	20%
HT5	D30	100%	100%	100%	0%	0%	100%	100%	100%	100%
HT6	F20, F21	100%	50%	100%	50%	0%	100%	0%	50%	0%
HT7	F28	100 %	100%	100%	100%	0%	100%	0%	0%	0%

Phenotypic characterization of the isolates indicated that strains of different species had different phenotypic features. When the genotypic results were compared with the results of enzyme screening and phsyical tests, a correlation between a few groups were observed. Most of the isolates produced lipase (Tween 20 and Tween 80) at all NaCI concentrations used grouped in HT4. All members of HT2, HT3, HT5, HT6, HT7 were also able to grow in the presence of 20% NaCI. Most of the isolates that could not grow at pH7 were placed in HT6. Isolates that could not grow at pH12 were placed in HT1, HT5, and HT6.

3.4.4 Analysis of Sequencing Reactions

16S partial sequencing results of six representative isolates are as listed below.

Table 3.8. Sequencing results

Genotypic Group	Isolate Name-Accession Number	Length of bp.-Similarity	Accession Number - Organism	Name of the Family
HT2	D7-AY604872	-89%	*AY345480.1-Bacterium K-29*	Unclassified *Bacillaceae*
HT3	D12-AY601901	716-93%	AF237976.1-*Salinococcus roseus*	Unclassified *Bacillaceae*
HT4	D23-AY601902	323-92%	AJ640133.1-*Halomonas* sp.18bAG	*Halomonadaceae*
HT5	D30-AY601903	-92%	AY383044.1-*Halomonas meridiana*	*Halomonadaceae*
HT6	F20 (C1)-AY604871	-89%	AJ29397-*Nestenkonia* sp.EL-30	*Micrococcaceae*
HT7	F28 (15B)-AY601904	-91%	AY3091336-Bacterium K-2	Unclassified *Bacillaceae*

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, haloalkalophilic bacteria were isolated from samples of waste soil and leather processing. They were first characterized phenotypically (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope and extracellular enzyme screening, physiological tests such as growth at pH 7 and pH 12 , different NaCl concentrations and at different temperatures). Extracellular enzyme screening was performed on solid agar media. Molecular characterization was performed by using 16S-ITS rDNA RFLP.

All the isolated strains were Gram positive but they were not sporeforming. All the strains were catalase positive. Most of the isolates contained oxidase. Four strains could not grow at pH7. They were thus obligately alkalophilic. Twentyfive isolated strain were alkalitolerant because they could not grow at pH 12. Nine isolated strain could not grow with in 0% NaCl they were thus obligately halophilic. Eight of the isolates could not grow at 20% NaCl concentration.

Extracellular enzyme screening resulted in 21 protease and 72 lipase activity. Isolates produced protease were grouped in HT1, HT2, HT3, HT4.Different concentrations of NaCl were used for lipase screening. Seventeen of the isolated strains which could utilize Tween 20 in the presence of 12% NaCI were all grouped in HT4. Isolated strains, which could not utilize T20, were grouped in HT2 except E47, F26, and F27. Seven of the isolated strains were not produce lipase in the presence of NaCl were grouped in HT1, HT2 and HT3.

For restriction analysis of 16S-ITS rDNA fragments of isolated strains, two restriction enzymes, *Taq* I and Hae III, were used. Both enzymes produced similar RFLP groups. Representative group members were subjected to 16S partial sequencing. They had high similarity with *Bacillaceae*, *Halomonadaceae* and *Micrococcaceae* families.

Characterization of the extracellular enzymes from different isolated strains is the essential work for these Haloalkalophilic bacteria because of their unique futures.

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APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Experiments

NO	CHEMICAL	CODE
1	Agar Merck	1.01613
2	Bacteriological pepton Oxoid	LP037
3	D-Glucose AppliChem	A3666
4	Yeast Extract Merck	1.03753
5	Skimmed milk LabM	MC27
6	Glycerol AppliChem	A2926
7	NaCI AppliChem	A2942
8	K2HPO4 AppliChem	A2945
9	MgSO4.7H2O Merck	1.05886
10	Tween80 Applichem	A1390
11	Ammonium sulfate Applichem	A3485
12	Nutrient broth Merck	1.05443
13	Sodium carbonate Merck 1.06392	
14	KH2PO4 Merck	1.04871
15	Disodium hydrogen phosphate Applichem	A2943
16	Immersion oil Applichem	A0699
17	Calcium chloride Applichem	A3652
18	Crystal violet Sigma	C3886
19	Safranin Merck	1.15948
20	N,N,N.,N.- Tetramethyl-p-phenylenediamine Sigma	T3134
21	Congo Red Sigma	C6767
22	Potassium Iodide Sigma	P8256
23	Tris Base Sigma	T6066
24	EDTA AppliChem	A2937
24	Isopropanol AppliChem	A3928
25	Proteinase K AppliChem	A3830
24	Ethidium bromide AppliChem	A1151
25	Ethanol AppliChem	A3678
26	Taq DNA polymerase Promega	M1865
27	Primers: Ege 1 and L1 Promega	
28	dNTP set MBI, Fermentas,	R0181
29	Standard agarose (low electroendoosmosis) AppliChem	A2114
30	Chloroform AppliChem	A3633
31	Isoamyl alcohol AppliChem	A2610
32	Bromophenol blue Merck	1.08122
33	Boric acid AppliChem	A2940

APPENDIX B

RECIPIES FOR MEDIA USED IN EXPERIMENTS

B.1 HORIKOSHI BROTH AND AGAR

HORIKOSHI-I BROTH

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water. Medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

HORIKOSHI-I AGAR (pH=10.2)

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

B.2 NUTRIENT AGAR (pH:10.3) USED FOR GROWTH TESTS AT DIFFERENT TEMPERATURES AND SODIUM CHLORIDE CONCENTRATIONS

Nutrient Broth	8
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 was sterilized separately and added into medium.

B.5 MEDIUM USED FOR LIPASE SCREENING (pH: 10.3)

	g/l
Nutrient Broth	8
$CaCl_2.H_2O$	0.1
Agar	13
Tween20/Tween80	10ml
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 and Tween20/80 was sterilized separately and added into medium.

APPENDIX C

BUFFERS AND STOCK SOLUTIONS

C.1 ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

Ethidium bromide (0.5 g) was dissolved in 50 ml of deionized water.

C.2 CHLOROFORM-ISOAMYL ALCOHOL SOLUTION (24:1)

Forty eight milliliters of chloroform was mixed with 2 ml of isoamyl alcohol.

C.3 50 X TAE

Two hundred and fifty-two grams Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

C.4 1XTAE

Twenty milliliters of 50X TAE buffer was taken and the volume was adjusted to1000 ml with deionized water to obtain 1 liter 1X TAE buffer.

C.5 1X TE BUFFER

10 mM Tris pH 8.0
1 mM EDTA pH 8.0

C.6 10 X TBE

One hundred and eight grams Tris Base and 55 g boric acid were weighed. They

were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was added. The volume was brought to 1000 ml with deionized water.

C.7 1X TBE

Hundred milliliters, 10X TBE was taken and the volume was brought to 1000 ml with deionized water to obtain 1X TBE buffer.

C.8 CTAB/ NaCl (10% CTAB in 0,7 M NaCl)

NaCl (4.1 g) was dissolved in 80 ml water and CTAB was added slowly while heating and stirring. Final volume was adjusted to 100ml.

APPENDIX D

STAINS AND INDICATORS

D.1. BROMTYMOL BLUE SOLUTION

Bromtymol blue	1 g
N/10 NaOH	25 ml
Deionized water	475 ml

D.2. SOLUTIONS FOR GRAM STAINING

D.2.1. CRYSTAL VIOLET STAINING REAGENT

Solution A

Crystal violet	2g
Ethanol (95%)	20ml 83

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

D.2.2. IODINE SOLUTION

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Iodine and potassium iodide were grinded in a mortar, water was added slowly with continuous grinding until the iodine was dissolved. The solution was stored in amber bottle.

D.2.3. SAFRANIN SOLUTION

Safranin (2.5% in 95% alcohol)	10ml
Distilled water	100ml

APPENDIX E

PCR RECIPIES

E.1. PCR MIX

Mg free Taq DNA polymerase buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Sterile deionized water	32 µl
Oligo forward (10 picomole/µl)	1 µl
Oligo reverse (10 picomole/ µl)	1 µl
dNTP (2mM each) 10X	5 µl

E.2. 6X GEL LOADING BUFFER (20 ML)

10x TBE	2 ml
Glycerol	6 ml
Deionized water	12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

E.3. dNTP (10X)

Ten microliters of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at .20 °C.

E.4. RESTRICTION ENZYME MIX

Restriction enzyme buffer	5 µl
DNA	8 µl
Restriction enzyme (10U/ µl)	1.5 µl
Sterile deionized water	35.5 µl

APPENDIX F

OLIGONUCLEOTIDE PRIMERS

L1: 5.- CAAGGCATCCACCGT -3.

Three hundred and fifty micrograms primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliters of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

EGE 1 5.- AGAGTTTGATCCTGGCTCAG -3.

Five hundred and ninty micrograms primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain final concentration of 2 µg / µl stock solutions. Five microliters of stock solution were then taken and mixed with 95 µl sterile deionized water. Therefore, 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

APPENDIX G

RECOGNITION SITES OF RESTRICTION ENZYMES

G1. ***Taq*** **I**

5.- T▼CG A -3.
5.- A GC ▲T -3.

G2. ***Hae*** **III**

5.-GG▼CC-3.
5.-CC▲GG-3.

APPENDIX H

ISOLATE NAMES

DI- C12a	F26- Ü4z
D2- Ş6a	F27- Ü6a
D5- E15	F28- 15b
D6-C15a	F29- F1
D7- 20a	F30- 24a
D10-19a	F31- 27b
D11- B5c	F32- B4c
D12- B1a	F33- T1b
D14- 23a	F34- E6z
D18- C4	F35- B8a
D20- B8b0	E42- C8
D22- H2	E43- C14a
D23- H1a	E47- C19y
D24- P5z	F1- E11x
D25- C17	F2- F2x
D26- 2b	F3- F2y
D29- D3c	F4- F2z
D30- B9	F5- G5
D31- A9	F6- G8
D32- D2b	F7- H3x
E1- A2	F8- H3y
E2- P2y	F9- P1x
E3- C6	F10- P4a
E4- C2a	F11- P5y
E6- G9a	F12- S1
E7- 3a	F13- S2
E9-24a	F14- Ş1

E10- 13b	F15- V3
E12- 29c	F16- A1
E13- 22c	F17- A4
E14- 12c	F18- A6
E15- 27c	F19- A7
E16- 1a	F20- B10
E17- 2d	F21- C1
E20- B2	F22- C2
E23- B8c	F23- C3
E24- 18b	F24- C24
E25- 11a	F25- C10

ISOLATION OF HALOALKALIPHILIC MICROORGANISMS FROM LEATHER INDUSTRY

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By

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ABSTRACT

Haloalkalophiles are extremophilic microorganisms that are adapted to saline and alkaline conditions. Different species of them have been isolated so far from soda lakes and soil samples. Haloalkalophilic microorganisms have significant adaptive mechanisms to avoid denaturing effect of salts and to balance their interior pH. Extracellular enzymes that are produced by these halophilic and alkalophilic microorganism are applicable for industrial purposes. Therefore isolation of these organisms from their habitats and study on genotypic characterization constitute initial steps for furter biotechnological studies.

In this study, processing steps of leather factories and their wastewater were chosen for sampling. In order to isolate target microorganisms Horikoshi-I medium including 12% NaCl was used. After isolation microorganisms were purified. Phenotypic tests were applied (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope, sensitivity to antibiotics and extracellular enzyme screenings). For genotypic characterization, genomic DNA was isolated and 16S-ITS rDNA region was amplified.

Differentiation was achieved according to Restriction Fragment Length Polymorphism (RFLP) method by using *Hae* III and *Taq* I endonucleases. Isolates, which represented the different RFLP patterns, were chosen for building up the haplotype groups.

As a result of the study seven different RFLP haplotypes were identified. Moreover, 16S ribosomal DNA partial sequencing was also performed on some of the strains in. These haloalkalophilic microorganisms and their enzymes could be used in different biotechnological studies in the future for various industrial applications.

ÖZ

Haloalkalofilik mikroorganizmalar yüksek alkali ve tuzlu koşullara adapte olmuş ekstremofilik canlılardır. Birçok farklı tür toprak ve soda gölü örneklerinden izole edilmiştir. Haloalkalofilik mikroorganizmalar tuzun zararlı etkilerin ve hücre içi pH değerlerini düzenleme yeteneğine sahiptirler. Halofilik ve alkalofilik olan bu organizmaların ürettiği ekstraselüler enzimler endüstride farklı amaçlar için kullanılanılabilir. Haloalkalofilik mikroorganizmaların yaşadıkları habitatlardan izole edilmeleri ve genotipik tanımlamalarının yapılması endüstri alanında kullanım amaçlı olarak genetik potansiyellerinin tanınması ve ileride yapılacak biyoteknolojik çalışmalar adına atılacak ilk adımı oluşturmaktadır.

Deri fabrikaları işlem basamakları ve atık suyu bu çalışma için örnekleme alanı olarak belirlenmiştir. Bu amaç için Horikoshi-I besi yerine %12 tuz ilave edilerek kullanılmıştır. İzolasyon aşamasının ardından organizmaların saflaştırma işlemi tamamlanmıştır. Fenotipik testler uygulanmıştır. Bunlar;Gram boyama, katalaz ve oksidaz testi, faz-kontrast mikroskobu ile spor gözlemi, ekstraselüler enzim taraması, farklı tuz konsantrasyonlarında (%0, %20, %25) gelişme, farklı sıcaklılarda (45-50 °C) gelişme, farklı pH (7-10-12) değerlerinde gelişme özellikleri). Genotipik karakterizasyon için genomik DNA izolasyonunun ardından 16S-ITS rDNA amplifikasyonu yapılmıştır. RFLP için iki farklı restriksiyon enzimi kullanıldı (*Hae* III and *Taq* I). Kesim ürünlerinin analizi yapılmıştır. Farklı jel profillerine sahip olanların oluşturduğu gruplar her iki enzim için de belirlendikten sonra grup temsilcileri kullanılarak dendogramlar oluşturulmuştur. Hae III restiriksiyon enzim kesimi sonucunda elde edilen grup temsilcilerinin 16S rDNA - PCR amplifikasyon ürünleri kısmi dizi analizleri için kullanıldı.

Haloalkalofilik mikroorganizmalar ve enzimleri daha ileride yapılacak biyoteknolojik çalışmalarda kullanılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES..... xi

LIST OF ABREVIATIONS xii

Chapter1 INTRODUCTION..... 1

1.1.Definition of Haloalkalophiles..... 1

1.2.Haloalkalophilic Microorganisms..... 1

1.2.1. Eubacterial Haloalkalophiliphiles..... 2

1.2.2. Haloalkalophilic Bacillus..... 2

1.2.3. Gram NegativeHaloalkalophilicSpecies..... 3

1.2.4. Archaeal Haloalkalophilic Species..... 3

1.2.5. Distribution and Isolation..... 5

1.2.6. Biotechnological and Industrial Applications..... 5

1.2.7. Extracellular Enzymes and Their Industrial Application..... 6

1.2.8. Proteases from Haloalkalophilic Bacteria..... 6

1.2.9. Lipases from Haloalkalophiles..... 8

1.3. Identification of the Haloalkaolphilic Organisms..... 9

1.3.1. Phenotypic Methods..... 9

1.3.2. Molecular Characterization Methods..... 10

1.3.2.1. Pulsed Field Gel Electrophoresis..... 10

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)..... 11

1.3.2.3. 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP..... 11

1.3.2.4. Plasmid Profiling..... 12

1.3.2.5. Ribotyping..... 12

1.3.2.6. DNA Sequencing..... 15

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir) 15
1.5.Thesis Objective 16

Chapter 2 MATERIAL METHODS 17
2.1. Materials 17
2.1.1. Chemicals 17
2.1.2. Samples used for isolation 17
2.2. Methods 17
2.2.1. Isolation and Preservation of the Isolates 17
2.2.1.2. Isolation of Haloalkalophilic Bacteria 17
2.2.1.3. Preservation and Activation of Isolates 18
2.2.2. Determination of Phenotypic Characteristics 18
2.2.2.1. Gram Staining 18
2.2.2.2. Examination of Endospores 19
2.2.2.3. Catalase Test 19
2.2.2.4. Oxidase Test 19
2.2.2.5. Growth at Different Temperatures 19
2.2.2.6. Growth at Different NaCl Concentrations 19
2.2.2.7. Growth at Different pH Ranges 20
2.2.3. Screening for Extracellular Enzymes 20
2.2.3.1. Screening for Protease Activity 20
2.2.3.2. Screening for Lipase Activity 20
2.2.4. Preparation of Genomic DNA 20
2.2.5. Genotypic Characterization 21
2.2.5.1. Identification of Isolates by 16SrDNA–ITS Region 21
2.2.5.1.1. Amplification of 16S rDNA – ITS Region 21
2.2.5.1.2. Electrophoresis of Amplified 16S-ITS Fragments 22
2.2.5.1.3. Chloroform Extraction of PCR Products 23
2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP) 23

2.2.5.1.5. Electrophoresis of Restriction Fragments 23
2.2.6. Sequencing 24
2.2.6.1. Amplification of 16SrRNA Gene 24
2.2.6.2 Extraction of DNA Fragments from Agarose Gel 25
2.2.6.3 Sequencing Reactions 24
2.2.6.4. Purification of Sequencing Reactions 25
2.2.6.4.1. Removal of unincorporated dye terminators using ethanol presipitation 26
2.2.6.4.2. Assembling the SEQ Sequencer 26

Chapter 3 RESULTS AND DISCUSSION 28
3.1. Isolation of Haloalkalophilic Bacteria 28
3.2. Determination of Phenotypic Characteristics 28
3.2.1. Gram staining 28
3.2.2. Examination of Endospore 28
3.2.3. Catalase and oxidase tests 28
3.2.4. Physiological Tests 29
3.3.Extracellular Enzyme Screening 32
3.3.1. Extracellular Enzyme Profiles of Isolates 33
3.4. Genotypic Characterization 36
3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon 36
3.4.2. 16S-ITS rDNA Based RFLP Profiling 37
3.4.3. Analysis of the Genotypic and Phenotypic Groups 40
3.4.4 Analysis of Sequencing Reactions 41

Chapter 4 CONCLUSION AND FUTURE PERSPECTIVE 42
REFERENCES 43
APENDICES AA1
APENDIX A. CHEMICHALS USED AA1
APENDIX B. RECIPIES MEDIA USED FOR EXPERIMENTS AB1
APPENDIX C. BUFFERS AND STOCK SOLUTIONS AC1
APPENDIX D. STAINS AND INDICATORS AD1

APPENDIX E. PCR RECIPIES AE1
APPENDIX F. OLIGONUCLEOTIDE PRIMERS.. AF1
APPENDIX G. RECOGNITION SITES OF RESTRICTION ENZYMES................AG1
APPENDIX H. ISOLATE NAMES AH1

LIST OF FIGURES

Figure 1.1. Archaea kingdom 4

Figure1.2. Rybotyping 14

Figure3.1. Apperance of extracellular protease 36

Figure 3.2. Apperance of extracellular lipase 36

Figure 3.3. 16S rDNA-ITS Amplification products of some isolates 36

Figure 3.4. *Hae* III and *Taq* I Digests of 16S-ITS rDNA Fragments of Representative Isolates 37

Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles of Isolates 38

Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles of Isolates 38

LIST OF TABLES

Table 1.1. Tolerance for pH, NaCl of selected species of the genus Bacillus 2

Table 1.2. "Natro group" of Halobacteria 5

Table 1.3. Detergent Proteases 7

Table.1.4. Lipolytic activity 9

Table 2.1. Samples used for isolation 17

Table 3.1. Results of the Physiologic test 29

Table.3.2. Tolerance for pH, NaCl of isolated strains 30

Table 3.3 Extracellular Enzyme Tests 33

Table 3.4 Extracellular Enzyme Profiles of isolates 34

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA 38

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups 39

Table 3.7. Analysis of the Genotypic and Phenotypic Groups 40

Table 3.8. Sequencing Results 41

ABBREVIATIONS

A	Adenine
bp	Base pair
C	Cytosine
CGT	Cyclodextrin Glucosyltransferase
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
G	Guanine
EDTA	Ethylenediamine Tetra Acetic Acid
ITS	Internal Transcribed Spacer
ISR	Internal Spacer Region
kb	Kilo base
PAGE	Polyacrylamide Gel Electrophoresis
P.E.	Pectinolytic Enzyme
PGA	Polygalacturonic Acid
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
T	Thymine
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA

CHAPTER 1

INTRODUCTION

1.1. Definition of Haloalkalophiles

Haloalkalophiles are one of the main physiological groups of microorganisms classified in alkaliphiles. Lake Magadii in Kenya, Wadi Natrun in Egypt have been defined as the most stable alkaline environments on earth. Therefore the first ecological studies related to haloalkalophilic microorganisms have been performed in these places. The conditions of high mineralization and/or alkalinity in such environments regulate their prokaryotic communities (Trotsenko and Khmelenia, 2001). Both alkalinity (pH 9) and salinity up to saturation (33% (wt/vol)) are needed for the survival of these microorganisms (Koki Horikoshi, 1999).

Alkaliphiles can be classified as obligately and facultatively alkaliphilic organisms. Obligately alkaliphilic microorganisms cannot grow below pH 9. Facultatively alkaliphilic organisms show optimal growth at pH 10.0 or above but they also have the ability to grow at neutral pH.

Halophilicity divided the organisms into three categories: slightly halophilic, moderately halophilic and extremely halophilic. Halophilic microorganisms require certain concentrations of NaCI for their growth, while the halotolerant ones have the ability to grow in the absence or presence of higher concentrations of NaCl (Mimura and Nagata, 2000).

1.2. Haloalkalophilic Microorganisms

Haloalkalophilic microorganisms inhabiting extremely alkaline and saline environments are found in both Archaea and Eubacteria. Most of the extremely salt tolerant haloalkaliphiles belong to *Halobacteriaceae*.

1.2.1. Eubacterial Haloalkalophilic

1.1.2.1 Haloalkalophilic *Bacillus*

Genus *Bacillus* can be characterized by unique physiological characteristics such as Gram staining, spore examination, catalase and oxidase tests. *Bacillus* is a Gram positive usually catalase and oxidase positive and spore producing organism that classified in Firmiculates. It is well known that *Bacillus* strains do not form endospores under many environmental conditions, and the ability to form spores is often difficult to demonstrate. This is very important for their taxonomy. There have been organisms described as non-spore-formers, which had to be reclassified after the detection of spores (Fritze and Claus, 1992). Recent taxonomic studies have separated some of the *Bacillus* members and grouped into new genera: *Alicyclobacillus, Aneurinibacillus, Brevibacillus, Gracilibacillus, Halobacillus, Paenibacillus, Virgibacillus* (Baumgart, 2003)

Table1.1. Tolerance for pH and NaCl of selected species of the genus *Bacillus.*

Species	Source	pH Tolerance	NaCl Tolerance
B. cohnii	Horse meadow soil	obligate alkalophilic	5%
B. horikoshii	Soil	obligate alkalophilic	17%
B. marismortui	Dead sea water	6.0-9.0, pH optimum 7.5	5-25%
B. agaradhaerens	Soil	obligate alkalophilic	16%
B. aclarkii	Soil	obligate alkalophilic	16%
B. horti	Soil, Japan	alkali tolerant	10%
B. vedderi	Bauxite waste	obligate alkalophilic	7.5%
B. alkalophilus	Soil and faeces	obligate alkalophilic	8%
B. clausii	Garden soil	alkali tolerant	10%
B. haloalkaliphilus	Brine/mud, Wadi Natrun	obligate alkalophilic	25%
B. halodurans	Soil	obligate alkalophilic	12%
B. pseudoalkaliphilus	Soil	obligate alkalophilic	10%
B. pseudofirmus	Lake bank soil	obligate alkalophilic	17%
Bacillus sp. DSM8714	River bank soil	alkalitolerant	10%
Bacillus sp. DSM8717	Horse and elephant manure	alkalitolerant	10%
Halobacillus halophilus	Salt marsh soil and solar salterns	7.0-9.0	15%

1.2.3. Gram Negative Haloalkalophilic Microorganisms

Halomonadaceae members are slightly and moderately halophilic bacteria with a broad pH tolerance. They are isolated from saline water, soil samples, leather processing, seawater and estuarine water. Species belong to the Halomonadaceae are Gram negative, rod shaped, non-spore forming, aerobic and also facultatively anaerobic in the presence of nitrate. (Dobzon and Franzman 1996; Franzman et al., 1988). By 16S rDNA sequence analysis, chemotaxonomic and physiological characteristics *Halomonas* have been defined as the only genus in Halomonadaceae (Dobson et al., 1996; Franzman and Tindall 1990). However, recently it has been proposed that Halomonadaceae has four different genera; *Halomonas*, *Alkanivorax, Carnivoras, Chromohalobacter* and *Zymobacte* (Bromguarte, 2003).

1.2.4. Haloalkalophilic Archea

Bacteria and archaea have been first recognized as two distinct groups of Prokarya by Woese et al. (1997). The original classification based on 16S ribosomal RNA gene sequence has been confirmed by extensive phylogenetic studies (Rosa et al., 2002). Extreme halophiles or Euryarchaeota require high salt concentrations, up to 20% NaCl for growth.

Archaea possess genes with recognizable counterparts in the Bacteria, showing that the two groups have functional similarities. Archaea also possess genes that also found in Eukarya (Rosa et al., 2002).

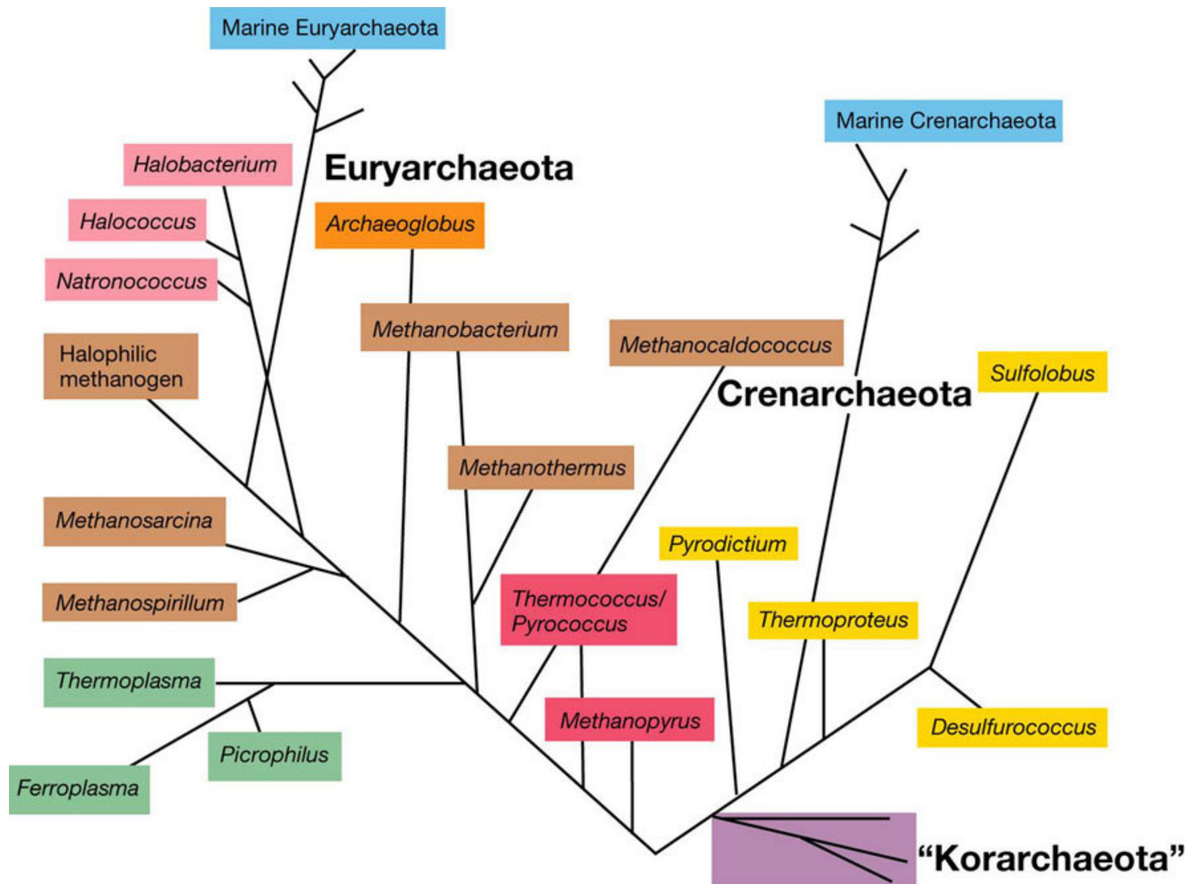


Figure1.1 Archaea kingdom

Euryarchaeaota (*Natronobacterium, Natronococcus, Natronomonas, Natrialba*, *Natronorubrum* genera are classified in "Natro group" of *Halobacteria*. These microorganism are extremely halophilic and alkaliphilic.

Table1.2. "Natro group" of Halobacteria.

Genus	Morphology	Number of species	Habitat
Natronobacterium	Rods	1	Highly saline soda lakes
Natrinema	Rods	2	Salted fish;hides
Natrialba	Rods	2	Soda lakes; beach sand
Natronomonas	Rods	1	Soda lakes
Natronococcus	Cocci	2	Soda lakes
Natronorubrum	Flattened cells	2	Soda lakes

1.2.5. Distribution and Isolation

Isolation of haloalkalophilic microorganisms is performed using different medium ingredients with respect to the group of target organism and the target environment. Actually the salt tolerance range plays an important role because the extremely halophilic organisms can not grow in low concentrations of salt although they have the ability to grow in minimal media. These microorganisms can live at high concentrations of different salts therefore they are populated in salt lakes. Limnological studies focus on the life conditions for extremely halophilic members during seasonal cycles. Anaerobic conditions are suitable in the presence of nitrate for facultatively anaerobes of eucaryotic members.

1.2.6. Biotechnological and Industrial Applications

Extremophilic microorganisms produce cell-associated products that can be used in different industrial applications. First of all their stability and activity in harsh conditions such as alkaline, acidic, high temperature, high concentration of salinity enables broad applications. Extracellular enzymes produced by these organisms are of great interest in biotechnology. Many *Bacillus* species are known to produce alkaline and saline stable proteases, amylases, cellulases, lipases, pectinases and xylanases (Martins et al., 2001)

Enzyme characterization studies with amylase from *Halobacillus* sp. strain MA-2, (Amoozegar et al., 2003) and *Halomonas mediteridiana* (J.Nieto et al., 1999) have

been known. Also the extracellular enzymes from archaeal members have unique features in their molecular structures.

Halophilic microorganisms also used for biological treatment (Kargı and Uygur, 1995).

Halophilic exopolysaccharides have been used in food manufacturing. Furthermore, there is a mereaning demand for non-toxic, biodegradable, environmentally friendly substances. *Halomonas maura* have been studied with novel composition exopolysaccharide (Arias et al., 2003).

1.2.7. Extracellular Enzymes and Their Industrial Application

Extracellular enzymes produced by haloalkalophilic microorganisms have unique properties. They have activity and stability at broad pH ranges and high concentrations of salts in that many other proteins are denaturated. Enzymes from archeal and eubacterial haloalkalophilic organisms have different characteristics. Archaeal enzymes need high concentrations of salt for their activity and stability (Mevarech et al., 2000). This is explained by the comparison of molecular structures from halophilic enzymes with those of nonhalophilic enzymes, this feature is related with adaptation mechanisms used for the protecting of cell from the denaturing effects of salt molecules (Ventosa et al., 1998)

1.2.8. Proteases from Haloalkalophilic Bacteria

Proteases are the enzymes that cleave protein molecules by hydrolysis. The first classification is made by using their cleavage mechanism. Exopeptidases work on the peptide bonds near the amino or carboxy-termini but endopeptidases are active on the peptide bonds distant from the termini of the substrate. The subclassification is based on their catalytic mechanism. Chemically different groups are found on active sites. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao et al., 1998). Subtilisins were grouped in serine type proteases. Catalytic triad of subtilisins consists of aspartic acid, histidine and serine. Although the size of subtilisins varies from 18 kDa to 90 kDa, all the subtilisins used in detergents have a size of

approximately 27 kDa. Most of the alkaline proteases were subtilisins. At present, less than 15 different enzyme molecules are used in detergents worldwide. These enzymes originate from *B. amyloliquefaciens, B. licheniformis, Bacillus clausii, Bacillus lentus, Bacillus alkaloophilus,* and *Bacillus halodurans*

The success of subtilisins is based on several factors, including their high stability and relatively low substrate specificity, features common in extracellular proteases. Their production as extracellular enzymes is of course an important factor in itself, as this greatly simplifies the separation of the enzyme from the biomass and facilitates other downstream processing steps. Another important point is the ability of *Bacillus* strains to secrete enzymes over a very short period of time into the fermentation broth.

Detergent industry prefers the enzymes, which are stable at high pH and compatible with chelating and oxidizing agents (Rao et al., 1998). Table represents the detergent proteases available (Maurer, 2004).

Table1.3. Detergent proteases (Maurer, 2004).

Trade mark	Origin	WT/PE	Production strain	Synonym
Genencor	*B. amyloliquefacies*	PE	*B. subtilis*	
Novozymes	*B. clausii*	WT	*B. clausii*	Subtilisin 309
Genencor	*B. lentus*	WT	*B. subtilis*	
Kao	*B. alkalophilus*	WT	*B. alkalophilus*	
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Novozymes	*B. halodurans*	WT	*B. halodurans*	Subtilisin 147
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. alkalophilus* PB92	PE	*B. alkaliphilus*	

PE, protein engineered; WT, wild type.

Since 1997, several gene-shuffling approaches have been performed with subtilisins. Interesting results from the shuffling of 26 protease genes have been described for properties such as activity in organic solvents, temperature stability, and activity at high or low pH (Minshull et al., 1999). Little, however, has been published on stain removal. Owing to the large number of variant molecules generated by

shuffling and other random techniques, screening methods with high-throughput and increased relevance have had to be developed. Unfortunately, these methods are still not entirely satisfactory, which might explain why no outstanding new subtilisin variant created by one of the gene shuffling technologies is yet present in detergents. Subtilisins for detergents are produced in *Bacillus*, because these species are able to secrete large a mounts of extracellular enzymes. The control mechanisms involved in the production of proteases in Bacillus are extremely complex and still not fully understood. An example is the two-component regulatory system that acts as a quorum sensing mechanism in *B. Subtilis* and which has been found to control the expression of the alkaline protease. This regulatory system is encoded on the chromosome and on endogenous plasmids. (Maurer, 2004)

Arhaea members have proteolytic activity in high salt concentrations (Eichler, 2001). Proteases from *Natronococcus occultus* and *Natrialba magadi* have been characterized (De Castro et al., 2001).

Leather industry uses proteases at dehairing and baiting steps (Kumar and Takagi, 1999). Stable proteases under alkaline and saline conditions would be most effective enzymes for such purposes.

It has been reported that Halophilic bacteria destroied brine-cured hides with their high proteoliytic activity (Bailey et al., 1998).

The studies about using proteases and gelatinases for the pretreatment of leather waste including cromium are important for the convertion of waste material to animal feed (Taylor, 2002).

Silver recovery has been achieved with different types of alkaline proteases at high alkaline conditions (Singh et al., 1999, Kumar and Takagi, 1999).

1.2.9. Lipases from Haloalkalophililes

Lipases, (triacylglycerol acylhydrolases, EC 3.1.1.3), are able to catalyse both the hydrolysis and the synthesis of esters bonds in lipid molecules. Lipid molecules are composed of glycerol and long-chain fatty acid molecules. Esterification and hydrolysis reactions that can occur in organic solvents are very important (Ishıkawa and Ogino,

2000). Most of the lipolytic enzymes used commercially are produced by alkalophilic microorganisms and they are used as detergent addiditives (Sharma et al. 2001).

Table.1.4. Lipolytic activity

Active site	Catalytic triad consisting of the amino acids serine, aspartic (or glutamic acid) and histidine; homologue to the triad of serine proteases.
Substrates	Oils (olive, soybean, mineral oils),Triglycerides (triolein, tributyrin), Synthetic surfactants (Tweens 20 and 40).
Other Activities	Phospholipase, lysophospholipase, cholesterol esterase, cutinase, amidase.

Lipases from *Halomonas* strains have been characterized (Mattiasson et al., 2004).

Alkaline lipases are mainly used in detergent industry. Approximately 1000 tons of lipases are added to the 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Alkaline lipases to be used in detergents should meet certain requirements. They should hydrolyze fats of various compositions, be stable in harsh washing conditions (pH 10-11, 30-60 °C) and in the presence of surfactants (linear alkyl benzene sulfonates (LAS) and proteases) (Sharma *et al.,* 2001).

1.3. Identification of the Haloalkalophilic Organisms

Identification of the haloalkalophilic microorganisms are performed by using the phenotypic and genotypic characteristics.

1.3.1. Phenotypic Methods

Morphological characteristics can be examined by direct observation, which give simple information on the organism for example colony formation that includes colony shape, diameter and color. Furthermore, microscopic observations after different chemical treatments give detailed information on the cell morphology. These are cell shape, Gram behavior and sporulation type. Phase contrast microscopy gives

information on the cell shape and sporulation type without any chemical treatment. Some tests can provide information about the adaptive mechanisms and relation between interior of the cell and the medium. These tests conditions are: growth at different pH ranges, temperatures, pressure and salt concentrations. Bergey's Manual of Systematic Bacteriology a major taxonomic compilation of prokaryotes gives information in all recognized species of prokaryotes and contains number of keys to evaluate the properties given above (Madigan *et al.,* 1997).

Phenotypic methods also include biotyping, antibiogram, phage typing, serotyping, PAGE/immunoblotting and multilocus enzyme electrophoresis (Busch and Nitschko, 1999).

Although molecular characterization methods provide clear differentiation and precise identification, using of phenotypic characterization methods combined with molecular techniques are required for taxonomic studies and functional purposes.

1.3.2. Molecular Characterization Methods

1.3.2.1. Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is used for the analysis of microbial genomes and molecular characterization of the microorganisms below species level. It enables to the seperation of large DNA molecules and therefore whole genomic DNA can be analysed. Analysis of chromosomal macrorestriction patterns by PFGE is a powerful method to produce fingerprints of closely related strains (Farber, 1996). Application of the method can differ for different type of organism. Different organism can require different cell disruption treatments for the releasing the genomic DNA. Also the size and content of genomic DNA can differ among the organisms. Thus, optimization parameters such as lysis of cells, electrophoresis conditions (pulse time, voltage) are the main points for obtaining fine discriminative PFGE results.

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)

DNA fragments are amplified by the polymerase chain reaction (PCR) using short (generally 10 bp) synthetic primers of random sequence. These oligonucleotides serve as both forward and reverse primer at the same time, and are usually able to amplify fragments at 3-10 genomic sites simultaneously. Amplified fragments (within the 0.5-5 kb range) are separated by gel-electrophoresis and polymorphisms are detected as the presence or absence of bands of particular size. These polymorphisms are considered to be primarily due to variation in the primer annealing sites. Some advantages of RAPD are as follows: low quantities of template DNA are required (5-50 ng per reaction), no sequence data for primer construction are required, easy and quick to assay, low costs involved, generation of multiple bands per reaction, and it suitable for automation.

1.3.2.3 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP (Restriction Fragment Length Polymorphism)

In most prokaryotes the ribosomal RNA genes reside sequentially in the chromosome (16S-23S-5S) and they are transcribed as a single polycistronic RNA molecule (Luz et al., 1998). These ordered genes have long been served as a genetic marker to study microbial diversity. The 16S rRNA gene sequence has been used for assessing bacterial phylogenies (Abd-El-Haleem et al., 2002). However, it has only provided information useful for identifications at genus level (Shaver et al., 2002).

Below the genus level, additional rDNA sequences have been necessary. The use of 23S rDNA has also been limited by its larger size and insufficient sequence data (Abd- El-Haleem et al., 2002). The intergenic spacer region (ISR) or also known as intergenic/internal transcribed spacer (ITS) (Abd-El-Haleem et al., 2002; Daffonchio et al., 2000; Shaver et al., 2002; Fisher and Triplett, 1999; Toth et al., 2001) between 16S and 23S rDNA genes, includes both conserved and highly variable sequence motifs, such as tRNA genes, and anti-terminators (boxA) (Abd-El Haleem et al., 2002). ISRs are generally found in multiple copies in most bacteria genomes. Since they are

hypervariable with respect to adjacent genes, they can differentiate between multiple operons in the cell (Daffonchio et al., 1998). The size of the spacer has been found to differ considerably among different species, and even among the different operons within a single cell (Garcia-Martinez et al., 1999).

Figure 1.8 Schematic representation of a 16S-23S spacer and organization of ITS functional regions (Garcia-Martinez et al., 1999).

The method is based on the amplification of 16S rDNA and ISR regions as singleamplicon. The resulting amplicon is then digested with frequent cutting enzymes. Finally restriction fragments obtained are separated by agarose gel electrophoresis and restriction patterns are then compared. The method could discriminate different groups of bacteria at species level.

1.3.2.4. Plasmid Profiling

Plasmids are the genetic materials of bacteria carried as extracromosomal elements. They are self-replicative and they can be transferred naturally between the microorganisms. Genes coded in plasmid DNA structures give different characteristics to microorganisms.

Plasmids isolated from the microorganisms give variable profiles with respect to the organism type and the habitats. Therefore the gel profiles of plasmids for closely related organism would be useful in taxonomic studies (Farber, 1996).

1.3.2.5. Ribotyping

Nucleic acid probes that recognize ribosomal genes are used for ribotying. Ribosomal RNA (rRNA) genes are composed of 23S, 16S and 5S regions. They are known to be highly conserved. Whereas most of the bacterial genes present in one copy, rRNA operons can be present in anywhere more than one copy (2-11 copies per bacterial cell). Thus the more copies of rRNA operon enables more discriminatory riboyping. In practice, first bacterial DNA is isolated and digested by restriction endonucleases. After agarose gel electrophoresing, DNA is transferred onto nylon or nitrocellulose membrane by capillary system or electrophoresis. The nylon membranes

are used as a solid support for probing. Probes specific to 23S, 16S, 5S rRNA genes are labeled using radioactive or non radioactive labeling techniques (non isotopic cold-labelling systems). Hence fingerprint patterns of bacterial DNA containing ribosomal genes are created. These patterns are varied from 1 to 15 bands and compared among the isolates. For another ribotying technique called as chemiluminescence ribotyping, digoxigenin-labeled cDNA (DNA which has been made by reverse transcription of rRNA) can be seen in figure 2. One of the major advantages of ribotyping is that because of the similarity of ribosomal genes, universal probes can be used. Reproducibility is also another advantage of this genotypic method.

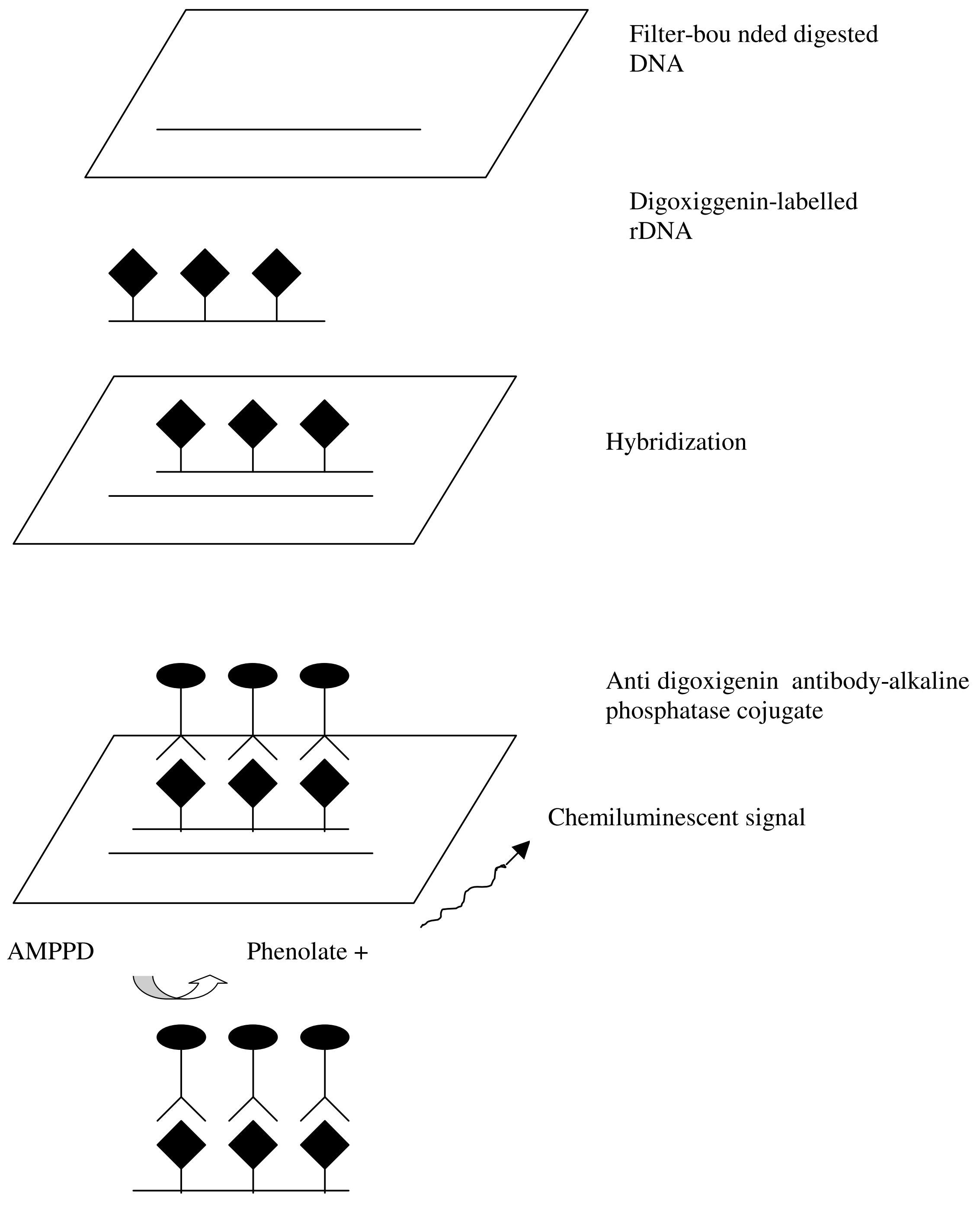


Figure1.2. Schematic representation of chemiluminescence ribotyping. AMPPD, adamantly-1, 2-dioxetane phosphate (Bingen et al., 1994)

1.3.2.6. DNA Sequencing

DNA sequencing is used for investigation of the phylogenetic relationship of organisms. This tecnique is based on the exact order of nucleotides in a region of the DNA molecule Automated DNA sequencing generally includes PCR amplification of a DNA region of interest, computer-aided sequence analysis and interpretation. For DNA sequencing the region of the chromosome to be analysed must be short consisting of a variable sequence flanked by highly conserved region. The variability within the selected sequence must be sufficient to differentiate microorganisms. And also the selected sequence should not be horizontally transmissible to other strains of species (Olive and Bean, 1999).

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir)

Leather factories of Ege Region are populated in Menemen – İzmir. twenty different companies have leather factories in Menemen region. It is known that industrial activities bring the most important pollution problems. For this reason leather factories were placed far from the centre of the city. Leather processing steps are as fallows:

- Curing
- Beamhouse operations which wash and soak the hides or skins and (at most tanneries) remove the attached hair.
- Tanyard processes in which the tanning agent (primarily chromium) reacts with and stabilizes the proteinaceous matter in the hides or skins.
- Finishing or post-tanning processes.

Waste Water of the leather factories are removed from the other factories at Menemen region and a simple waste water treatment is applied. The classical scheme is given below (UNEP/IEO, 1994).

- Pre-treatment: mechanical screening to remove coarse material.

- Primary treatment: sulphide removal from beamhouse effluents; chrome removal from tanning effluents; flow equalization; physical-chemical treatment for

-BOD removal and neutralization.

- Secondary treatment, usually biological.

- Tertiary treatment, including nitrification and denitrification.

- Sedimentation and sludge handling

1.5Thesis Objectives

The purpose of this study was,

- Isolation of haloalkalophilic strains
- Screening for their extracellular enzymes: protease and lipase
- Characterization of these isolates by phenotypic and genotypic methods.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used were listed in Appendix A.

2.1.2. Samples used for isolation

Table 2.1. Samples used for isolation

Sample Type	Location
Polluted Soil	Leather Factories waste (İzmir – Menemen)
Leather processing	Leather Factories (Izmir-Menemen)

2.2. Methods

2.2.1. Isolation and Preservation of the Isolates

2.2.1.2. Isolation of Haloalkalophilic Bacteria

Dilution plate method was used for the isolation of haloalkalophilic bacteria (Mora et al., 1998). Ten g soil, leather or 10 ml of water samples were transferred in 90 ml of 0.85% saline water. In order to homogenize the samples 6h incubation was performed in orbital shaker at room temperature. The samples were pasteurized for 10 m. at 80°C in a water bath in order to kill most of the vegetative cells and eliminate non-

spore forming bacteria (Mora et al., 1998). After the heat treatment, for dilution, 1ml aliquots from each sample was transferred in 9 ml of 0.85 % saline water and 6 fold dilutions were prepared. One ml of the dilutions was plated on appropriate solid media (Appendix B.1) and incubated for 5 days at 37 °C. Single colonies were picked and they were purified using streak plate method.

2.2.1.3. Preservation and Activation of Isolates

Cultures were grown in Horikoshi-I (Apendix B.1) agar plates containing 12% NaCl and incubated for 2 days at 37°C they were then transferred into the isolation broth. After turbidity formation in isolation broth, 0.5ml of each culture were transferred into cryotubes and 0.5ml of isolation broth containing 20% glycerol was added. These steps were all performed on ice. Then tubes were mixed gently by pipetting. Cultures were stored at -20°C for an hour they were then stored at -80°C. For the phenotypic test culture activation was applied from 20% glycerol stocks. For phenotypic and genotypic identification of the isolates, cultures were prepared from these stocks.

2.2.2. Determination of Phenotypic Characteristics

2.2.2.1. Gram Staining

Isolates were stained by modified Gram method for halophilic bacteria. Eighty µl of sterile 0.85% saline water were pipetted onto slides the organisms were then suspended and mixed thoroughly. Overnight culture was spread onto the microscope slides until a thin film formed by using tooth stick. After being air dried, the slides were fixed and desalted simultaneously by immersing in 2% acetic acid for 5 min. Then they were dried again. The smears were covered with 0.25% aqueous solution of crystal violet (Apendix D.2.1) for 1 min. After rinsing under the tap water, the slides were transferred into iodine (Apendix D.2.2) solution and were kept for 1 min. Following this step, slides were washed in alcohol for 6 s. They were then stained with safranin (Apendix D.2.3) for 30 s. After staining, the slides were dried on paper towels and the

cells were examined under light microscope. Gram (+) cells assumed purple color while Gram (-) cells appeared pink or red.

2.2.2.2. Examination of Endospores

Isolates were grown on Horikoshi-I (Apendix B.1) agar plates and 0.0005% $MnCl_2$ (Travers, 1987) were added in to the medium. Observations for spore formation were monitored at 3-4- 5-6th day.

2.2.2.3. Catalase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. Catalase activity was observed after dropping 3% hydrogen peroxide solution onto the target colony. Formation of the air bubbles indicated the presence of catalase activity.

2.2.2.4. Oxidase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. 1% solution of tetramethyl-p-phenylenediamine was poured onto a filter paper placed in a petri dish. Then the target colony was taken from the agar plate and spotted onto wet filter paper. Observation of the blue-purple color indicated the oxsidase activity.

2.2.2.5. Growth at Different Temperatures

The isolates were grown on nutrient agar (Apendix B.2) 45°C and 50°C. Observations for growth were noted during the 5-day incubation.

2.2.2.6. Growth at Different NaCl Concentrations

Cultures were tested for their salt tolerance on Horikoshi-I (Apendix B.1) agar plates containing 0%, 20%, 25 % NaCl during the 5-day incubation.

2.2.2.7. Growth at Different pH Ranges

Cultures were tested for pH tolerance in nutrient agar plates at different pH points (7, 10, and 12). Growth behaviors were observed during the 5-day incubation

2.2.3. Screening for Extracellular Enzymes

2.2.3.1. Screening for Protease Activity

Proteteolytic activity of the isolates observed by adding casein into Horikoshi–I (Apendix B.1) medium adding sikimmed milk. Different concentrations of NaCl (12% and 20%) were added. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete proteases extracellularly, give clear zones around the colony (Horikoshi, 1999).

2.2.3.2. Screening for Lipase Activity

Lipolytic activity of the isolates was observed by using the medium described in Appendix B.5. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete lipase extracellularly, gave opaque zones around the colony (Haba et al., 2000).

2.2.4. Preparation of Genomic DNA

Cultures were grown on solid Horikoshi – I (Apendix B.1) medium. Cells were suspended in 1.5 ml of sterile water in petri dishes. Then cell suspension was transferred into eppendorf tubes. In order to prepare cell pellets, tubes were centrifugated for 5 min at 5000 rpm. Pellets were resuspended in 567 µl 1Xte (Apendix C.4). Thirty µl 10% SDS and 3µl proteinase K solution (20 mg/ml) were added and the solution was mixed thoroughly. Cell lysis was performed with 1h incubation at 37°C. Afterwards 0.1 ml of 5 M NaCl were added and mixed well. Then 80µl of CTAB/NaCl (Appendix C.8) were added, the tubes were then mixed and incubated for 10 min at 65°C. Following the incubation, 0.7 ml of chloroform/isoamyl alcohol (Appendix C.2) were added, mixed thoroughly and centrifuged for 5 min at 10000 rpm. Aqueous, viscous supernatant was

transferred into a fresh eppendorf tube. An equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged for 5 min. The supernatant was again transferred into a fresh eppendorf tube and 0.6 volume of isopropanol were added to precipitate the nucleic acids. The tube was then shaken back and forth until a white DNA precipitate became visible. DNA wool were taken with a pipet tip and placed into a new tube, contain 300µl 70% ethanol. The tubes were stored at -20°C for 1h. Afterwards tubes were centrifuged for 15 min at 6000 rpm. Then the supernatant was discarded and DNA pellet was washed with 500 µl 70% ethanol. It was centrifuged for 10 min at 8000 rpm. After this, ethanol was discarded. The pellet was dried and dissolved in 100 µl 1xTE containing 100 µg/ml RNAase. Then tubes were incubated for 1h at 37°C. After the incubation the sample volume was adjusted to 400 µl with 1xTE. DNA was dissolved by alternating cold-heat shocks (for 10 min at 80°C and 20 min at -20°C, repeated twice). To further purify DNA an equal volume of phenol was added and mixed well. An equal volume of chloroform was added and mixed well. Then the tubes were centrifuged for 5 min at 6000 rpm. The aqueous phase was transferred into a fresh eppendorf tube. Equal volume of chloroform /isoamyl alcohol was added and mixed well. Samples were centrifuged for 5 min at 6000 rpm. Chloroform /isoamyl alcohol step was repeated once again and DNA was precipitated by adding 1/10 sample volume of 5 M NaCl and 2 volumes of 99% ethanol. Pellet was washed in 500 µl 70% ethanol by centrifugation for 5 min at 6000 rpm. After that, ethanol was removed and samples were dried for 10 min at 37°C. DNA was dissolved in 20µl, 50µl, 100µl or 200µl 1xTE according to the pellet size. Then the samples were preserved at -20 °C.

2.2.5. Genotypic Characterization

2.2.5.1. Identification of isolates by 16S rDNA - ITS rDNA - RFLP

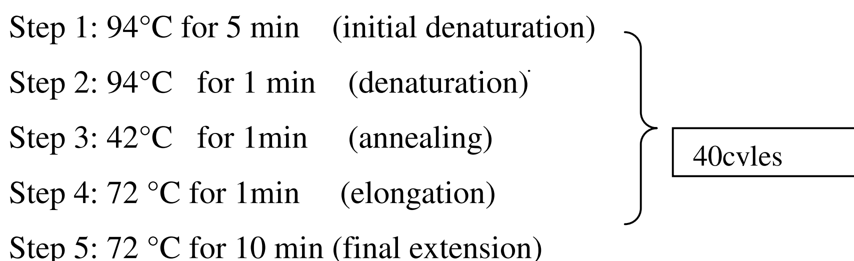
2.2.5.1.1. Amplification of 16S ITS rDNA

PCR amplification of 16S-ITS rDNA of the isolates was performed by using two DNA primers (Apendix F). The forward primer was complementary to the upstream of 16S rRNA gene and the reverse was complementary to the upstream of 23S rRNA gene.

Forward: 5. -AGAGTTTGATCCTGGCTCAG-3 (Mora et al., 1998).

Reverse: 5. -CAAGGCATCCACCGT-3 (Jensen et al., 1993).

Amplifications were performed in a Mini Cycler System. PCR conditions were as follows:



The final reaction volume was 50µl. PCR mix (Appendix E.1) was prepared after 2 µl -4µl of genomic DNA (approx. 500 µg) was placed into 1.5ml eppendorf tubes. During the preperation of the mix, tubes were kept on icebox. Taq DNA polymerase (1.25U) was added and mixed gently and then tubes were centrifuged for 2-3 s. The mix were added to each of the tube. Then 60 µl of mineral oil was added into the tubes while they were on ice. Tubes were centrifuged for 2-3 s then they were placed into the wells of Mini Cycler System (MJ Research INC, USA).

2.2.5.1.2. Electrophoresis of Amplified 16S-ITS rDNA Fragments

Amplified fragments were separated in 0.8 % agarose gel. First, 0.96 g of agarose was dissolved in 120 ml of 1×TAE buffer (Appendix C.4) by boiling (15 µl of ethidium bromide solution (10 mg/ml) were added after cooling the solution to 40°C). The gel was then poured into the gel-casting stand and the combs were placed. 1×TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. Five µl of PCR products were taken and mixed with 1 µl, 6 × gel-loading buffers. The samples were then loaded into the wells of agarose gel starting from the second well. Finally, 5 µl of DNA molecular weight marker were loaded into the first well and they

were electrophoresed at 40 mA until bromophenol blue have reached 2/3 of the gel. DNA fragments were visualized in a photodocumentation system (Vilber-Lourmat, France).

2.2.5.1.3. Chloroform Extraction of PCR Products

Final volumes of the PCR products were adjusted to 100 µl with 1×TE buffer. Then two volume of chloroform were added. After centrifugation for 2 min at 6000 rpm, organic phase was discarded and chloroform extraction was repeated. Then the tubes were centrifuged for 2 min at 6000 rpm. The aqueous phase was transferred into new eppendorf tubes containing 10 µl of 3 M sodium acetate (pH 5.2) and mixed well. After that, 450 µl of 99% ethanol were added and mixed. The samples were centrifuged for 15 min at 7000 rpm. Pellet was washed with 500 µl 70% ethanol. After centrifugation for 5 min at 6000 rpm, alcohol was removed. Pellet was dried for 10 min at 37°C, and they were dissolved in 25 µl 1xTE, and stored at 20°C.

2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP)

Purified PCR products were separated in 1% agarose gel in order to estimate DNA concentrations for each sample. Then 12.5µl of PCR products were aliquoted into new tubes for digestion. *Taq* I and *Hae* III were the enzymes chosen for this purpose. Reaction mix (Appendix F2) was added on PCR products while the tubes were stored on ice. 5 U of enzyme was used for each reaction. The final volume of the reaction was adjusted to 50 µl. Incubation period was 5 h for both of the enzymes. But the optimum reaction temperatures were 65°C for *Taq I* and 37°C for *Hae* III. Mineral oil was added over the reaction mixtures to avoid evaporations at 65°C. After the incubation DNA was extracted as described in section 2.2.6.1.3. and dissolved in 15 µl of 1xTE.

2.2.5.1.5. Electrophoresis of Restriction Fragments

Restriction fragments were first separated in 2 % agarose gel containing 20 µl of ethidium bromide solution (10mg/ml). Electrophoresis was carried out at 60 mA for the

first 20 min, then the current was adjusted to 80 mA and the electrophoresis was continued further for 2.5 h. Some isolates produced similar restriction fragment patterns. These were contained within distinct groups. One digested sample from each group was picked and re-electrophoresed in order to produce representative restriction patterns for further restriction profile analyses.

2.2.6 Sequencing

2.2.6.1. Amplification of 16SrRNA Gene

16SrDNA region of rRNA genes of the isolates were amplified using the following DNA oligoprimers for sequencing reactions

EGE 1. 5. - AGAGTTTGATCCTGGCTCAG –3 (Jensen *et al.,* 1993)

EGE 2. 5. - CTACGGCTACCTTGTTACGA –3 (Jensen *et al.,* 1993)

Two µl of genomic DNA (approx. 500 µg) were transferred into 0.5 ml eppendorf tubes, as the template. PCR mix was prepared and after the addition of *Taq* DNA polymerase (1.25 U), it was mixed gently and centrifuged for 2-3 s. Forty-eight ml of the mix were distributed into the tubes. All the manipulations were performed on ice. The tubes were then overlaid with 60µl mineral oil, centrifuged for 2-3 s at 6000 rpm. Finally, they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) using the following program:

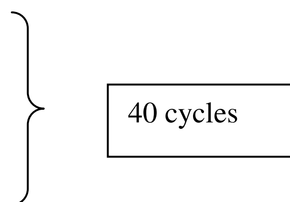
Step1: 95°C for 5 min

Step2: 95°C for 1 min

Step3: 56°C for 1 min

Step4: 72°C for 1 min

Step4: 72°C for 10 min



2.2.6.2 Extraction of DNA Fragments from Agarose Gel

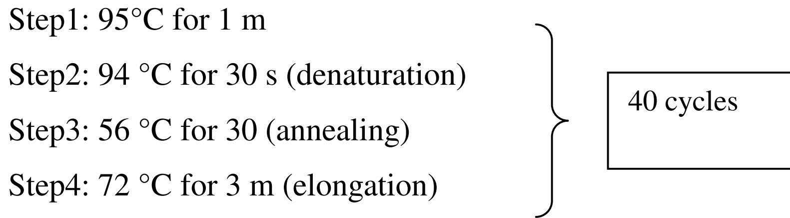
DNA isolation kit (Applichem A3421) was used for extraction of amplification products from agarose gel.

DNA bands (approximately 1600 bp) were excised from the agarose gel by a rasor blade. Three gel slice volume of 6 M NaI (for TAE gels) was added. The samples were incubated for 5 min at 55°C, mixed and continued for incubation for 2 min. Ten µl of well mixed glass powder suspension was then added, mixed well and incubated for 5 min at 55°C by occasional mixing. The samples were then centrifuged for 10 s at 10.000 rpm and the supernatant was discharged. The glass pellet was rinsed with 500 µl wash buffer. And resusupended by pipetting or gently flicking the tube. The samples were then centrifuged for 10 s at 10,000 rpm. The washing step was repeated twice. After the last wash, the supernatant was removed as much as possible. The pellet was suspended in 25 µl of 1xTE buffer. The samples were eluted for 5 min at 55°C with occasional mixing. Centrifugation was performed for 45 s at 10,000 rpm. The supernatant was finally transferred carefully into a new eppendorf tube. The eluted DNA samples were stored at -20°C until use.

2.2.6.3 Sequencing Reactions

The sequencing reactions were performed by using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit.

Four eppendorf tubes were firstly labeled as “A”, “C”, “G”, and “T” for respective termination reactions. Two µl of corresdonding Cy5.5 ddNTP termination mix were then dispensed into the appropriately labelled tubes. The tubes were then capped to prevent evaporation. The master mix (Appendix I) was prepared in an eppendorf tube for each template to be sequenced. The content of master mix was mixed thoroughly and 7µl of the mix was dispensed into each tube labelled “A”, “C”, “G”, and “T”. Each sequencing reaction was mixed thoroughly and overlaid with one drop of mineral oil. Each tube was capped and placed into the thermal cycler. The sequencing condition reastions were:



40 amplification cycles of Step 2, 3 and 4 were performed
The primer used was ;
EGE 1; 5. - AGAGTTTGATCCTGGCTCAG -3. (Jensen *et al.,* 1993)

2.2.6.4 Purification of Sequencing Reactions

2.2.6.4.1 Removal of unincorporated dye terminators using ethanol precipitation

The volume of the sample was adjusted to 50 µl by adding 1xTE. The samples under the mineral oil were then transferred into fresh eppendorf tubes. Twenty µl of 7.5 M ammonium acetate and 200 µl (3 times the reaction volume) of chilled 100% ethanol were added to each tube. The tubes were then mixed thoroughly by vortexing and placed on ice for 20 min to precipitate the DNA. Centrifugation was then performed at 12, 000 rpm for 30 min at 4°C. Supernatants were removed carefully and the pellets were washed with 200 µl of chilled 70% ethanol. After the centrifugation for 5 min, the supernatants were removed and the pellets were dried. Each pellet was then resuspended in 6 µl of formamide loading dye and vortexed vigorously (2-3 min) to ensure complete resuspension. Just prior to loading the samples onto gel, each sample was heated at 70 °C for 2-3 min for denaturation and then placed on ice. One and a half or 2µl of each sample were then loaded into the lanes of sequencing gel.

2.2.6.4.2 Assembling the SEQ Sequencer

The sequencing reactions were performed with SEQ personal sequencing system (Amercham Pharmacia Biotech). The system consists of SEQ personal sequencer, SEQ

Rapidset gel polymerizer, RapidGel QuickFill cassettes, RapidGel-XL-6% cartridge and filling gun and filling fixture.

Preparing a gel involves attaching the RapidGel-XL cartridge to the casting gun, filling the RapidGel cassettes, polymerizing the acyrlamide in the gel polymerizer. After the polymerization the comb was removed from the cassette. After cleaning the cassette, it was placed properly into the buffer chamber. At this step pre-run was performed to measure the backgraound redding. Once the gel cassette was aligned and the pre-run was complete, 1xTE buffer was loaded into the upper and lower buffer chambers until it reached the fill lines. The prepared samples (1-2 µl) were then loaded into the gel with a micropipette. Then the analysis was started. After the run was finished, data were analysed by SEQ software.

The sequences obteined were evaluated in and submitted to GenBank.

Chapter 3

RESULTS AND DISCUSSION

3.1. Isolation of Haloalkalophilic Bacteria

Haloalkalophilic bacteria were isolated from Menemen Region. Target places were leather factories and the wastes of this industry. Therefore the samples were taken from both the processing steps and waste.

3.2. Determination of Phenotypic Characteristics

3.2.1. Gram staining

The modified Gram staining method specific for halophilic bacteria gave desired results with these isolates. All the isolates were gram positive, rod shaped except one cocci (C1).

3.2.2. Examination of Endospore

Isolates were grown on agar plates (Appendix) for 5 days. Isolates were non-sporforming.

3.2.3. Catalase and oxidase tests

All the isolates were catalase and oxsidase positive.

3.2.4. Physiological Tests

Growth at pH 7 was observed to determine whether the isolates were obligate alkalophilic (Table 3.2). Four isolates could not grow at this pH value. Twenty-five of the isolates were alkali tolerant and could not grow at pH 12. Isolates were not thermotolerant since they could not grow at 45ºC and 50ºC on alkaline nutrient agar plates.

Table 3.1. Results of the Physiological Test

Physiologic test	Isolate number
No Growth at 20%NaCl	D2, D10, D20. D26, E4, E16, E24, F22
No Growth at PH 7	D1, D20, F9, F21
No Growth at 0%NaCl pH 10	D2, D6, D18, D23, E4, E43, F5, F7, F29
No Growth at pH 12	D1, D6, D10, D18, D20, D23, D24, D30, D32, E2, E4, E10, E12, E14, E16, E23, E24, E36, F7, F10, F13, F16, F20, F22, F29,

Growth of the isolates was also observed at 0% and 20% NaCl concentrations. Nine of the isolates were oblitely halophilic. Eight of the isolates were not extremely halophilic.

Table3.2. Differentiation of Isolates according to growth at different NaCl concentrations and pH ranges.

İsolate Name	20% NaCl	PH 7	0%NaCl pH 10	pH 12
D1	+	-	+	-
D2	-	+	-	+
D5	+	+	+	+
D6	+	+	-	-
D7	+	+	+	+
D10	-	+	+	-
D11	+	+	+	+
D12	+	+	+	+
D14	+	+	+	+
D18	+	+	-	-
D20	-	-	+	-
D23	+	+	-	-
D24	+	+	+	-
D25	+	+	+	+
D26	-	+	+	+
D29	+	+	+	+
D30	+	+	+	-
D31	+	+	+	+
D32	+	+	+	-
E1	+	+	+	+
E2	+	+	+	-
E3	+	+	+	+
E4	-	+	-	-
E6	+	+	+	+
E7	+	+	+	+
E9	+	+	+	+
E10	+	+	+	-
E12	+	+	+	-
E13	+	+	+	+
E14	+	+	+	-
E15	+	+	+	+

E16	-	+	+	-
E17	+	+	+	+
E20	+	+	+	+
E23	+	+	+	-
E24	-	+	+	-
E25	+	+	+	+
E26	+	+	+	+
E27	+	+	+	+
E28	+	+	+	+
E29	+	+	+	+
E30	+	+	+	+
E33	+	+	+	+
E34	+	+	+	+
E35	+	+	+	+
E36	+	+	+	-
E39	+	+	+	+
E42	+	+	+	+
E43	+	+	-	+
E47	+	+	+	+
F1	+	+	+	+
F2	+	+	+	+
F3	+	+	+	+
F4	+	+	+	+
F5	+	+	-	+
F7	+	+	-	-
F8	+	+	+	+
F9	+	-	+	+
F10	+	+	+	-
F11	+	+	+	+
F12	+	+	+	+
F13	+	+	+	-
F14	+	+	+	+
F15	+	+	+	+
F16	+	+	+	-
F17	+	+	+	+
F18	+	+	+	+

F19	+	+	+	+
F20	+	+	+	-
F21	+	-	+	+
F22	-	+	+	-
F23	+	+	+	+
F24	+	+	+	+
F25	+	+	+	+
F26	+	+	+	+
F27	+	+	+	+
F28	+	+	+	+
F29	+	+	-	-
F30	+	+	+	+
F32	+	+	+	+
F34	+	+	+	+
F35	+	+	+	+

3.3. Extracellular Enzyme Screening

Extracellular enzyme screening was performed on solid agar plates by using different substrates for each enzyme activity. Protease screening based on the observation of clear zones around the colonies (Figure). Lipase activity gave opaque zones around the colonies (Figure). Different NaCI concentratios were used for lipase screening. The enzyme screening studies were repeated performed twice.

3.3.1 Extracellular Enzyme Profiles of the Isolates

Table 3.3 Extracellular Enzyme Tests

Extracellular Enzyme Type	Isolate Name
Did not produce lipase	D5, D10, D20, D26 D31, E23, E43, F11, F29 F33
Utilized Tween80 at 0% NaCl	D1, D2, D30, F25, F15
Utilized Tween 20 at 12% NaCl	D23, D24, D25, D30, E1, E2, E3, E42, F5, F12, F13, F14, F15, F16, F18, F23, F25
Did not Utilized Tween 20	D7, E7, E9, E10, E13, E14, E15, E16, E23, E24, E25, E26, E27, E28, E29, E30, E39, E47, F26, F27, F31
Did not utilase Tween80	D12, E12, E34, F1, F20, F30, F32,
Did not produce lipase without NaCI	D12, D14, E12, E34, F1, F30, F32
Utilase casein	F17, D5, F29, D32, E34, F1, F15, E36, D24, F10, F13, F12, E20, F32, E1, E47, E33, E14, E25, E42, F22

Table 3.4 Extracellular Enzyme Profiles of isolates

Isolate Name	Protease	0%NaCl Tween20	12% NaCl Tween20	20% NaCl Tween20	0% NaCl Tween80	12% NaCl Tween80	20% NaCl Tween 80
D1	-	-	-	-	+	+	-
D2	-	-	-	+	+	-	-
D5	+	-	-	-	-	-	-
D6	-	+	-	-	-	+	-
D7	-	-	-	-	-	+	-
D10	-	-	-	-	-	-	-
D11	-	+	-	+	-	+	+
D12	-	+	-	-	-	-	-
D14	-	+	-	-	-	-	-
D18	-	+	-	+	-	-	+
D20	-	-	-	-	-	-	-
D23	-	+	+	-	-	+	+
D24	+	+	+	-	-	+	+
D25	-	+	+	-	-	-	+
D26	-	-	-	-	-	-	-
D29	-	+	-	-	-	+	+
D30	-	+	+	+	+	+	+
D31	-	-	-	-	-	-	-
D32	+	-	-	-	-	+	+
E1	+	+	+	-	-	+	+
E2	-	+	+	-	-	+	
E3	-	+	+	-	-	+	+
E4	-	+	-	-	-	+	-
E6	-	+	-	-	-	+	+
E7	-	-	-	-	-	+	-
E9	-	-	-	-	-	+	-
E10	-	-	-	-	-	-	+
E12	-	+	-	-	-	-	-
E13	-	-	-	-	-	+	-
E14	+	-	-	-	-	+	-
E15	-	-	-	-	-	+	-
E16	-	-	-	-	-	+	-
E17	-	-	-	-	-	+	-
E20	+	+	-	-	-	+	+
E23	-	-	-	-	-	+	-
E24	-	-	-	-	-	+	-
E25	+	-	-	-	-	+	-
E26	-	-	-	-	-	+	-
E27	-	-	-	-	-	+	-
E28	-	-	-	-	-	+	-
E29	-	-	-	-	-	+	-
E30	-	-	-	-	-	-	+
E33	+	-	-	+	-	+	+
E34	+	+	-	-	-	-	-
E35	-	-	-	+	-	-	+

E36	+	+	-	-	-	+	+
E39	-	-	-	-	-	+	-
E42	+	+	+	-	-	-	+
E43	-	-	-	-	-	-	-
E47	+	-	-	-	-	-	+
F1	+	+	-	-	-	-	-
F2	-	+	-	+	-	+	+
F3	-	+	-	+	-	+	-
F4	-	+	-	-	-	+	+
F5	-	+	+	+	-	+	+
F7	-	+	-	+	-	+	+
F8	-	+	-	-	-	+	+
F9	-	+	-	-	-	-	+
F10	+	+	-	+	-	+	+
F11	-	-	-	-	-	-	-
F12	+	+	+	+	-	+	+
F13	+	+	+	-	-	+	+
F14	-	-	+	-	-	+	+
F15	+	+	+	+	+	-	+
F16	-	+	+	-	-	+	+
F17	+	+	-	-	-	+	+
F18	-	-	+	-	-	+	+
F19	-	+	-	-	-	+	+
F20	-	+	-	+	-	-	-
F21	-	+	-	-	-	+	-
F22	+	+	-	-	-	+	-
F23	-	+	+	+	-	+	+
F24	-	-	-	-	-	-	+
F25	-	+	+	+	+	+	+
F26	-	-	-	-	-	+	+
F27	-	-	-	-	-	-	+
F28	-	+	-	-	-	-	+
F29	+	-	-	-	-	-	-
F30	-	+	-	-	-	-	-
F32	+	+	-	-	-		-
F35	-	-	-	-	-	-	-
F34	-		-	-	-	-	-

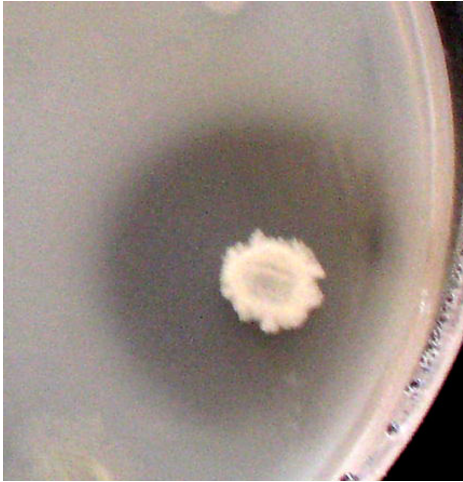


Figure 3.1. Apperance of extracellular protease

Figure 3.2. Apperance of extracellular lipase

3.4. Genotypic Characterization

3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon

The amplification product size differed between 1800 and 2000 bp (Figure)

.

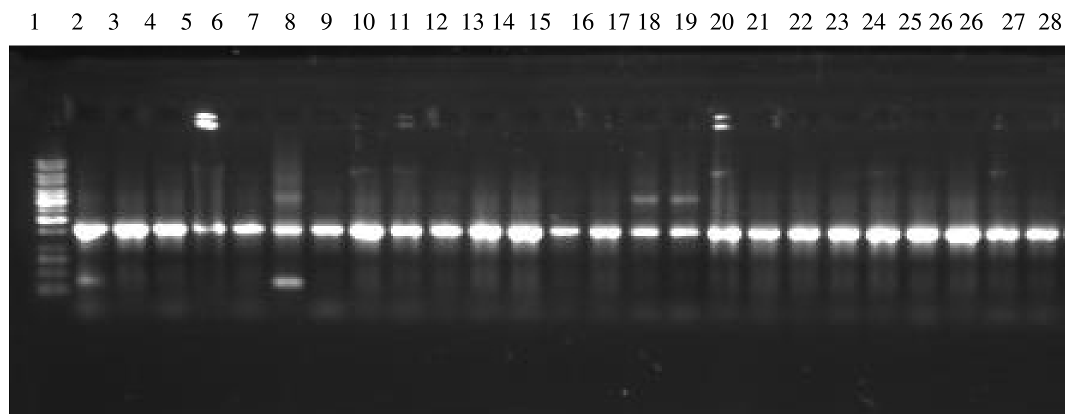


Figure 3.3. 16S rDNA-ITS Amplification products of some isolates. Lanes 1. 1 kb DNA Lac D1, 3. Isolate F2, 4. Isolate F1 5. Isolate D8, 6. Isolate D10, 7. Isolate 12,8. Is Isolate E15, 10. Isolate E17, 11. Isolate E19, 12. Isolate D23 lane 13. Isolate D24

3.4.2. 16S-ITS rDNA - RFLP Profiling

In order to identify the isolates amplification products of 16S-ITS rDNA region was digested with restriction endonucleases. *Taq* I and *Hae* III endonucleases were chosen for digestion. Digestion products were first run in 2% agarose gel to see all different profiles and to make groups within the digestion products. For a specific RFLP group, one representative digestion product was chosen and electrophoresed together in 2.5% agarose gel (Figure 3.3).

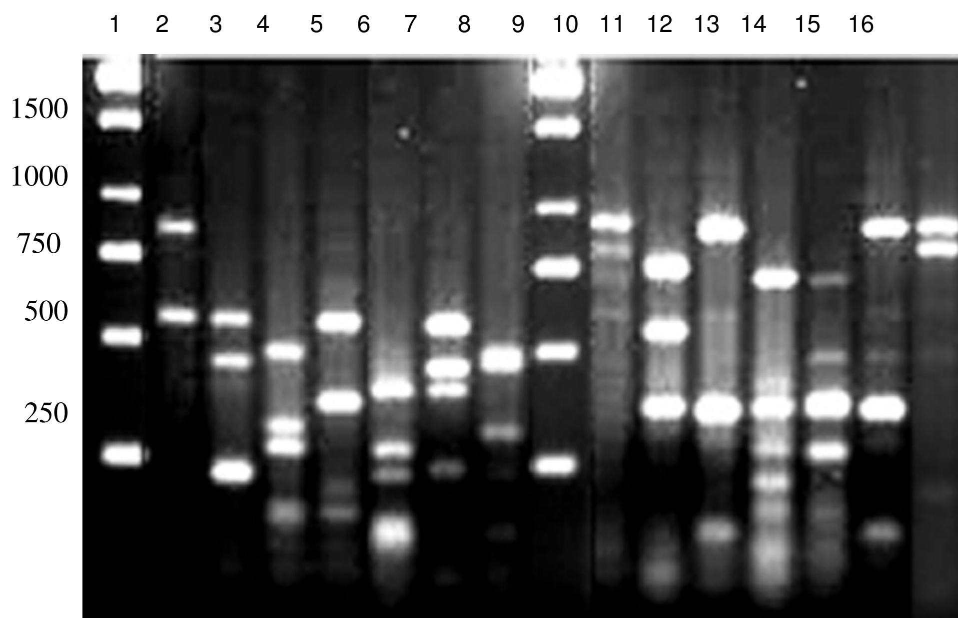


Figure 3.4 16S-ITS rDNA RFLP profiles of *Hae* III groups. Lanes 1. 1 kb DNA ladder, 2. Isolate D5, 3. Isolate D7, 4. Isolate D30, 5. Isolate D12, 6. Isolate D23, 7. Isolate F28, 8. Isolate, F21 16S-ITS rDNA RFLP profiles of *Taq*I groups. Lanes 9. 1 kb DNA ladder,10. Isolate D2, 11. Isolate D18, 12. Isolate D12, 13. Isolate F6, 14. Isolate F20, 15. Isolate F28, 16. IsolateE30,

Resulting gel profiles were analyzed using the program BIO-1D++. Dendrogram of representative strains were carried out using 15% homology coefficient. (Figure 3.3 and 3.4). Fragment sizes were given in Table 3.6.

Hae III and *Taq* I restriction analysis of 16S-ITS rDNA fragments of strains revealed 7 distinct groups (Table 3.3.).

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2000	854	546	465	546	384	546	465	2000	886	673	808	661	361	838	870
1500	555	440	309	354	257	440	432	1473		509	338	354	338	338	778
1000		219	272	125	211	377	294	934		354	78	265	257	78	
750			125		94	219		685				195			
500					70			465				133			
250								227				31			

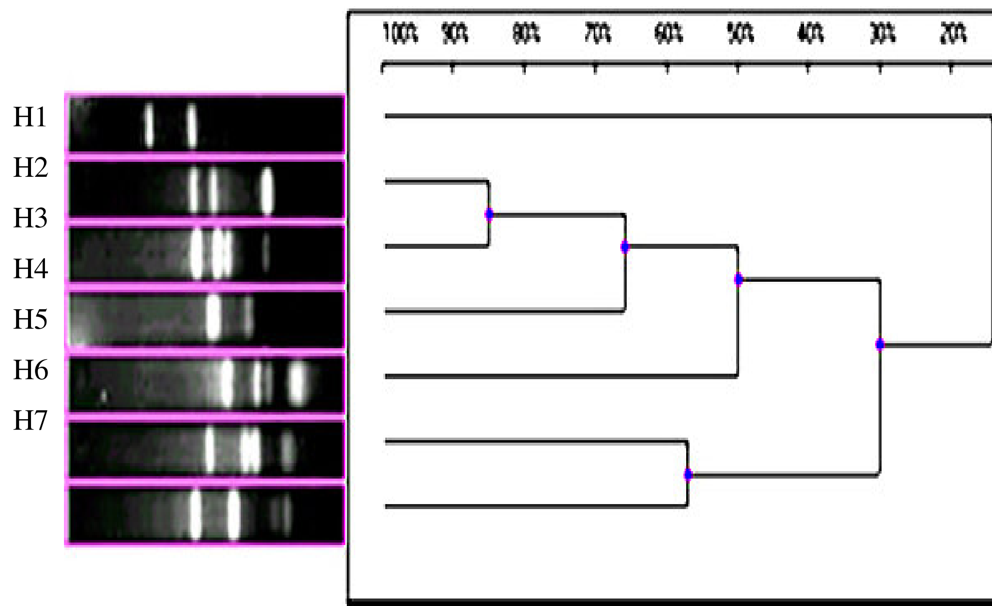


Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles.

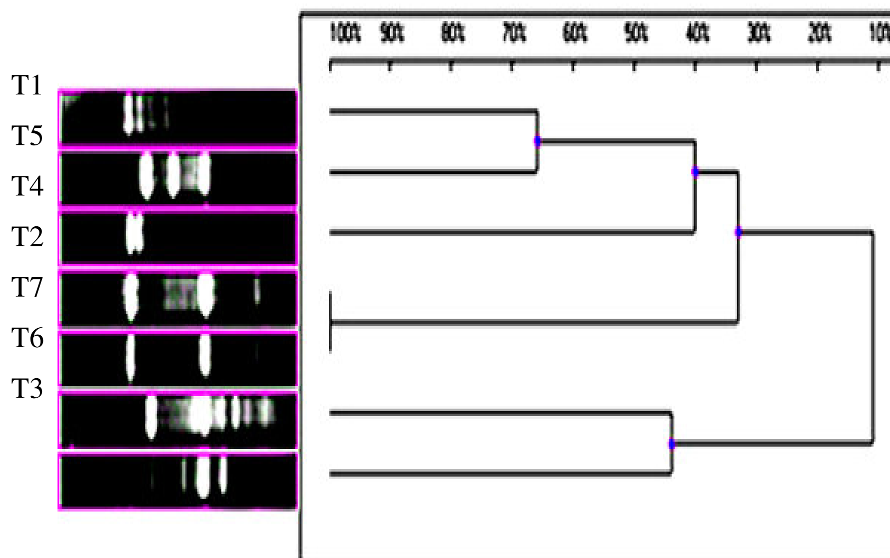


Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles.

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups.

Genotypic groups	Name of the Isolates
HT1	D1, D2, D5, E43,E47,F1
HT2	D6, D7, D10, D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E30, E34, E35
HT3	D12, F32
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E20, E33, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F19, F22, F23, F24, F25, F26, F27
HT5	D30
HT6	F20, F21
HT7	F28

The use of two restriction enzymes enabled the revelation of diversity among the isolates. When the results of both digestion were combined, 7 distinct genotypes were obtained (Table 3.10). The results indicated that isolate group members were differentiated by each restriction enzyme, were the same.

In this study, 16S ITS and rDNA regions were used as a single amplicon for RFLP analysis. No report was found in literature on genotypic characterization of haloalkalophiles using this method. Similar studies have been performed in this institute for genotypic characterization of alkalophilic bacilli (Akbalik, 2004), thermophilic bacilli, (Yavuz, 2004), lactic acid bacteria (Yavuz, 2003, Bulut, 2003.

3.4.3. Analysis of the Genotypic and Phenotypic Groups

Table 3.7. Analysis of the Genotypic and Phenotypic Groups

	Isolate number	20%NaCI	pH 7	0%NaCl	pH 12	Protease	0%NaCl Tween 20	12%NaCl Tween 20	20%NaCl Tween 20	0%NaCl Tween 80
HT1	D1,D2,D5,E34, E43, E47, F1	%80	80%	70%	80%	40%	0%20	0%	20%	%30
HT2	D6,D7,D10,D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E39, F29, F30, F34, F35,	100%	100%	100%	100%	10%	10%	0%	0%	0%
HT3	D12, F32	100%	100%	100%	100%	50%	100%	0%	0%	0%
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E30, E33, E35, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18, F19, F22, F23, F24, F25, F26, F27	90%	100%	90%	70%	30%	90%	40%	30%	20%
HT5	D30	100%	100%	100%	0%	0%	100%	100%	100%	100%
HT6	F20, F21	100%	50%	100%	50%	0%	100%	0%	50%	0%
HT7	F28	100 %	100%	100%	100%	0%	100%	0%	0%	0%

Phenotypic characterization of the isolates indicated that strains of different species had different phenotypic features. When the genotypic results were compared with the results of enzyme screening and phsyical tests, a correlation between a few groups were observed. Most of the isolates produced lipase (Tween 20 and Tween 80) at all NaCI concentrations used grouped in HT4. All members of HT2, HT3, HT5, HT6, HT7 were also able to grow in the presence of 20% NaCI. Most of the isolates that could not grow at pH7 were placed in HT6. Isolates that could not grow at pH12 were placed in HT1, HT5, and HT6.

3.4.4 Analysis of Sequencing Reactions

16S partial sequencing results of six representative isolates are as listed below.

Table 3.8. Sequencing results

Genotypic Group	Isolate Name-Accession Number	Length of bp.-Similarity	Accession Number - Organism	Name of the Family
HT2	D7-AY604872	-89%	*AY345480.1-Bacterium K-29*	Unclassified *Bacillaceae*
HT3	D12-AY601901	716-93%	AF237976.1-*Salinococcus roseus*	Unclassified *Bacillaceae*
HT4	D23-AY601902	323-92%	AJ640133.1-*Halomonas* sp.18bAG	*Halomonadaceae*
HT5	D30-AY601903	-92%	AY383044.1-*Halomonas meridiana*	*Halomonadaceae*
HT6	F20 (C1)-AY604871	-89%	AJ29397-*Nestenkonia* sp.EL-30	*Micrococcaceae*
HT7	F28 (15B)-AY601904	-91%	AY3091336-Bacterium K-2	Unclassified *Bacillaceae*

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, haloalkalophilic bacteria were isolated from samples of waste soil and leather processing. They were first characterized phenotypically (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope and extracellular enzyme screening, physiological tests such as growth at pH 7 and pH 12 , different NaCl concentrations and at different temperatures). Extracellular enzyme screening was performed on solid agar media. Molecular characterization was performed by using 16S-ITS rDNA RFLP.

All the isolated strains were Gram positive but they were not sporeforming. All the strains were catalase positive. Most of the isolates contained oxidase. Four strains could not grow at pH7. They were thus obligately alkalophilic. Twentyfive isolated strain were alkalitolerant because they could not grow at pH 12. Nine isolated strain could not grow with in 0% NaCl they were thus obligately halophilic. Eight of the isolates could not grow at 20% NaCl concentration.

Extracellular enzyme screening resulted in 21 protease and 72 lipase activity. Isolates produced protease were grouped in HT1, HT2, HT3, HT4.Different concentrations of NaCl were used for lipase screening. Seventeen of the isolated strains which could utilize Tween 20 in the presence of 12% NaCI were all grouped in HT4. Isolated strains, which could not utilize T20, were grouped in HT2 except E47, F26, and F27. Seven of the isolated strains were not produce lipase in the presence of NaCl were grouped in HT1, HT2 and HT3.

For restriction analysis of 16S-ITS rDNA fragments of isolated strains, two restriction enzymes, *Taq* I and Hae III, were used. Both enzymes produced similar RFLP groups. Representative group members were subjected to 16S partial sequencing. They had high similarity with *Bacillaceae*, *Halomonadaceae* and *Micrococcaceae* families.

Characterization of the extracellular enzymes from different isolated strains is the essential work for these Haloalkalophilic bacteria because of their unique futures.

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APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Experiments

NO	CHEMICAL	CODE
1	Agar Merck	1.01613
2	Bacteriological pepton Oxoid	LP037
3	D-Glucose AppliChem	A3666
4	Yeast Extract Merck	1.03753
5	Skimmed milk LabM	MC27
6	Glycerol AppliChem	A2926
7	NaCI AppliChem	A2942
8	K2HPO4 AppliChem	A2945
9	MgSO4.7H2O Merck	1.05886
10	Tween80 Applichem	A1390
11	Ammonium sulfate Applichem	A3485
12	Nutrient broth Merck	1.05443
13	Sodium carbonate Merck 1.06392	
14	KH2PO4 Merck	1.04871
15	Disodium hydrogen phosphate Applichem	A2943
16	Immersion oil Applichem	A0699
17	Calcium chloride Applichem	A3652
18	Crystal violet Sigma	C3886
19	Safranin Merck	1.15948
20	N,N,N.,N.- Tetramethyl-p-phenylenediamine Sigma	T3134
21	Congo Red Sigma	C6767
22	Potassium Iodide Sigma	P8256
23	Tris Base Sigma	T6066
24	EDTA AppliChem	A2937
24	Isopropanol AppliChem	A3928
25	Proteinase K AppliChem	A3830
24	Ethidium bromide AppliChem	A1151
25	Ethanol AppliChem	A3678
26	Taq DNA polymerase Promega	M1865
27	Primers: Ege 1 and L1 Promega	
28	dNTP set MBI, Fermentas,	R0181
29	Standard agarose (low electroendoosmosis) AppliChem	A2114
30	Chloroform AppliChem	A3633
31	Isoamyl alcohol AppliChem	A2610
32	Bromophenol blue Merck	1.08122
33	Boric acid AppliChem	A2940

APPENDIX B

RECIPIES FOR MEDIA USED IN EXPERIMENTS

B.1 HORIKOSHI BROTH AND AGAR

HORIKOSHI-I BROTH

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water. Medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

HORIKOSHI-I AGAR (pH=10.2)

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

B.2 NUTRIENT AGAR (pH:10.3) USED FOR GROWTH TESTS AT DIFFERENT TEMPERATURES AND SODIUM CHLORIDE CONCENTRATIONS

Nutrient Broth	8
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 was sterilized separately and added into medium.

B.5 MEDIUM USED FOR LIPASE SCREENING (pH: 10.3)

	g/l
Nutrient Broth	8
$CaCl_2.H_2O$	0.1
Agar	13
Tween20/Tween80	10ml
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 and Tween20/80 was sterilized separately and added into medium.

APPENDIX C

BUFFERS AND STOCK SOLUTIONS

C.1 ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

Ethidium bromide (0.5 g) was dissolved in 50 ml of deionized water.

C.2 CHLOROFORM-ISOAMYL ALCOHOL SOLUTION (24:1)

Forty eight milliliters of chloroform was mixed with 2 ml of isoamyl alcohol.

C.3 50 X TAE

Two hundred and fifty-two grams Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

C.4 1XTAE

Twenty milliliters of 50X TAE buffer was taken and the volume was adjusted to1000 ml with deionized water to obtain 1 liter 1X TAE buffer.

C.5 1X TE BUFFER

10 mM Tris pH 8.0
1 mM EDTA pH 8.0

C.6 10 X TBE

One hundred and eight grams Tris Base and 55 g boric acid were weighed. They

were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was added. The volume was brought to 1000 ml with deionized water.

C.7 1X TBE

Hundred milliliters, 10X TBE was taken and the volume was brought to 1000 ml with deionized water to obtain 1X TBE buffer.

C.8 CTAB/ NaCl (10% CTAB in 0,7 M NaCl)

NaCl (4.1 g) was dissolved in 80 ml water and CTAB was added slowly while heating and stirring. Final volume was adjusted to 100ml.

APPENDIX D

STAINS AND INDICATORS

D.1. BROMTYMOL BLUE SOLUTION

Bromtymol blue	1 g
N/10 NaOH	25 ml
Deionized water	475 ml

D.2. SOLUTIONS FOR GRAM STAINING

D.2.1. CRYSTAL VIOLET STAINING REAGENT

Solution A

Crystal violet	2g
Ethanol (95%)	20ml 83

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

D.2.2. IODINE SOLUTION

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Iodine and potassium iodide were grinded in a mortar, water was added slowly with continuous grinding until the iodine was dissolved. The solution was stored in amber bottle.

D.2.3. SAFRANIN SOLUTION

Safranin (2.5% in 95% alcohol)	10ml
Distilled water	100ml

APPENDIX E

PCR RECIPIES

E.1. PCR MIX

Mg free Taq DNA polymerase buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Sterile deionized water	32 µl
Oligo forward (10 picomole/µl)	1 µl
Oligo reverse (10 picomole/ µl)	1 µl
dNTP (2mM each) 10X	5 µl

E.2. 6X GEL LOADING BUFFER (20 ML)

10x TBE	2 ml
Glycerol	6 ml
Deionized water	12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

E.3. dNTP (10X)

Ten microliters of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at .20 °C.

E.4. RESTRICTION ENZYME MIX

Restriction enzyme buffer	5 µl
DNA	8 µl
Restriction enzyme (10U/ µl)	1.5 µl
Sterile deionized water	35.5 µl

APPENDIX F

OLIGONUCLEOTIDE PRIMERS

L1: 5.- CAAGGCATCCACCGT -3.

Three hundred and fifty micrograms primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliters of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

EGE 1 5.- AGAGTTTGATCCTGGCTCAG -3.

Five hundred and ninty micrograms primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain final concentration of 2 µg / µl stock solutions. Five microliters of stock solution were then taken and mixed with 95 µl sterile deionized water. Therefore, 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

APPENDIX G

RECOGNITION SITES OF RESTRICTION ENZYMES

G1. ***Taq*** **I**

5.- T▼CG A -3.
5.- A GC ▲T -3.

G2. ***Hae*** **III**

5.-GG▼CC-3.
5.-CC▲GG-3.

APPENDIX H

ISOLATE NAMES

DI- C12a	F26- Ü4z
D2- Ş6a	F27- Ü6a
D5- E15	F28- 15b
D6-C15a	F29- F1
D7- 20a	F30- 24a
D10-19a	F31- 27b
D11- B5c	F32- B4c
D12- B1a	F33- T1b
D14- 23a	F34- E6z
D18- C4	F35- B8a
D20- B8b0	E42- C8
D22- H2	E43- C14a
D23- H1a	E47- C19y
D24- P5z	F1- E11x
D25- C17	F2- F2x
D26- 2b	F3- F2y
D29- D3c	F4- F2z
D30- B9	F5- G5
D31- A9	F6- G8
D32- D2b	F7- H3x
E1- A2	F8- H3y
E2- P2y	F9- P1x
E3- C6	F10- P4a
E4- C2a	F11- P5y
E6- G9a	F12- S1
E7- 3a	F13- S2
E9-24a	F14- Ş1

E10- 13b F15- V3

E12- 29c F16- A1

E13- 22c F17- A4

E14- 12c F18- A6

E15- 27c F19- A7

E16- 1a F20- B10

E17- 2d F21- C1

E20- B2 F22- C2

E23- B8c F23- C3

E24- 18b F24- C24

E25- 11a F25- C10

ISOLATION OF HALOALKALIPHILIC MICROORGANISMS FROM LEATHER INDUSTRY

Hatice Sevgi ÇOBAN

October, 2004

Isolation of Haloalkaliphilic Microorganisms from Leather Industry

By

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ABSTRACT

Haloalkalophiles are extremophilic microorganisms that are adapted to saline and alkaline conditions. Different species of them have been isolated so far from soda lakes and soil samples. Haloalkalophilic microorganisms have significant adaptive mechanisms to avoid denaturing effect of salts and to balance their interior pH. Extracellular enzymes that are produced by these halophilic and alkalophilic microorganism are applicable for industrial purposes. Therefore isolation of these organisms from their habitats and study on genotypic characterization constitute initial steps for furter biotechnological studies.

In this study, processing steps of leather factories and their wastewater were chosen for sampling. In order to isolate target microorganisms Horikoshi-I medium including 12% NaCl was used. After isolation microorganisms were purified. Phenotypic tests were applied (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope, sensitivity to antibiotics and extracellular enzyme screenings). For genotypic characterization, genomic DNA was isolated and 16S-ITS rDNA region was amplified.

Differentiation was achieved according to Restriction Fragment Length Polymorphism (RFLP) method by using *Hae* III and *Taq* I endonucleases. Isolates, which represented the different RFLP patterns, were chosen for building up the haplotype groups.

As a result of the study seven different RFLP haplotypes were identified. Moreover, 16S ribosomal DNA partial sequencing was also performed on some of the strains in. These haloalkalophilic microorganisms and their enzymes could be used in different biotechnological studies in the future for various industrial applications.

ÖZ

Haloalkalofilik mikroorganizmalar yüksek alkali ve tuzlu koşullara adapte olmuş ekstremofilik canlılardır. Birçok farklı tür toprak ve soda gölü örneklerinden izole edilmiştir. Haloalkalofilik mikroorganizmalar tuzun zararlı etkilerin ve hücre içi pH değerlerini düzenleme yeteneğine sahiptirler. Halofilik ve alkalofilik olan bu organizmaların ürettiği ekstraselüler enzimler endüstride farklı amaçlar için kullanılanılabilir. Haloalkalofilik mikroorganizmaların yaşadıkları habitatlardan izole edilmeleri ve genotipik tanımlamalarının yapılması endüstri alanında kullanım amaçlı olarak genetik potansiyellerinin tanınması ve ileride yapılacak biyoteknolojik çalışmalar adına atılacak ilk adımı oluşturmaktadır.

Deri fabrikaları işlem basamakları ve atık suyu bu çalışma için örnekleme alanı olarak belirlenmiştir. Bu amaç için Horikoshi-I besi yerine %12 tuz ilave edilerek kullanılmıştır. İzolasyon aşamasının ardından organizmaların saflaştırma işlemi tamamlanmıştır. Fenotipik testler uygulanmıştır. Bunlar;Gram boyama, katalaz ve oksidaz testi, faz-kontrast mikroskobu ile spor gözlemi, ekstraselüler enzim taraması, farklı tuz konsantrasyonlarında (%0, %20, %25) gelişme, farklı sıcaklılarda (45-50 °C) gelişme, farklı pH (7-10-12) değerlerinde gelişme özellikleri). Genotipik karakterizasyon için genomik DNA izolasyonunun ardından 16S-ITS rDNA amplifikasyonu yapılmıştır. RFLP için iki farklı restriksiyon enzimi kullanıldı (*Hae* III and *Taq* I). Kesim ürünlerinin analizi yapılmıştır. Farklı jel profillerine sahip olanların oluşturduğu gruplar her iki enzim için de belirlendikten sonra grup temsilcileri kullanılarak dendogramlar oluşturulmuştur. Hae III restiriksiyon enzim kesimi sonucunda elde edilen grup temsilcilerinin 16S rDNA - PCR amplifikasyon ürünleri kısmi dizi analizleri için kullanıldı.

Haloalkalofilik mikroorganizmalar ve enzimleri daha ileride yapılacak biyoteknolojik çalışmalarda kullanılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xi

LIST OF ABREVIATIONS xii

Chapter1 INTRODUCTION 1

1.1.Definition of Haloalkalophiles 1

1.2.Haloalkalophilic Microorganisms 1

1.2.1. Eubacterial Haloalkalophiliphiles 2

1.2.2. Haloalkalophilic Bacillus 2

1.2.3. Gram NegativeHaloalkalophilicSpecies 3

1.2.4. Archaeal Haloalkalophilic Species 3

1.2.5. Distribution and Isolation 5

1.2.6. Biotechnological and Industrial Applications 5

1.2.7. Extracellular Enzymes and Their Industrial Application 6

1.2.8. Proteases from Haloalkalophilic Bacteria 6

1.2.9. Lipases from Haloalkalophiles 8

1.3. Identification of the Haloalkaolphilic Organisms 9

1.3.1. Phenotypic Methods 9

1.3.2. Molecular Characterization Methods 10

1.3.2.1. Pulsed Field Gel Electrophoresis 10

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD) 11

1.3.2.3. 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP 11

1.3.2.4. Plasmid Profiling 12

1.3.2.5. Ribotyping 12

1.3.2.6. DNA Sequencing 15

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir) 15
1.5.Thesis Objective 16

Chapter 2 MATERIAL METHODS 17
2.1. Materials 17
2.1.1. Chemicals 17
2.1.2. Samples used for isolation 17
2.2. Methods 17
2.2.1. Isolation and Preservation of the Isolates 17
2.2.1.2. Isolation of Haloalkalophilic Bacteria 17
2.2.1.3. Preservation and Activation of Isolates 18
2.2.2. Determination of Phenotypic Characteristics 18
2.2.2.1. Gram Staining 18
2.2.2.2. Examination of Endospores 19
2.2.2.3. Catalase Test 19
2.2.2.4. Oxidase Test 19
2.2.2.5. Growth at Different Temperatures 19
2.2.2.6. Growth at Different NaCl Concentrations 19
2.2.2.7. Growth at Different pH Ranges 20
2.2.3. Screening for Extracellular Enzymes 20
2.2.3.1. Screening for Protease Activity 20
2.2.3.2. Screening for Lipase Activity 20
2.2.4. Preparation of Genomic DNA 20
2.2.5. Genotypic Characterization 21
2.2.5.1. Identification of Isolates by 16SrDNA–ITS Region 21
2.2.5.1.1. Amplification of 16S rDNA – ITS Region 21
2.2.5.1.2. Electrophoresis of Amplified 16S-ITS Fragments 22
2.2.5.1.3. Chloroform Extraction of PCR Products 23
2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP) 23

2.2.5.1.5. Electrophoresis of Restriction Fragments 23
2.2.6. Sequencing 24
2.2.6.1. Amplification of 16SrRNA Gene 24
2.2.6.2 Extraction of DNA Fragments from Agarose Gel 25
2.2.6.3 Sequencing Reactions 24
2.2.6.4. Purification of Sequencing Reactions 25
2.2.6.4.1. Removal of unincorporated dye terminators using ethanol presipitation 26
2.2.6.4.2. Assembling the SEQ Sequencer 26

Chapter 3 RESULTS AND DISCUSSION 28
3.1. Isolation of Haloalkalophilic Bacteria 28
3.2. Determination of Phenotypic Characteristics 28
3.2.1. Gram staining 28
3.2.2. Examination of Endospore 28
3.2.3. Catalase and oxidase tests 28
3.2.4. Physiological Tests 29
3.3.Extracellular Enzyme Screening 32
3.3.1. Extracellular Enzyme Profiles of Isolates 33
3.4. Genotypic Characterization 36
3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon 36
3.4.2. 16S-ITS rDNA Based RFLP Profiling 37
3.4.3. Analysis of the Genotypic and Phenotypic Groups 40
3.4.4 Analysis of Sequencing Reactions 41

Chapter 4 CONCLUSION AND FUTURE PERSPECTIVE 42
REFERENCES 43
APENDICES AA1
APENDIX A. CHEMICHALS USED AA1
APENDIX B. RECIPIES MEDIA USED FOR EXPERIMENTS AB1
APPENDIX C. BUFFERS AND STOCK SOLUTIONS AC1
APPENDIX D. STAINS AND INDICATORS AD1

APPENDIX E. PCR RECIPIES AE1
APPENDIX F. OLIGONUCLEOTIDE PRIMERS.. AF1
APPENDIX G. RECOGNITION SITES OF RESTRICTION ENZYMES................AG1
APPENDIX H. ISOLATE NAMES AH1

LIST OF FIGURES

Figure 1.1. Archaea kingdom 4

Figure1.2. Rybotyping 14

Figure3.1. Apperance of extracellular protease 36

Figure 3.2. Apperance of extracellular lipase 36

Figure 3.3. 16S rDNA-ITS Amplification products of some isolates 36

Figure 3.4. *Hae* III and *Taq* I Digests of 16S-ITS rDNA Fragments of Representative Isolates 37

Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles of Isolates 38

Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles of Isolates 38

LIST OF TABLES

Table 1.1. Tolerance for pH, NaCl of selected species of the genus Bacillus 2

Table 1.2. "Natro group" of Halobacteria 5

Table 1.3. Detergent Proteases 7

Table.1.4. Lipolytic activity 9

Table 2.1. Samples used for isolation 17

Table 3.1. Results of the Physiologic test 29

Table.3.2. Tolerance for pH, NaCl of isolated strains 30

Table 3.3 Extracellular Enzyme Tests 33

Table 3.4 Extracellular Enzyme Profiles of isolates 34

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA 38

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups 39

Table 3.7. Analysis of the Genotypic and Phenotypic Groups 40

Table 3.8. Sequencing Results 41

ABBREVIATIONS

A	Adenine
bp	Base pair
C	Cytosine
CGT	Cyclodextrin Glucosyltransferase
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
G	Guanine
EDTA	Ethylenediamine Tetra Acetic Acid
ITS	Internal Transcribed Spacer
ISR	Internal Spacer Region
kb	Kilo base
PAGE	Polyacrylamide Gel Electrophoresis
P.E.	Pectinolytic Enzyme
PGA	Polygalacturonic Acid
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
T	Thymine
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA

CHAPTER 1

INTRODUCTION

1.1. Definition of Haloalkalophiles

Haloalkalophiles are one of the main physiological groups of microorganisms classified in alkaliphiles. Lake Magadii in Kenya, Wadi Natrun in Egypt have been defined as the most stable alkaline environments on earth. Therefore the first ecological studies related to haloalkalophilic microorganisms have been performed in these places. The conditions of high mineralization and/or alkalinity in such environments regulate their prokaryotic communities (Trotsenko and Khmelenia, 2001). Both alkalinity (pH 9) and salinity up to saturation (33% (wt/vol)) are needed for the survival of these microorganisms (Koki Horikoshi, 1999).

Alkaliphiles can be classified as obligately and facultatively alkaliphilic organisms. Obligately alkaliphilic microorganisms cannot grow below pH 9. Facultatively alkaliphilic organisms show optimal growth at pH 10.0 or above but they also have the ability to grow at neutral pH.

Halophilicity divided the organisms into three categories: slightly halophilic, moderately halophilic and extremely halophilic. Halophilic microorganisms require certain concentrations of NaCI for their growth, while the halotolerant ones have the ability to grow in the absence or presence of higher concentrations of NaCl (Mimura and Nagata, 2000).

1.2. Haloalkalophilic Microorganisms

Haloalkalophilic microorganisms inhabiting extremely alkaline and saline environments are found in both Archaea and Eubacteria. Most of the extremely salt tolerant haloalkaliphiles belong to *Halobacteriaceae*.

1.2.1. Eubacterial Haloalkalophilic

1.1.2.1 Haloalkalophilic *Bacillus*

Genus *Bacillus* can be characterized by unique physiological characteristics such as Gram staining, spore examination, catalase and oxidase tests. *Bacillus* is a Gram positive usually catalase and oxidase positive and spore producing organism that classified in Firmiculates. It is well known that *Bacillus* strains do not form endospores under many environmental conditions, and the ability to form spores is often difficult to demonstrate. This is very important for their taxonomy. There have been organisms described as non-spore-formers, which had to be reclassified after the detection of spores (Fritze and Claus, 1992). Recent taxonomic studies have separated some of the *Bacillus* members and grouped into new genera: *Alicyclobacillus, Aneurinibacillus, Brevibacillus, Gracilibacillus, Halobacillus, Paenibacillus, Virgibacillus* (Baumgart, 2003)

Table1.1. Tolerance for pH and NaCl of selected species of the genus *Bacillus.*

Species	Source	pH Tolerance	NaCl Tolerance
B. cohnii	Horse meadow soil	obligate alkalophilic	5%
B. horikoshii	Soil	obligate alkalophilic	17%
B. marismortui	Dead sea water	6.0-9.0, pH optimum 7.5	5-25%
B. agaradhaerens	Soil	obligate alkalophilic	16%
B. aclarkii	Soil	obligate alkalophilic	16%
B. horti	Soil, Japan	alkali tolerant	10%
B. vedderi	Bauxite waste	obligate alkalophilic	7.5%
B. alkalophilus	Soil and faeces	obligate alkalophilic	8%
B. clausii	Garden soil	alkali tolerant	10%
B. haloalkaliphilus	Brine/mud, Wadi Natrun	obligate alkalophilic	25%
B. halodurans	Soil	obligate alkalophilic	12%
B. pseudoalkaliphilus	Soil	obligate alkalophilic	10%
B. pseudofirmus	Lake bank soil	obligate alkalophilic	17%
Bacillus sp. DSM8714	River bank soil	alkalitolerant	10%
Bacillus sp. DSM8717	Horse and elephant manure	alkalitolerant	10%
Halobacillus halophilus	Salt marsh soil and solar salterns	7.0-9.0	15%

1.2.3. Gram Negative Haloalkalophilic Microorganisms

Halomonadaceae members are slightly and moderately halophilic bacteria with a broad pH tolerance. They are isolated from saline water, soil samples, leather processing, seawater and estuarine water. Species belong to the Halomonadaceae are Gram negative, rod shaped, non-spore forming, aerobic and also facultatively anaerobic in the presence of nitrate. (Dobzon and Franzman 1996; Franzman et al., 1988). By 16S rDNA sequence analysis, chemotaxonomic and physiological characteristics *Halomonas* have been defined as the only genus in Halomonadaceae (Dobson et al., 1996; Franzman and Tindall 1990). However, recently it has been proposed that Halomonadaceae has four different genera; *Halomonas*, *Alkanivorax, Carnivoras, Chromohalobacter* and *Zymobacte* (Bromguarte, 2003).

1.2.4. Haloalkalophilic Archea

Bacteria and archaea have been first recognized as two distinct groups of Prokarya by Woese et al. (1997). The original classification based on 16S ribosomal RNA gene sequence has been confirmed by extensive phylogenetic studies (Rosa et al., 2002). Extreme halophiles or Euryarchaeota require high salt concentrations, up to 20% NaCl for growth.

Archaea possess genes with recognizable counterparts in the Bacteria, showing that the two groups have functional similarities. Archaea also possess genes that also found in Eukarya (Rosa et al., 2002).

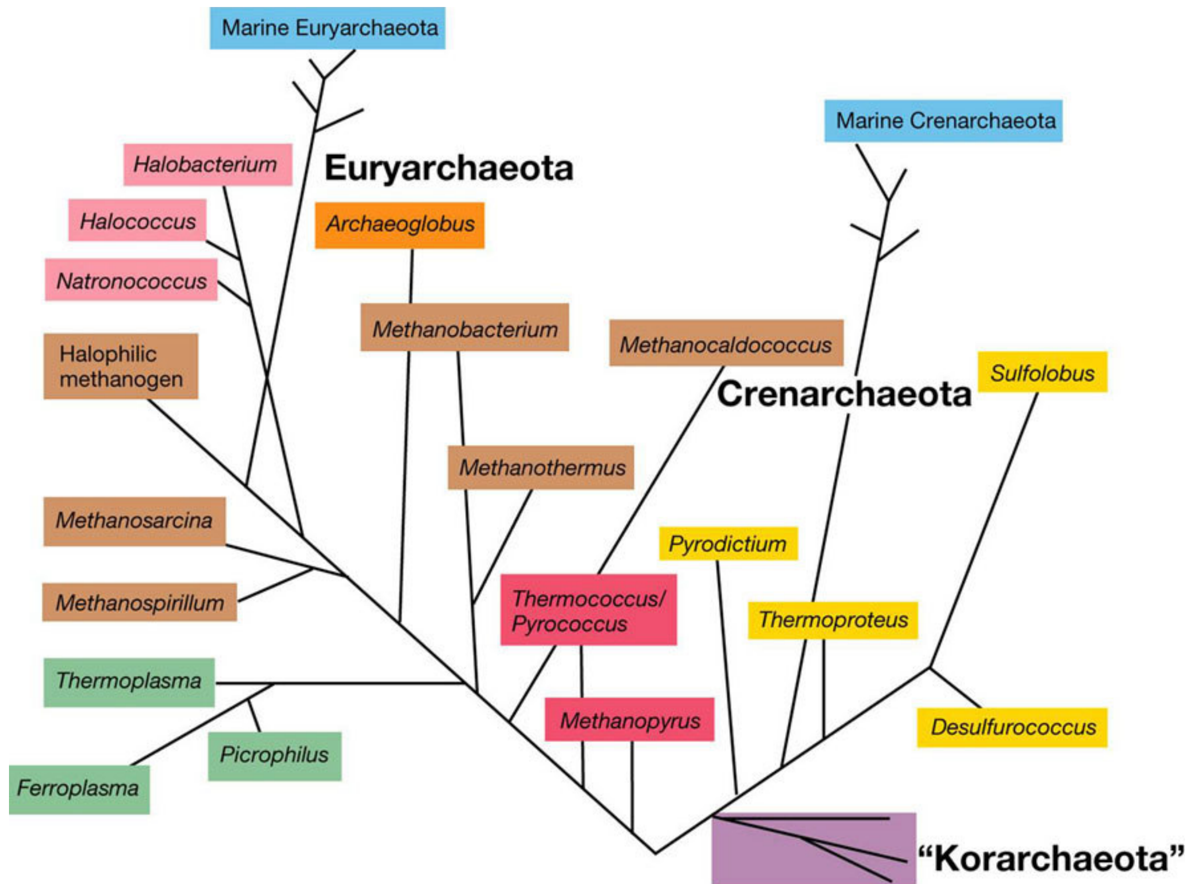


Figure1.1 Archaea kingdom

Euryarchaeaota (*Natronobacterium, Natronococcus, Natronomonas, Natrialba*, *Natronorubrum* genera are classified in "Natro group" of *Halobacteria*. These microorganism are extremely halophilic and alkaliphilic.

Table1.2. "Natro group" of Halobacteria.

Genus	Morphology	Number of species	Habitat
Natronobacterium	Rods	1	Highly saline soda lakes
Natrinema	Rods	2	Salted fish;hides
Natrialba	Rods	2	Soda lakes; beach sand
Natronomonas	Rods	1	Soda lakes
Natronococcus	Cocci	2	Soda lakes
Natronorubrum	Flattened cells	2	Soda lakes

1.2.5. Distribution and Isolation

Isolation of haloalkalophilic microorganisms is performed using different medium ingredients with respect to the group of target organism and the target environment. Actually the salt tolerance range plays an important role because the extremely halophilic organisms can not grow in low concentrations of salt although they have the ability to grow in minimal media. These microorganisms can live at high concentrations of different salts therefore they are populated in salt lakes. Limnological studies focus on the life conditions for extremely halophilic members during seasonal cycles. Anaerobic conditions are suitable in the presence of nitrate for facultatively anaerobes of eucaryotic members.

1.2.6. Biotechnological and Industrial Applications

Extremophilic microorganisms produce cell-associated products that can be used in different industrial applications. First of all their stability and activity in harsh conditions such as alkaline, acidic, high temperature, high concentration of salinity enables broad applications. Extracellular enzymes produced by these organisms are of great interest in biotechnology. Many *Bacillus* species are known to produce alkaline and saline stable proteases, amylases, cellulases, lipases, pectinases and xylanases (Martins et al., 2001)

Enzyme characterization studies with amylase from *Halobacillus* sp. strain MA-2, (Amoozegar et al., 2003) and *Halomonas mediteridiana* (J.Nieto et al., 1999) have

been known. Also the extracellular enzymes from archaeal members have unique features in their molecular structures.

Halophilic microorganisms also used for biological treatment (Kargı and Uygur, 1995).

Halophilic exopolysaccharides have been used in food manufacturing. Furthermore, there is a mereaning demand for non-toxic, biodegradable, environmentally friendly substances. *Halomonas maura* have been studied with novel composition exopolysaccharide (Arias et al., 2003).

1.2.7. Extracellular Enzymes and Their Industrial Application

Extracellular enzymes produced by haloalkalophilic microorganisms have unique properties. They have activity and stability at broad pH ranges and high concentrations of salts in that many other proteins are denaturated. Enzymes from archeal and eubacterial haloalkalophilic organisms have different characteristics. Archaeal enzymes need high concentrations of salt for their activity and stability (Mevarech et al., 2000). This is explained by the comparison of molecular structures from halophilic enzymes with those of nonhalophilic enzymes, this feature is related with adaptation mechanisms used for the protecting of cell from the denaturing effects of salt molecules (Ventosa et al., 1998)

1.2.8. Proteases from Haloalkalophilic Bacteria

Proteases are the enzymes that cleave protein molecules by hydrolysis. The first classification is made by using their cleavage mechanism. Exopeptidases work on the peptide bonds near the amino or carboxy-termini but endopeptidases are active on the peptide bonds distant from the termini of the substrate. The subclassification is based on their catalytic mechanism. Chemically different groups are found on active sites. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao et al., 1998). Subtilisins were grouped in serine type proteases. Catalytic triad of subtilisins consists of aspartic acid, histidine and serine. Although the size of subtilisins varies from 18 kDa to 90 kDa, all the subtilisins used in detergents have a size of

approximately 27 kDa. Most of the alkaline proteases were subtilisins. At present, less than 15 different enzyme molecules are used in detergents worldwide. These enzymes originate from *B. amyloliquefaciens, B. licheniformis, Bacillus clausii, Bacillus lentus, Bacillus alkaloophilus,* and *Bacillus halodurans*

The success of subtilisins is based on several factors, including their high stability and relatively low substrate specificity, features common in extracellular proteases. Their production as extracellular enzymes is of course an important factor in itself, as this greatly simplifies the separation of the enzyme from the biomass and facilitates other downstream processing steps. Another important point is the ability of *Bacillus* strains to secrete enzymes over a very short period of time into the fermentation broth.

Detergent industry prefers the enzymes, which are stable at high pH and compatible with chelating and oxidizing agents (Rao et al., 1998). Table represents the detergent proteases available (Maurer, 2004).

Table1.3. Detergent proteases (Maurer, 2004).

Trade mark	Origin	WT/PE	Production strain	Synonym
Genencor	*B. amyloliquefacies*	PE	*B. subtilis*	
Novozymes	*B. clausii*	WT	*B. clausii*	Subtilisin 309
Genencor	*B. lentus*	WT	*B. subtilis*	
Kao	*B. alkalophilus*	WT	*B. alkalophilus*	
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Novozymes	*B. halodurans*	WT	*B. halodurans*	Subtilisin 147
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. alkalophilus* PB92	PE	*B. alkaliphilus*	

PE, protein engineered; WT, wild type.

Since 1997, several gene-shuffling approaches have been performed with subtilisins. Interesting results from the shuffling of 26 protease genes have been described for properties such as activity in organic solvents, temperature stability, and activity at high or low pH (Minshull et al., 1999). Little, however, has been published on stain removal. Owing to the large number of variant molecules generated by

shuffling and other random techniques, screening methods with high-throughput and increased relevance have had to be developed. Unfortunately, these methods are still not entirely satisfactory, which might explain why no outstanding new subtilisin variant created by one of the gene shuffling technologies is yet present in detergents. Subtilisins for detergents are produced in *Bacillus*, because these species are able to secrete large a mounts of extracellular enzymes. The control mechanisms involved in the production of proteases in Bacillus are extremely complex and still not fully understood. An example is the two-component regulatory system that acts as a quorum sensing mechanism in *B. Subtilis* and which has been found to control the expression of the alkaline protease. This regulatory system is encoded on the chromosome and on endogenous plasmids. (Maurer, 2004)

Arhaea members have proteolytic activity in high salt concentrations (Eichler, 2001). Proteases from *Natronococcus occultus* and *Natrialba magadi* have been characterized (De Castro et al., 2001).

Leather industry uses proteases at dehairing and baiting steps (Kumar and Takagi, 1999). Stable proteases under alkaline and saline conditions would be most effective enzymes for such purposes.

It has been reported that Halophilic bacteria destroied brine-cured hides with their high proteoliytic activity (Bailey et al., 1998).

The studies about using proteases and gelatinases for the pretreatment of leather waste including cromium are important for the convertion of waste material to animal feed (Taylor, 2002).

Silver recovery has been achieved with different types of alkaline proteases at high alkaline conditions (Singh et al., 1999, Kumar and Takagi, 1999).

1.2.9. Lipases from Haloalkalophililes

Lipases, (triacylglycerol acylhydrolases, EC 3.1.1.3), are able to catalyse both the hydrolysis and the synthesis of esters bonds in lipid molecules. Lipid molecules are composed of glycerol and long-chain fatty acid molecules. Esterification and hydrolysis reactions that can occur in organic solvents are very important (Ishıkawa and Ogino,

2000). Most of the lipolytic enzymes used commercially are produced by alkalophilic microorganisms and they are used as detergent addiditives (Sharma et al. 2001).

Table.1.4. Lipolytic activity

Active site	Catalytic triad consisting of the amino acids serine, aspartic (or glutamic acid) and histidine; homologue to the triad of serine proteases.
Substrates	Oils (olive, soybean, mineral oils),Triglycerides (triolein, tributyrin), Synthetic surfactants (Tweens 20 and 40).
Other Activities	Phospholipase, lysophospholipase, cholesterol esterase, cutinase, amidase.

Lipases from *Halomonas* strains have been characterized (Mattiasson et al., 2004).

Alkaline lipases are mainly used in detergent industry. Approximately 1000 tons of lipases are added to the 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Alkaline lipases to be used in detergents should meet certain requirements. They should hydrolyze fats of various compositions, be stable in harsh washing conditions (pH 10-11, 30-60 °C) and in the presence of surfactants (linear alkyl benzene sulfonates (LAS) and proteases) (Sharma *et al.,* 2001).

1.3. Identification of the Haloalkalophilic Organisms

Identification of the haloalkalophilic microorganisms are performed by using the phenotypic and genotypic characteristics.

1.3.1. Phenotypic Methods

Morphological characteristics can be examined by direct observation, which give simple information on the organism for example colony formation that includes colony shape, diameter and color. Furthermore, microscopic observations after different chemical treatments give detailed information on the cell morphology. These are cell shape, Gram behavior and sporulation type. Phase contrast microscopy gives

information on the cell shape and sporulation type without any chemical treatment. Some tests can provide information about the adaptive mechanisms and relation between interior of the cell and the medium. These tests conditions are: growth at different pH ranges, temperatures, pressure and salt concentrations. Bergey's Manual of Systematic Bacteriology a major taxonomic compilation of prokaryotes gives information in all recognized species of prokaryotes and contains number of keys to evaluate the properties given above (Madigan *et al.,* 1997).

Phenotypic methods also include biotyping, antibiogram, phage typing, serotyping, PAGE/immunoblotting and multilocus enzyme electrophoresis (Busch and Nitschko, 1999).

Although molecular characterization methods provide clear differentiation and precise identification, using of phenotypic characterization methods combined with molecular techniques are required for taxonomic studies and functional purposes.

1.3.2. Molecular Characterization Methods

1.3.2.1. Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is used for the analysis of microbial genomes and molecular characterization of the microorganisms below species level. It enables to the seperation of large DNA molecules and therefore whole genomic DNA can be analysed. Analysis of chromosomal macrorestriction patterns by PFGE is a powerful method to produce fingerprints of closely related strains (Farber, 1996). Application of the method can differ for different type of organism. Different organism can require different cell disruption treatments for the releasing the genomic DNA. Also the size and content of genomic DNA can differ among the organisms. Thus, optimization parameters such as lysis of cells, electrophoresis conditions (pulse time, voltage) are the main points for obtaining fine discriminative PFGE results.

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)

DNA fragments are amplified by the polymerase chain reaction (PCR) using short (generally 10 bp) synthetic primers of random sequence. These oligonucleotides serve as both forward and reverse primer at the same time, and are usually able to amplify fragments at 3-10 genomic sites simultaneously. Amplified fragments (within the 0.5-5 kb range) are separated by gel-electrophoresis and polymorphisms are detected as the presence or absence of bands of particular size. These polymorphisms are considered to be primarily due to variation in the primer annealing sites. Some advantages of RAPD are as follows: low quantities of template DNA are required (5-50 ng per reaction), no sequence data for primer construction are required, easy and quick to assay, low costs involved, generation of multiple bands per reaction, and it suitable for automation.

1.3.2.3 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP (Restriction Fragment Length Polymorphism)

In most prokaryotes the ribosomal RNA genes reside sequentially in the chromosome (16S-23S-5S) and they are transcribed as a single polycistronic RNA molecule (Luz et al., 1998). These ordered genes have long been served as a genetic marker to study microbial diversity. The 16S rRNA gene sequence has been used for assessing bacterial phylogenies (Abd-El-Haleem et al., 2002). However, it has only provided information useful for identifications at genus level (Shaver et al., 2002).

Below the genus level, additional rDNA sequences have been necessary. The use of 23S rDNA has also been limited by its larger size and insufficient sequence data (Abd- El-Haleem et al., 2002). The intergenic spacer region (ISR) or also known as intergenic/internal transcribed spacer (ITS) (Abd-El-Haleem et al., 2002; Daffonchio et al., 2000; Shaver et al., 2002; Fisher and Triplett, 1999; Toth et al., 2001) between 16S and 23S rDNA genes, includes both conserved and highly variable sequence motifs, such as tRNA genes, and anti-terminators (boxA) (Abd-El Haleem et al., 2002). ISRs are generally found in multiple copies in most bacteria genomes. Since they are

hypervariable with respect to adjacent genes, they can differentiate between multiple operons in the cell (Daffonchio et al., 1998). The size of the spacer has been found to differ considerably among different species, and even among the different operons within a single cell (Garcia-Martinez et al., 1999).

Figure 1.8 Schematic representation of a 16S-23S spacer and organization of ITS functional regions (Garcia-Martinez et al., 1999).

The method is based on the amplification of 16S rDNA and ISR regions as singleamplicon. The resulting amplicon is then digested with frequent cutting enzymes. Finally restriction fragments obtained are separated by agarose gel electrophoresis and restriction patterns are then compared. The method could discriminate different groups of bacteria at species level.

1.3.2.4. Plasmid Profiling

Plasmids are the genetic materials of bacteria carried as extracromosomal elements. They are self-replicative and they can be transferred naturally between the microorganisms. Genes coded in plasmid DNA structures give different characteristics to microorganisms.

Plasmids isolated from the microorganisms give variable profiles with respect to the organism type and the habitats. Therefore the gel profiles of plasmids for closely related organism would be useful in taxonomic studies (Farber, 1996).

1.3.2.5. Ribotyping

Nucleic acid probes that recognize ribosomal genes are used for ribotying. Ribosomal RNA (rRNA) genes are composed of 23S, 16S and 5S regions. They are known to be highly conserved. Whereas most of the bacterial genes present in one copy, rRNA operons can be present in anywhere more than one copy (2-11 copies per bacterial cell). Thus the more copies of rRNA operon enables more discriminatory riboyping. In practice, first bacterial DNA is isolated and digested by restriction endonucleases. After agarose gel electrophoresing, DNA is transferred onto nylon or nitrocellulose membrane by capillary system or electrophoresis. The nylon membranes

are used as a solid support for probing. Probes specific to 23S, 16S, 5S rRNA genes are labeled using radioactive or non radioactive labeling techniques (non isotopic cold-labelling systems). Hence fingerprint patterns of bacterial DNA containing ribosomal genes are created. These patterns are varied from 1 to 15 bands and compared among the isolates. For another ribotying technique called as chemiluminescence ribotyping, digoxigenin-labeled cDNA (DNA which has been made by reverse transcription of rRNA) can be seen in figure 2. One of the major advantages of ribotyping is that because of the similarity of ribosomal genes, universal probes can be used. Reproducibility is also another advantage of this genotypic method.

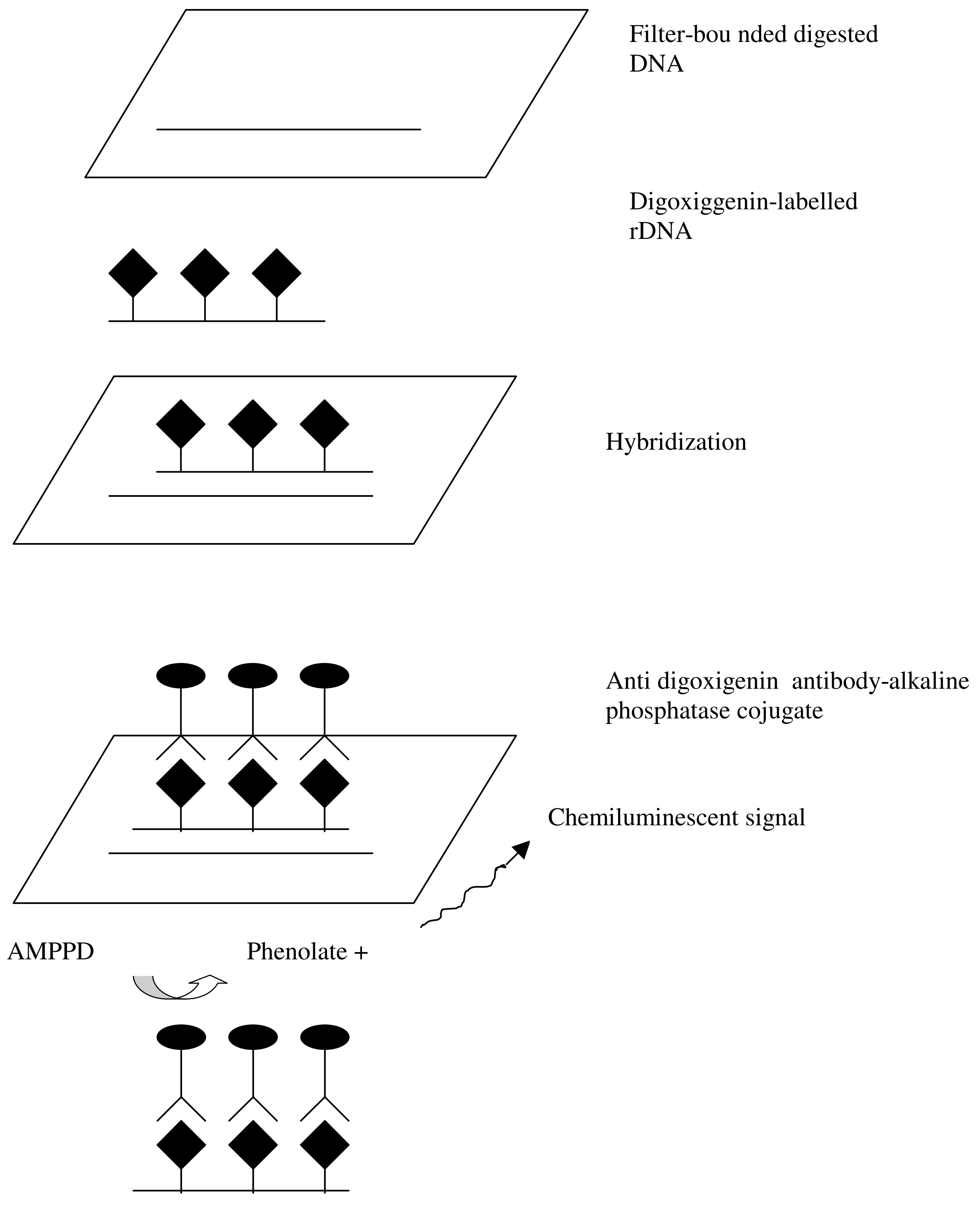


Figure1.2. Schematic representation of chemiluminescence ribotyping. AMPPD, adamantly-1, 2-dioxetane phosphate (Bingen et al., 1994)

1.3.2.6. DNA Sequencing

DNA sequencing is used for investigation of the phylogenetic relationship of organisms. This tecnique is based on the exact order of nucleotides in a region of the DNA molecule Automated DNA sequencing generally includes PCR amplification of a DNA region of interest, computer-aided sequence analysis and interpretation. For DNA sequencing the region of the chromosome to be analysed must be short consisting of a variable sequence flanked by highly conserved region. The variability within the selected sequence must be sufficient to differentiate microorganisms. And also the selected sequence should not be horizontally transmissible to other strains of species (Olive and Bean, 1999).

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir)

Leather factories of Ege Region are populated in Menemen – İzmir. twenty different companies have leather factories in Menemen region. It is known that industrial activities bring the most important pollution problems. For this reason leather factories were placed far from the centre of the city. Leather processing steps are as fallows:

- Curing
- Beamhouse operations which wash and soak the hides or skins and (at most tanneries) remove the attached hair.
- Tanyard processes in which the tanning agent (primarily chromium) reacts with and stabilizes the proteinaceous matter in the hides or skins.
- Finishing or post-tanning processes.

Waste Water of the leather factories are removed from the other factories at Menemen region and a simple waste water treatment is applied. The classical scheme is given below (UNEP/IEO, 1994).

- Pre-treatment: mechanical screening to remove coarse material.

- Primary treatment: sulphide removal from beamhouse effluents; chrome removal from tanning effluents; flow equalization; physical-chemical treatment for

-BOD removal and neutralization.

- Secondary treatment, usually biological.

- Tertiary treatment, including nitrification and denitrification.

- Sedimentation and sludge handling

1.5Thesis Objectives

The purpose of this study was,

- Isolation of haloalkalophilic strains
- Screening for their extracellular enzymes: protease and lipase
- Characterization of these isolates by phenotypic and genotypic methods.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used were listed in Appendix A.

2.1.2. Samples used for isolation

Table 2.1. Samples used for isolation

Sample Type	Location
Polluted Soil	Leather Factories waste (İzmir – Menemen)
Leather processing	Leather Factories (Izmir-Menemen)

2.2. Methods

2.2.1. Isolation and Preservation of the Isolates

2.2.1.2. Isolation of Haloalkalophilic Bacteria

Dilution plate method was used for the isolation of haloalkalophilic bacteria (Mora et al., 1998). Ten g soil, leather or 10 ml of water samples were transferred in 90 ml of 0.85% saline water. In order to homogenize the samples 6h incubation was performed in orbital shaker at room temperature. The samples were pasteurized for 10 m. at 80°C in a water bath in order to kill most of the vegetative cells and eliminate non-

spore forming bacteria (Mora et al., 1998). After the heat treatment, for dilution, 1ml aliquots from each sample was transferred in 9 ml of 0.85 % saline water and 6 fold dilutions were prepared. One ml of the dilutions was plated on appropriate solid media (Appendix B.1) and incubated for 5 days at 37 °C. Single colonies were picked and they were purified using streak plate method.

2.2.1.3. Preservation and Activation of Isolates

Cultures were grown in Horikoshi-I (Apendix B.1) agar plates containing 12% NaCl and incubated for 2 days at 37°C they were then transferred into the isolation broth. After turbidity formation in isolation broth, 0.5ml of each culture were transferred into cryotubes and 0.5ml of isolation broth containing 20% glycerol was added. These steps were all performed on ice. Then tubes were mixed gently by pipetting. Cultures were stored at -20°C for an hour they were then stored at -80°C. For the phenotypic test culture activation was applied from 20% glycerol stocks. For phenotypic and genotypic identification of the isolates, cultures were prepared from these stocks.

2.2.2. Determination of Phenotypic Characteristics

2.2.2.1. Gram Staining

Isolates were stained by modified Gram method for halophilic bacteria. Eighty µl of sterile 0.85% saline water were pipetted onto slides the organisms were then suspended and mixed thoroughly. Overnight culture was spread onto the microscope slides until a thin film formed by using tooth stick. After being air dried, the slides were fixed and desalted simultaneously by immersing in 2% acetic acid for 5 min. Then they were dried again. The smears were covered with 0.25% aqueous solution of crystal violet (Apendix D.2.1) for 1 min. After rinsing under the tap water, the slides were transferred into iodine (Apendix D.2.2) solution and were kept for 1 min. Following this step, slides were washed in alcohol for 6 s. They were then stained with safranin (Apendix D.2.3) for 30 s. After staining, the slides were dried on paper towels and the

cells were examined under light microscope. Gram (+) cells assumed purple color while Gram (-) cells appeared pink or red.

2.2.2.2. Examination of Endospores

Isolates were grown on Horikoshi-I (Apendix B.1) agar plates and 0.0005% $MnCl_2$ (Travers, 1987) were added in to the medium. Observations for spore formation were monitored at 3-4- 5-6th day.

2.2.2.3. Catalase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. Catalase activity was observed after dropping 3% hydrogen peroxide solution onto the target colony. Formation of the air bubbles indicated the presence of catalase activity.

2.2.2.4. Oxidase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. 1% solution of tetramethyl-p-phenylenediamine was poured onto a filter paper placed in a petri dish. Then the target colony was taken from the agar plate and spotted onto wet filter paper. Observation of the blue-purple color indicated the oxsidase activity.

2.2.2.5. Growth at Different Temperatures

The isolates were grown on nutrient agar (Apendix B.2) 45°C and 50°C. Observations for growth were noted during the 5-day incubation.

2.2.2.6. Growth at Different NaCl Concentrations

Cultures were tested for their salt tolerance on Horikoshi-I (Apendix B.1) agar plates containing 0%, 20%, 25 % NaCl during the 5-day incubation.

2.2.2.7. Growth at Different pH Ranges

Cultures were tested for pH tolerance in nutrient agar plates at different pH points (7, 10, and 12). Growth behaviors were observed during the 5-day incubation

2.2.3. Screening for Extracellular Enzymes

2.2.3.1. Screening for Protease Activity

Proteteolytic activity of the isolates observed by adding casein into Horikoshi–I (Apendix B.1) medium adding sikimmed milk. Different concentrations of NaCl (12% and 20%) were added. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete proteases extracellularly, give clear zones around the colony (Horikoshi, 1999).

2.2.3.2. Screening for Lipase Activity

Lipolytic activity of the isolates was observed by using the medium described in Appendix B.5. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete lipase extracellularly, gave opaque zones around the colony (Haba et al., 2000).

2.2.4. Preparation of Genomic DNA

Cultures were grown on solid Horikoshi – I (Apendix B.1) medium. Cells were suspended in 1.5 ml of sterile water in petri dishes. Then cell suspension was transferred into eppendorf tubes. In order to prepare cell pellets, tubes were centrifugated for 5 min at 5000 rpm. Pellets were resuspended in 567 µl 1Xte (Apendix C.4). Thirty µl 10% SDS and 3µl proteinase K solution (20 mg/ml) were added and the solution was mixed thoroughly. Cell lysis was performed with 1h incubation at 37°C. Afterwards 0.1 ml of 5 M NaCl were added and mixed well. Then 80µl of CTAB/NaCl (Appendix C.8) were added, the tubes were then mixed and incubated for 10 min at 65°C. Following the incubation, 0.7 ml of chloroform/isoamyl alcohol (Appendix C.2) were added, mixed thoroughly and centrifuged for 5 min at 10000 rpm. Aqueous, viscous supernatant was

transferred into a fresh eppendorf tube. An equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged for 5 min. The supernatant was again transferred into a fresh eppendorf tube and 0.6 volume of isopropanol were added to precipitate the nucleic acids. The tube was then shaken back and forth until a white DNA precipitate became visible. DNA wool were taken with a pipet tip and placed into a new tube, contain 300µl 70% ethanol. The tubes were stored at -20°C for 1h. Afterwards tubes were centrifuged for 15 min at 6000 rpm. Then the supernatant was discarded and DNA pellet was washed with 500 µl 70% ethanol. It was centrifuged for 10 min at 8000 rpm. After this, ethanol was discarded. The pellet was dried and dissolved in 100 µl 1xTE containing 100 µg/ml RNAase. Then tubes were incubated for 1h at 37°C. After the incubation the sample volume was adjusted to 400 µl with 1xTE. DNA was dissolved by alternating cold-heat shocks (for 10 min at 80°C and 20 min at -20°C, repeated twice). To further purify DNA an equal volume of phenol was added and mixed well. An equal volume of chloroform was added and mixed well. Then the tubes were centrifuged for 5 min at 6000 rpm. The aqueous phase was transferred into a fresh eppendorf tube. Equal volume of chloroform /isoamyl alcohol was added and mixed well. Samples were centrifuged for 5 min at 6000 rpm. Chloroform /isoamyl alcohol step was repeated once again and DNA was precipitated by adding 1/10 sample volume of 5 M NaCl and 2 volumes of 99% ethanol. Pellet was washed in 500 µl 70% ethanol by centrifugation for 5 min at 6000 rpm. After that, ethanol was removed and samples were dried for 10 min at 37°C. DNA was dissolved in 20µl, 50µl, 100µl or 200µl 1xTE according to the pellet size. Then the samples were preserved at -20 °C.

2.2.5. Genotypic Characterization

2.2.5.1. Identification of isolates by 16S rDNA - ITS rDNA - RFLP

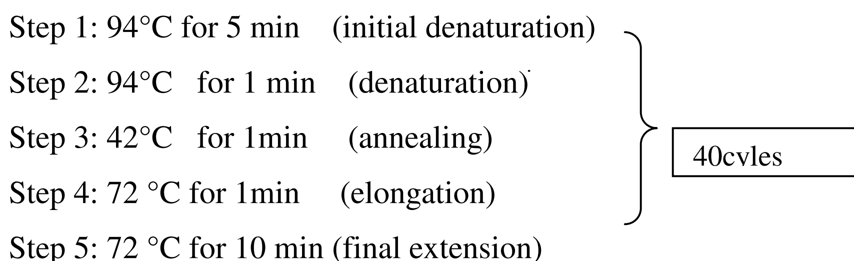
2.2.5.1.1. Amplification of 16S ITS rDNA

PCR amplification of 16S-ITS rDNA of the isolates was performed by using two DNA primers (Apendix F). The forward primer was complementary to the upstream of 16S rRNA gene and the reverse was complementary to the upstream of 23S rRNA gene.

Forward: 5. -AGAGTTTGATCCTGGCTCAG-3 (Mora et al., 1998).

Reverse: 5. -CAAGGCATCCACCGT-3 (Jensen et al., 1993).

Amplifications were performed in a Mini Cycler System. PCR conditions were as follows:



The final reaction volume was 50µl. PCR mix (Appendix E.1) was prepared after 2 µl -4µl of genomic DNA (approx. 500 µg) was placed into 1.5ml eppendorf tubes. During the preperation of the mix, tubes were kept on icebox. Taq DNA polymerase (1.25U) was added and mixed gently and then tubes were centrifuged for 2-3 s. The mix were added to each of the tube. Then 60 µl of mineral oil was added into the tubes while they were on ice. Tubes were centrifuged for 2-3 s then they were placed into the wells of Mini Cycler System (MJ Research INC, USA).

2.2.5.1.2. Electrophoresis of Amplified 16S-ITS rDNA Fragments

Amplified fragments were separated in 0.8 % agarose gel. First, 0.96 g of agarose was dissolved in 120 ml of 1×TAE buffer (Appendix C.4) by boiling (15 µl of ethidium bromide solution (10 mg/ml) were added after cooling the solution to 40°C). The gel was then poured into the gel-casting stand and the combs were placed. 1×TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. Five µl of PCR products were taken and mixed with 1 µl, 6 × gel-loading buffers. The samples were then loaded into the wells of agarose gel starting from the second well. Finally, 5 µl of DNA molecular weight marker were loaded into the first well and they

were electrophoresed at 40 mA until bromophenol blue have reached 2/3 of the gel. DNA fragments were visualized in a photodocumentation system (Vilber-Lourmat, France).

2.2.5.1.3. Chloroform Extraction of PCR Products

Final volumes of the PCR products were adjusted to 100 µl with 1×TE buffer. Then two volume of chloroform were added. After centrifugation for 2 min at 6000 rpm, organic phase was discarded and chloroform extraction was repeated. Then the tubes were centrifuged for 2 min at 6000 rpm. The aqueous phase was transferred into new eppendorf tubes containing 10 µl of 3 M sodium acetate (pH 5.2) and mixed well. After that, 450 µl of 99% ethanol were added and mixed. The samples were centrifuged for 15 min at 7000 rpm. Pellet was washed with 500 µl 70% ethanol. After centrifugation for 5 min at 6000 rpm, alcohol was removed. Pellet was dried for 10 min at 37°C, and they were dissolved in 25 µl 1xTE, and stored at 20°C.

2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP)

Purified PCR products were separated in 1% agarose gel in order to estimate DNA concentrations for each sample. Then 12.5µl of PCR products were aliquoted into new tubes for digestion. *Taq* I and *Hae* III were the enzymes chosen for this purpose. Reaction mix (Appendix F2) was added on PCR products while the tubes were stored on ice. 5 U of enzyme was used for each reaction. The final volume of the reaction was adjusted to 50 µl. Incubation period was 5 h for both of the enzymes. But the optimum reaction temperatures were 65°C for *Taq I* and 37°C for *Hae* III. Mineral oil was added over the reaction mixtures to avoid evaporations at 65°C. After the incubation DNA was extracted as described in section 2.2.6.1.3. and dissolved in 15 µl of 1xTE.

2.2.5.1.5. Electrophoresis of Restriction Fragments

Restriction fragments were first separated in 2 % agarose gel containing 20 µl of ethidium bromide solution (10mg/ml). Electrophoresis was carried out at 60 mA for the

first 20 min, then the current was adjusted to 80 mA and the electrophoresis was continued further for 2.5 h. Some isolates produced similar restriction fragment patterns. These were contained within distinct groups. One digested sample from each group was picked and re-electrophoresed in order to produce representative restriction patterns for further restriction profile analyses.

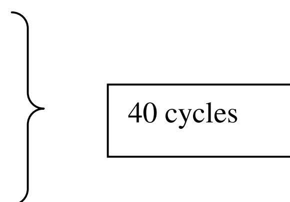
2.2.6 Sequencing

2.2.6.1. Amplification of 16SrRNA Gene

16SrDNA region of rRNA genes of the isolates were amplified using the following DNA oligoprimers for sequencing reactions
EGE 1. 5. - AGAGTTTGATCCTGGCTCAG –3 (Jensen *et al.,* 1993)
EGE 2. 5. - CTACGGCTACCTTGTTACGA –3 (Jensen *et al.,* 1993)

Two µl of genomic DNA (approx. 500 µg) were transferred into 0.5 ml eppendorf tubes, as the template. PCR mix was prepared and after the addition of *Taq* DNA polymerase (1.25 U), it was mixed gently and centrifuged for 2-3 s. Forty-eight ml of the mix were distributed into the tubes. All the manipulations were performed on ice. The tubes were then overlaid with 60µl mineral oil, centrifuged for 2-3 s at 6000 rpm. Finally, they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) using the following program:

Step1: 95°C for 5 min
Step2: 95°C for 1 min
Step3: 56°C for 1 min
Step4: 72°C for 1 min
Step4: 72°C for 10 min



2.2.6.2 Extraction of DNA Fragments from Agarose Gel

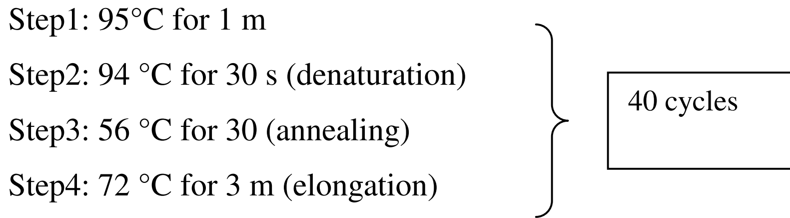
DNA isolation kit (Applichem A3421) was used for extraction of amplification products from agarose gel.

DNA bands (approximately 1600 bp) were excised from the agarose gel by a rasor blade. Three gel slice volume of 6 M NaI (for TAE gels) was added. The samples were incubated for 5 min at 55°C, mixed and continued for incubation for 2 min. Ten μl of well mixed glass powder suspension was then added, mixed well and incubated for 5 min at 55°C by occasional mixing. The samples were then centrifuged for 10 s at 10.000 rpm and the supernatant was discharged. The glass pellet was rinsed with 500 μl wash buffer. And resusupended by pipetting or gently flicking the tube. The samples were then centrifuged for 10 s at 10,000 rpm. The washing step was repeated twice. After the last wash, the supernatant was removed as much as possible. The pellet was suspended in 25 μl of 1xTE buffer. The samples were eluted for 5 min at 55°C with occasional mixing. Centrifugation was performed for 45 s at 10,000 rpm. The supernatant was finally transferred carefully into a new eppendorf tube. The eluted DNA samples were stored at -20°C until use.

2.2.6.3 Sequencing Reactions

The sequencing reactions were performed by using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit.

Four eppendorf tubes were firstly labeled as “A”, “C”, “G”, and “T” for respective termination reactions. Two μl of corresdonding Cy5.5 ddNTP termination mix were then dispensed into the appropriately labelled tubes. The tubes were then capped to prevent evaporation. The master mix (Appendix I) was prepared in an eppendorf tube for each template to be sequenced. The content of master mix was mixed thoroughly and 7μl of the mix was dispensed into each tube labelled “A”, “C”, “G”, and “T”. Each sequencing reaction was mixed thoroughly and overlaid with one drop of mineral oil. Each tube was capped and placed into the thermal cycler. The sequencing condition reastions were:



40 amplification cycles of Step 2, 3 and 4 were performed

The primer used was ;

EGE 1; 5. - AGAGTTTGATCCTGGCTCAG -3. (Jensen *et al.,* 1993)

2.2.6.4 Purification of Sequencing Reactions

2.2.6.4.1 Removal of unincorporated dye terminators using ethanol precipitation

The volume of the sample was adjusted to 50 µl by adding 1xTE. The samples under the mineral oil were then transferred into fresh eppendorf tubes. Twenty µl of 7.5 M ammonium acetate and 200 µl (3 times the reaction volume) of chilled 100% ethanol were added to each tube. The tubes were then mixed thoroughly by vortexing and placed on ice for 20 min to precipitate the DNA. Centrifugation was then performed at 12, 000 rpm for 30 min at 4°C. Supernatants were removed carefully and the pellets were washed with 200 µl of chilled 70% ethanol. After the centrifugation for 5 min, the supernatants were removed and the pellets were dried. Each pellet was then resuspended in 6 µl of formamide loading dye and vortexed vigorously (2-3 min) to ensure complete resuspension. Just prior to loading the samples onto gel, each sample was heated at 70 °C for 2-3 min for denaturation and then placed on ice. One and a half or 2µl of each sample were then loaded into the lanes of sequencing gel.

2.2.6.4.2 Assembling the SEQ Sequencer

The sequencing reactions were performed with SEQ personal sequencing system (Amercham Pharmacia Biotech). The system consists of SEQ personal sequencer, SEQ

Rapidset gel polymerizer, RapidGel QuickFill cassettes, RapidGel-XL-6% cartridge and filling gun and filling fixture.

Preparing a gel involves attaching the RapidGel-XL cartridge to the casting gun, filling the RapidGel cassettes, polymerizing the acyrlamide in the gel polymerizer. After the polymerization the comb was removed from the cassette. After cleaning the cassette, it was placed properly into the buffer chamber. At this step pre-run was performed to measure the backgraound redding. Once the gel cassette was aligned and the pre-run was complete, 1xTE buffer was loaded into the upper and lower buffer chambers until it reached the fill lines. The prepared samples (1-2 μl) were then loaded into the gel with a micropipette. Then the analysis was started. After the run was finished, data were analysed by SEQ software.

The sequences obteined were evaluated in and submitted to GenBank.

Chapter 3

RESULTS AND DISCUSSION

3.1. Isolation of Haloalkalophilic Bacteria

Haloalkalophilic bacteria were isolated from Menemen Region. Target places were leather factories and the wastes of this industry. Therefore the samples were taken from both the processing steps and waste.

3.2. Determination of Phenotypic Characteristics

3.2.1. Gram staining

The modified Gram staining method specific for halophilic bacteria gave desired results with these isolates. All the isolates were gram positive, rod shaped except one cocci (C1).

3.2.2. Examination of Endospore

Isolates were grown on agar plates (Appendix) for 5 days. Isolates were non-sporforming.

3.2.3. Catalase and oxidase tests

All the isolates were catalase and oxsidase positive.

3.2.4. Physiological Tests

Growth at pH 7 was observed to determine whether the isolates were obligate alkalophilic (Table 3.2). Four isolates could not grow at this pH value. Twenty-five of the isolates were alkali tolerant and could not grow at pH 12. Isolates were not thermotolerant since they could not grow at 45ºC and 50ºC on alkaline nutrient agar plates.

Table 3.1. Results of the Physiological Test

Physiologic test	Isolate number
No Growth at 20%NaCl	D2, D10, D20. D26, E4, E16, E24, F22
No Growth at PH 7	D1, D20, F9, F21
No Growth at 0%NaCl pH 10	D2, D6, D18, D23, E4, E43, F5, F7, F29
No Growth at pH 12	D1, D6, D10, D18, D20, D23, D24, D30, D32, E2, E4, E10, E12, E14, E16, E23, E24, E36, F7, F10, F13, F16, F20, F22, F29,

Growth of the isolates was also observed at 0% and 20% NaCl concentrations. Nine of the isolates were oblitely halophilic. Eight of the isolates were not extremely halophilic.

Table3.2. Differentiation of Isolates according to growth at different NaCl concentrations and pH ranges.

İsolate Name	20% NaCl	PH 7	0%NaCl pH 10	pH 12
D1	+	-	+	-
D2	-	+	-	+
D5	+	+	+	+
D6	+	+	-	-
D7	+	+	+	+
D10	-	+	+	-
D11	+	+	+	+
D12	+	+	+	+
D14	+	+	+	+
D18	+	+	-	-
D20	-	-	+	-
D23	+	+	-	-
D24	+	+	+	-
D25	+	+	+	+
D26	-	+	+	+
D29	+	+	+	+
D30	+	+	+	-
D31	+	+	+	+
D32	+	+	+	-
E1	+	+	+	+
E2	+	+	+	-
E3	+	+	+	+
E4	-	+	-	-
E6	+	+	+	+
E7	+	+	+	+
E9	+	+	+	+
E10	+	+	+	-
E12	+	+	+	-
E13	+	+	+	+
E14	+	+	+	-
E15	+	+	+	+

E16	-	+	+	-
E17	+	+	+	+
E20	+	+	+	+
E23	+	+	+	-
E24	-	+	+	-
E25	+	+	+	+
E26	+	+	+	+
E27	+	+	+	+
E28	+	+	+	+
E29	+	+	+	+
E30	+	+	+	+
E33	+	+	+	+
E34	+	+	+	+
E35	+	+	+	+
E36	+	+	+	-
E39	+	+	+	+
E42	+	+	+	+
E43	+	+	-	+
E47	+	+	+	+
F1	+	+	+	+
F2	+	+	+	+
F3	+	+	+	+
F4	+	+	+	+
F5	+	+	-	+
F7	+	+	-	-
F8	+	+	+	+
F9	+	-	+	+
F10	+	+	+	-
F11	+	+	+	+
F12	+	+	+	+
F13	+	+	+	-
F14	+	+	+	+
F15	+	+	+	+
F16	+	+	+	-
F17	+	+	+	+
F18	+	+	+	+

F19	+	+	+	+
F20	+	+	+	-
F21	+	-	+	+
F22	-	+	+	-
F23	+	+	+	+
F24	+	+	+	+
F25	+	+	+	+
F26	+	+	+	+
F27	+	+	+	+
F28	+	+	+	+
F29	+	+	-	-
F30	+	+	+	+
F32	+	+	+	+
F34	+	+	+	+
F35	+	+	+	+

3.3. Extracellular Enzyme Screening

Extracellular enzyme screening was performed on solid agar plates by using different substrates for each enzyme activity. Protease screening based on the observation of clear zones around the colonies (Figure). Lipase activity gave opaque zones around the colonies (Figure). Different NaCI concentratios were used for lipase screening. The enzyme screening studies were repeated performed twice.

3.3.1 Extracellular Enzyme Profiles of the Isolates

Table 3.3 Extracellular Enzyme Tests

Extracellular Enzyme Type	Isolate Name
Did not produce lipase	D5, D10, D20, D26 D31, E23, E43, F11, F29 F33
Utilized Tween80 at 0% NaCl	D1, D2, D30, F25, F15
Utilized Tween 20 at 12% NaCl	D23, D24, D25, D30, E1, E2, E3, E42, F5, F12, F13, F14, F15, F16, F18, F23, F25
Did not Utilized Tween 20	D7, E7, E9, E10, E13, E14, E15, E16, E23, E24, E25, E26, E27, E28, E29, E30, E39, E47, F26, F27, F31
Did not utilase Tween80	D12, E12, E34, F1, F20, F30, F32,
Did not produce lipase without NaCI	D12, D14, E12, E34, F1, F30, F32
Utilase casein	F17, D5, F29, D32, E34, F1, F15, E36, D24, F10, F13, F12, E20, F32, E1, E47, E33, E14, E25, E42, F22

Table 3.4 Extracellular Enzyme Profiles of isolates

Isolate Name	Protease	0%NaCl Tween20	12% NaCl Tween20	20% NaCl Tween20	0% NaCl Tween80	12% NaCl Tween80	20% NaCl Tween 80
D1	-	-	-	-	+	+	-
D2	-	-	-	+	+	-	-
D5	+	-	-	-	-	-	-
D6	-	+	-	-	-	+	-
D7	-	-	-	-	-	+	-
D10	-	-	-	-	-	-	-
D11	-	+	-	+	-	+	+
D12	-	+	-	-	-	-	-
D14	-	+	-	-	-	-	-
D18	-	+	-	+	-	-	+
D20	-	-	-	-	-	-	-
D23	-	+	+	-	-	+	+
D24	+	+	+	-	-	+	+
D25	-	+	+	-	-	-	+
D26	-	-	-	-	-	-	-
D29	-	+	-	-	-	+	+
D30	-	+	+	+	+	+	+
D31	-	-	-	-	-	-	-
D32	+	-	-	-	-	+	+
E1	+	+	+	-	-	+	+
E2	-	+	+	-	-	+	
E3	-	+	+	-	-	+	+
E4	-	+	-	-	-	+	-
E6	-	+	-	-	-	+	+
E7	-	-	-	-	-	+	-
E9	-	-	-	-	-	+	-
E10	-	-	-	-	-	-	+
E12	-	+	-	-	-	-	-
E13	-	-	-	-	-	+	-
E14	+	-	-	-	-	+	-
E15	-	-	-	-	-	+	-
E16	-	-	-	-	-	+	-
E17	-	-	-	-	-	+	-
E20	+	+	-	-	-	+	+
E23	-	-	-	-	-	+	-
E24	-	-	-	-	-	+	-
E25	+	-	-	-	-	+	-
E26	-	-	-	-	-	+	-
E27	-	-	-	-	-	+	-
E28	-	-	-	-	-	+	-
E29	-	-	-	-	-	+	-
E30	-	-	-	-	-	-	+
E33	+	-	-	+	-	+	+
E34	+	+	-	-	-	-	-
E35	-	-	-	+	-	-	+

E36	+	+	-	-	-	+	+
E39	-	-	-	-	-	+	-
E42	+	+	+	-	-	-	+
E43	-	-	-	-	-	-	-
E47	+	-	-	-	-	-	+
F1	+	+	-	-	-	-	-
F2	-	+	-	+	-	+	+
F3	-	+	-	+	-	+	-
F4	-	+	-	-	-	+	+
F5	-	+	+	+	-	+	+
F7	-	+	-	+	-	+	+
F8	-	+	-	-	-	+	+
F9	-	+	-	-	-	-	+
F10	+	+	-	+	-	+	+
F11	-	-	-	-	-	-	-
F12	+	+	+	+	-	+	+
F13	+	+	+	-	-	+	+
F14	-	-	+	-	-	+	+
F15	+	+	+	+	+	-	+
F16	-	+	+	-	-	+	+
F17	+	+	-	-	-	+	+
F18	-	-	+	-	-	+	+
F19	-	+	-	-	-	+	+
F20	-	+	-	+	-	-	-
F21	-	+	-	-	-	+	-
F22	+	+	-	-	-	+	-
F23	-	+	+	+	-	+	+
F24	-	-	-	-	-	-	+
F25	-	+	+	+	+	+	+
F26	-	-	-	-	-	+	+
F27	-	-	-	-	-	-	+
F28	-	+	-	-	-	-	+
F29	+	-	-	-	-	-	-
F30	-	+	-	-	-	-	-
F32	+	+	-	-	-		-
F35	-	-	-	-	-	-	-
F34	-		-	-	-	-	-

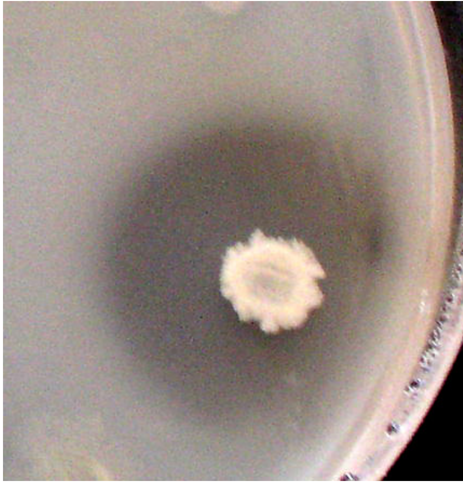


Figure 3.1. Apperance of extracellular protease

Figure 3.2. Apperance of extracellular lipase

3.4. Genotypic Characterization

3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon

The amplification product size differed between 1800 and 2000 bp (Figure)

.

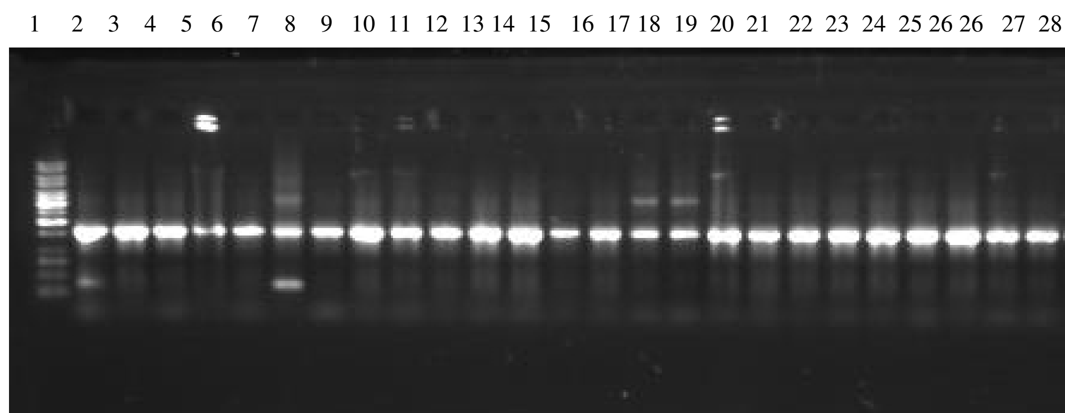


Figure 3.3. 16S rDNA-ITS Amplification products of some isolates. Lanes 1. 1 kb DNA Lac D1, 3. Isolate F2, 4. Isolate F1 5. Isolate D8, 6. Isolate D10, 7. Isolate 12,8. Is Isolate E15, 10. Isolate E17, 11. Isolate E19, 12. Isolate D23 lane 13. Isolate D24

3.4.2. 16S-ITS rDNA - RFLP Profiling

In order to identify the isolates amplification products of 16S-ITS rDNA region was digested with restriction endonucleases. *Taq* I and *Hae* III endonucleases were chosen for digestion. Digestion products were first run in 2% agarose gel to see all different profiles and to make groups within the digestion products. For a specific RFLP group, one representative digestion product was chosen and electrophoresed together in 2.5% agarose gel (Figure 3.3).

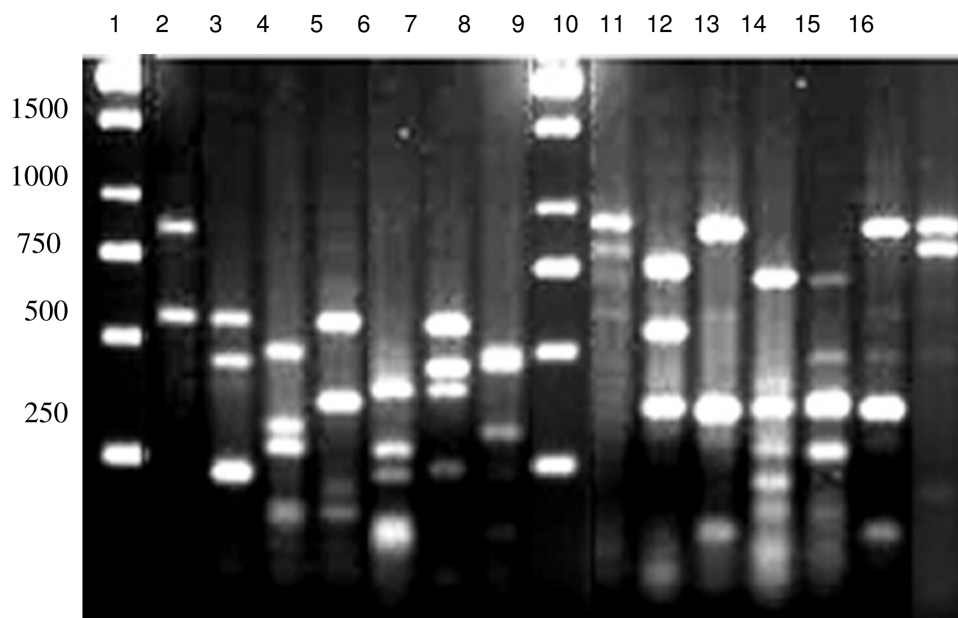


Figure 3.4 16S-ITS rDNA RFLP profiles of *Hae* III groups. Lanes 1. 1 kb DNA ladder, 2. Isolate D5, 3. Isolate D7, 4. Isolate D30, 5. Isolate D12, 6. Isolate D23, 7. Isolate F28, 8. Isolate, F21 16S-ITS rDNA RFLP profiles of *Taq*I groups. Lanes 9. 1 kb DNA ladder,10. Isolate D2, 11. Isolate D18, 12. Isolate D12, 13. Isolate F6, 14. Isolate F20, 15. Isolate F28, 16. IsolateE30,

Resulting gel profiles were analyzed using the program BIO-1D++. Dendrogram of representative strains were carried out using 15% homology coefficient. (Figure 3.3 and 3.4). Fragment sizes were given in Table 3.6.

Hae III and *Taq* I restriction analysis of 16S-ITS rDNA fragments of strains revealed 7 distinct groups (Table 3.3.).

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2000	854	546	465	546	384	546	465	2000	886	673	808	661	361	838	870
1500	555	440	309	354	257	440	432	1473		509	338	354	338	338	778
1000		219	272	125	211	377	294	934		354	78	265	257	78	
750			125		94	219		685				195			
500					70			465				133			
250								227				31			

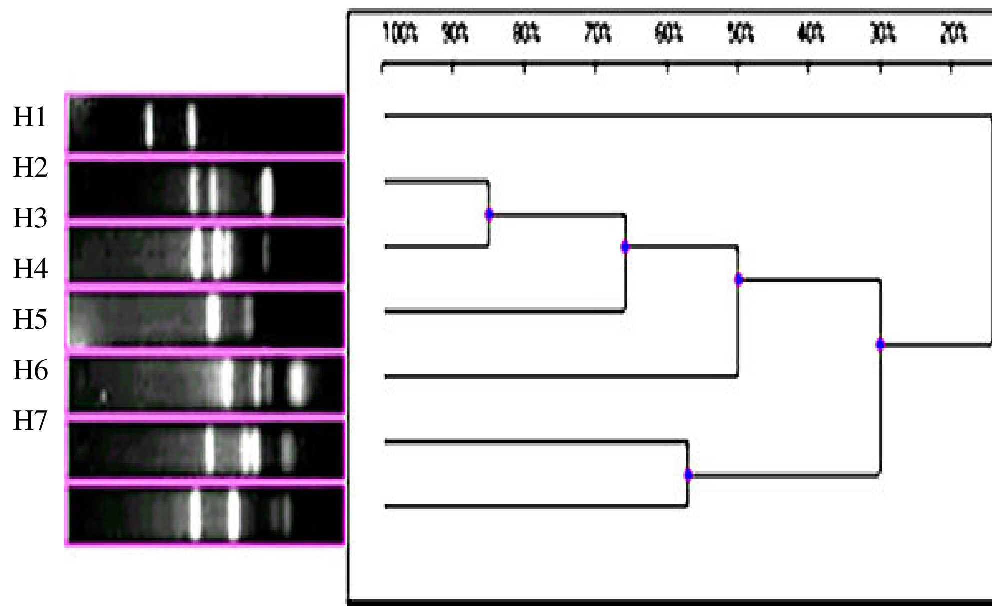


Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles.

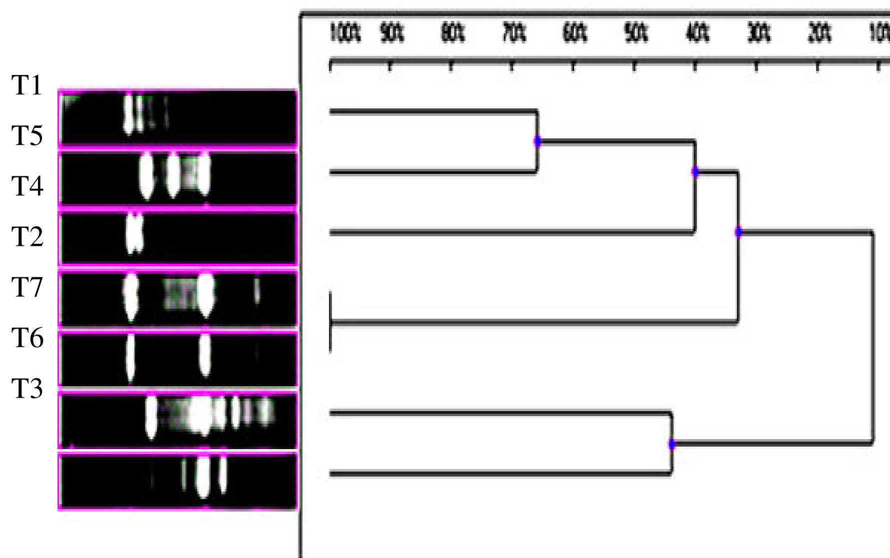


Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles.

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups.

Genotypic groups	Name of the Isolates
HT1	D1, D2, D5, E43,E47,F1
HT2	D6, D7, D10, D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E30, E34, E35
HT3	D12, F32
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E20, E33, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F19, F22, F23, F24, F25, F26, F27
HT5	D30
HT6	F20, F21
HT7	F28

The use of two restriction enzymes enabled the revelation of diversity among the isolates. When the results of both digestion were combined, 7 distinct genotypes were obtained (Table 3.10). The results indicated that isolate group members were differentiated by each restriction enzyme, were the same.

In this study, 16S ITS and rDNA regions were used as a single amplicon for RFLP analysis. No report was found in literature on genotypic characterization of haloalkalophiles using this method. Similar studies have been performed in this institute for genotypic characterization of alkalophilic bacilli (Akbalik, 2004), thermophilic bacilli, (Yavuz, 2004), lactic acid bacteria (Yavuz, 2003, Bulut, 2003.

3.4.3. Analysis of the Genotypic and Phenotypic Groups

Table 3.7. Analysis of the Genotypic and Phenotypic Groups

	Isolate number	20%NaCI	pH 7	0%NaCl	pH 12	Protease	0%NaCl Tween 20	12%NaCl Tween 20	20%NaCl Tween 20	0%NaCl Tween 80
HT1	D1,D2,D5,E34, E43, E47, F1	%80	80%	70%	80%	40%	0%20	0%	20%	%30
HT2	D6,D7,D10,D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E39, F29, F30, F34, F35,	100%	100%	100%	100%	10%	10%	0%	0%	0%
HT3	D12, F32	100%	100%	100%	100%	50%	100%	0%	0%	0%
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E30, E33, E35, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18, F19, F22, F23, F24, F25, F26, F27	90%	100%	90%	70%	30%	90%	40%	30%	20%
HT5	D30	100%	100%	100%	0%	0%	100%	100%	100%	100%
HT6	F20, F21	100%	50%	100%	50%	0%	100%	0%	50%	0%
HT7	F28	100 %	100%	100%	100%	0%	100%	0%	0%	0%

Phenotypic characterization of the isolates indicated that strains of different species had different phenotypic features. When the genotypic results were compared with the results of enzyme screening and phsyical tests, a correlation between a few groups were observed. Most of the isolates produced lipase (Tween 20 and Tween 80) at all NaCI concentrations used grouped in HT4. All members of HT2, HT3, HT5, HT6, HT7 were also able to grow in the presence of 20% NaCI. Most of the isolates that could not grow at pH7 were placed in HT6. Isolates that could not grow at pH12 were placed in HT1, HT5, and HT6.

3.4.4 Analysis of Sequencing Reactions

16S partial sequencing results of six representative isolates are as listed below.

Table 3.8. Sequencing results

Genotypic Group	Isolate Name-Accession Number	Length of bp.-Similarity	Accession Number - Organism	Name of the Family
HT2	D7-AY604872	-89%	*AY345480.1-Bacterium K-29*	Unclassified *Bacillaceae*
HT3	D12-AY601901	716-93%	AF237976.1-*Salinococcus roseus*	Unclassified *Bacillaceae*
HT4	D23-AY601902	323-92%	AJ640133.1-*Halomonas* sp.18bAG	*Halomonadaceae*
HT5	D30-AY601903	-92%	AY383044.1-*Halomonas meridiana*	*Halomonadaceae*
HT6	F20 (C1)-AY604871	-89%	AJ29397-*Nestenkonia* sp.EL-30	*Micrococcaceae*
HT7	F28 (15B)-AY601904	-91%	AY3091336-Bacterium K-2	Unclassified *Bacillaceae*

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, haloalkalophilic bacteria were isolated from samples of waste soil and leather processing. They were first characterized phenotypically (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope and extracellular enzyme screening, physiological tests such as growth at pH 7 and pH 12 , different NaCl concentrations and at different temperatures). Extracellular enzyme screening was performed on solid agar media. Molecular characterization was performed by using 16S-ITS rDNA RFLP.

All the isolated strains were Gram positive but they were not sporeforming. All the strains were catalase positive. Most of the isolates contained oxidase. Four strains could not grow at pH7. They were thus obligately alkalophilic. Twentyfive isolated strain were alkalitolerant because they could not grow at pH 12. Nine isolated strain could not grow with in 0% NaCl they were thus obligately halophilic. Eight of the isolates could not grow at 20% NaCl concentration.

Extracellular enzyme screening resulted in 21 protease and 72 lipase activity. Isolates produced protease were grouped in HT1, HT2, HT3, HT4.Different concentrations of NaCl were used for lipase screening. Seventeen of the isolated strains which could utilize Tween 20 in the presence of 12% NaCI were all grouped in HT4. Isolated strains, which could not utilize T20, were grouped in HT2 except E47, F26, and F27. Seven of the isolated strains were not produce lipase in the presence of NaCl were grouped in HT1, HT2 and HT3.

For restriction analysis of 16S-ITS rDNA fragments of isolated strains, two restriction enzymes, *Taq* I and Hae III, were used. Both enzymes produced similar RFLP groups. Representative group members were subjected to 16S partial sequencing. They had high similarity with *Bacillaceae*, *Halomonadaceae* and *Micrococcaceae* families.

Characterization of the extracellular enzymes from different isolated strains is the essential work for these Haloalkalophilic bacteria because of their unique futures.

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APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Experiments

NO	CHEMICAL	CODE
1	Agar Merck	1.01613
2	Bacteriological pepton Oxoid	LP037
3	D-Glucose AppliChem	A3666
4	Yeast Extract Merck	1.03753
5	Skimmed milk LabM	MC27
6	Glycerol AppliChem	A2926
7	NaCI AppliChem	A2942
8	K2HPO4 AppliChem	A2945
9	MgSO4.7H2O Merck	1.05886
10	Tween80 Applichem	A1390
11	Ammonium sulfate Applichem	A3485
12	Nutrient broth Merck	1.05443
13	Sodium carbonate Merck 1.06392	
14	KH2PO4 Merck	1.04871
15	Disodium hydrogen phosphate Applichem	A2943
16	Immersion oil Applichem	A0699
17	Calcium chloride Applichem	A3652
18	Crystal violet Sigma	C3886
19	Safranin Merck	1.15948
20	N,N,N.,N.- Tetramethyl-p-phenylenediamine Sigma	T3134
21	Congo Red Sigma	C6767
22	Potassium Iodide Sigma	P8256
23	Tris Base Sigma	T6066
24	EDTA AppliChem	A2937
24	Isopropanol AppliChem	A3928
25	Proteinase K AppliChem	A3830
24	Ethidium bromide AppliChem	A1151
25	Ethanol AppliChem	A3678
26	Taq DNA polymerase Promega	M1865
27	Primers: Ege 1 and L1 Promega	
28	dNTP set MBI, Fermentas,	R0181
29	Standard agarose (low electroendoosmosis) AppliChem	A2114
30	Chloroform AppliChem	A3633
31	Isoamyl alcohol AppliChem	A2610
32	Bromophenol blue Merck	1.08122
33	Boric acid AppliChem	A2940

APPENDIX B

RECIPIES FOR MEDIA USED IN EXPERIMENTS

B.1 HORIKOSHI BROTH AND AGAR

HORIKOSHI-I BROTH

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water. Medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

HORIKOSHI-I AGAR (pH=10.2)

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

B.2 NUTRIENT AGAR (pH:10.3) USED FOR GROWTH TESTS AT DIFFERENT TEMPERATURES AND SODIUM CHLORIDE CONCENTRATIONS

Nutrient Broth	8
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 was sterilized separately and added into medium.

B.5 MEDIUM USED FOR LIPASE SCREENING (pH: 10.3)

	g/l
Nutrient Broth	8
$CaCl_2.H_2O$	0.1
Agar	13
Tween20/Tween80	10ml
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 and Tween20/80 was sterilized separately and added into medium.

APPENDIX C

BUFFERS AND STOCK SOLUTIONS

C.1 ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

Ethidium bromide (0.5 g) was dissolved in 50 ml of deionized water.

C.2 CHLOROFORM-ISOAMYL ALCOHOL SOLUTION (24:1)

Forty eight milliliters of chloroform was mixed with 2 ml of isoamyl alcohol.

C.3 50 X TAE

Two hundred and fifty-two grams Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

C.4 1XTAE

Twenty milliliters of 50X TAE buffer was taken and the volume was adjusted to1000 ml with deionized water to obtain 1 liter 1X TAE buffer.

C.5 1X TE BUFFER

10 mM Tris pH 8.0
1 mM EDTA pH 8.0

C.6 10 X TBE

One hundred and eight grams Tris Base and 55 g boric acid were weighed. They

were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was added. The volume was brought to 1000 ml with deionized water.

C.7 1X TBE

Hundred milliliters, 10X TBE was taken and the volume was brought to 1000 ml with deionized water to obtain 1X TBE buffer.

C.8 CTAB/ NaCl (10% CTAB in 0,7 M NaCl)

NaCl (4.1 g) was dissolved in 80 ml water and CTAB was added slowly while heating and stirring. Final volume was adjusted to 100ml.

APPENDIX D

STAINS AND INDICATORS

D.1. BROMTYMOL BLUE SOLUTION

Bromtymol blue	1 g
N/10 NaOH	25 ml
Deionized water	475 ml

D.2. SOLUTIONS FOR GRAM STAINING

D.2.1. CRYSTAL VIOLET STAINING REAGENT

Solution A

Crystal violet	2g
Ethanol (95%)	20ml 83

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

D.2.2. IODINE SOLUTION

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Iodine and potassium iodide were grinded in a mortar, water was added slowly with continuous grinding until the iodine was dissolved. The solution was stored in amber bottle.

D.2.3. SAFRANIN SOLUTION

Safranin (2.5% in 95% alcohol)	10ml
Distilled water	100ml

APPENDIX E

PCR RECIPIES

E.1. PCR MIX

Mg free Taq DNA polymerase buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Sterile deionized water	32 µl
Oligo forward (10 picomole/µl)	1 µl
Oligo reverse (10 picomole/ µl)	1 µl
dNTP (2mM each) 10X	5 µl

E.2. 6X GEL LOADING BUFFER (20 ML)

10x TBE	2 ml
Glycerol	6 ml
Deionized water	12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

E.3. dNTP (10X)

Ten microliters of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at .20 °C.

E.4. RESTRICTION ENZYME MIX

Restriction enzyme buffer	5 µl
DNA	8 µl
Restriction enzyme (10U/ µl)	1.5 µl
Sterile deionized water	35.5 µl

APPENDIX F

OLIGONUCLEOTIDE PRIMERS

L1: 5.- CAAGGCATCCACCGT -3.

Three hundred and fifty micrograms primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliters of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

EGE 1 5.- AGAGTTTGATCCTGGCTCAG -3.

Five hundred and ninty micrograms primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain final concentration of 2 µg / µl stock solutions. Five microliters of stock solution were then taken and mixed with 95 µl sterile deionized water. Therefore, 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

APPENDIX G

RECOGNITION SITES OF RESTRICTION ENZYMES

G1. ***Taq*** **I**

5.- T▼CG A -3.
5.- A GC ▲T -3.

G2. ***Hae*** **III**

5.-GG▼CC-3.
5.-CC▲GG-3.

APPENDIX H

ISOLATE NAMES

DI- C12a	F26- Ü4z
D2- Ş6a	F27- Ü6a
D5- E15	F28- 15b
D6-C15a	F29- F1
D7- 20a	F30- 24a
D10-19a	F31- 27b
D11- B5c	F32- B4c
D12- B1a	F33- T1b
D14- 23a	F34- E6z
D18- C4	F35- B8a
D20- B8b0	E42- C8
D22- H2	E43- C14a
D23- H1a	E47- C19y
D24- P5z	F1- E11x
D25- C17	F2- F2x
D26- 2b	F3- F2y
D29- D3c	F4- F2z
D30- B9	F5- G5
D31- A9	F6- G8
D32- D2b	F7- H3x
E1- A2	F8- H3y
E2- P2y	F9- P1x
E3- C6	F10- P4a
E4- C2a	F11- P5y
E6- G9a	F12- S1
E7- 3a	F13- S2
E9-24a	F14- Ş1

E10- 13b	F15- V3
E12- 29c	F16- A1
E13- 22c	F17- A4
E14- 12c	F18- A6
E15- 27c	F19- A7
E16- 1a	F20- B10
E17- 2d	F21- C1
E20- B2	F22- C2
E23- B8c	F23- C3
E24- 18b	F24- C24
E25- 11a	F25- C10

ISOLATION OF HALOALKALIPHILIC MICROORGANISMS FROM LEATHER INDUSTRY

Hatice Sevgi ÇOBAN

October, 2004

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By

Hatice Sevgi ÇOBAN

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ABSTRACT

Haloalkalophiles are extremophilic microorganisms that are adapted to saline and alkaline conditions. Different species of them have been isolated so far from soda lakes and soil samples. Haloalkalophilic microorganisms have significant adaptive mechanisms to avoid denaturing effect of salts and to balance their interior pH. Extracellular enzymes that are produced by these halophilic and alkalophilic microorganism are applicable for industrial purposes. Therefore isolation of these organisms from their habitats and study on genotypic characterization constitute initial steps for furter biotechnological studies.

In this study, processing steps of leather factories and their wastewater were chosen for sampling. In order to isolate target microorganisms Horikoshi-I medium including 12% NaCl was used. After isolation microorganisms were purified. Phenotypic tests were applied (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope, sensitivity to antibiotics and extracellular enzyme screenings). For genotypic characterization, genomic DNA was isolated and 16S-ITS rDNA region was amplified.

Differentiation was achieved according to Restriction Fragment Length Polymorphism (RFLP) method by using *Hae* III and *Taq* I endonucleases. Isolates, which represented the different RFLP patterns, were chosen for building up the haplotype groups.

As a result of the study seven different RFLP haplotypes were identified. Moreover, 16S ribosomal DNA partial sequencing was also performed on some of the strains in. These haloalkalophilic microorganisms and their enzymes could be used in different biotechnological studies in the future for various industrial applications.

ÖZ

Haloalkalofilik mikroorganizmalar yüksek alkali ve tuzlu koşullara adapte olmuş ekstremofilik canlılardır. Birçok farklı tür toprak ve soda gölü örneklerinden izole edilmiştir. Haloalkalofilik mikroorganizmalar tuzun zararlı etkilerin ve hücre içi pH değerlerini düzenleme yeteneğine sahiptirler. Halofilik ve alkalofilik olan bu organizmaların ürettiği ekstraselüler enzimler endüstride farklı amaçlar için kullanılanılabilir. Haloalkalofilik mikroorganizmaların yaşadıkları habitatlardan izole edilmeleri ve genotipik tanımlamalarının yapılması endüstri alanında kullanım amaçlı olarak genetik potansiyellerinin tanınması ve ileride yapılacak biyoteknolojik çalışmalar adına atılacak ilk adımı oluşturmaktadır.

Deri fabrikaları işlem basamakları ve atık suyu bu çalışma için örnekleme alanı olarak belirlenmiştir. Bu amaç için Horikoshi-I besi yerine %12 tuz ilave edilerek kullanılmıştır. İzolasyon aşamasının ardından organizmaların saflaştırma işlemi tamamlanmıştır. Fenotipik testler uygulanmıştır. Bunlar;Gram boyama, katalaz ve oksidaz testi, faz-kontrast mikroskobu ile spor gözlemi, ekstraselüler enzim taraması, farklı tuz konsantrasyonlarında (%0, %20, %25) gelişme, farklı sıcaklılarda (45-50 °C) gelişme, farklı pH (7-10-12) değerlerinde gelişme özellikleri). Genotipik karakterizasyon için genomik DNA izolasyonunun ardından 16S-ITS rDNA amplifikasyonu yapılmıştır. RFLP için iki farklı restriksiyon enzimi kullanıldı (*Hae* III and *Taq* I). Kesim ürünlerinin analizi yapılmıştır. Farklı jel profillerine sahip olanların oluşturduğu gruplar her iki enzim için de belirlendikten sonra grup temsilcileri kullanılarak dendogramlar oluşturulmuştur. Hae III restiriksiyon enzim kesimi sonucunda elde edilen grup temsilcilerinin 16S rDNA - PCR amplifikasyon ürünleri kısmi dizi analizleri için kullanıldı.

Haloalkalofilik mikroorganizmalar ve enzimleri daha ileride yapılacak biyoteknolojik çalışmalarda kullanılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xi

LIST OF ABREVIATIONS xii

Chapter1 INTRODUCTION 1

1.1.Definition of Haloalkalophiles 1

1.2.Haloalkalophilic Microorganisms 1

1.2.1. Eubacterial Haloalkalophiliphiles 2

1.2.2. Haloalkalophilic Bacillus 2

1.2.3. Gram NegativeHaloalkalophilicSpecies 3

1.2.4. Archaeal Haloalkalophilic Species 3

1.2.5. Distribution and Isolation 5

1.2.6. Biotechnological and Industrial Applications 5

1.2.7. Extracellular Enzymes and Their Industrial Application 6

1.2.8. Proteases from Haloalkalophilic Bacteria 6

1.2.9. Lipases from Haloalkalophiles 8

1.3. Identification of the Haloalkaolphilic Organisms 9

1.3.1. Phenotypic Methods 9

1.3.2. Molecular Characterization Methods 10

1.3.2.1. Pulsed Field Gel Electrophoresis 10

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD) 11

1.3.2.3. 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP 11

1.3.2.4. Plasmid Profiling 12

1.3.2.5. Ribotyping 12

1.3.2.6. DNA Sequencing 15

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir) 15
1.5.Thesis Objective 16

Chapter 2 MATERIAL METHODS 17
2.1. Materials 17
2.1.1. Chemicals 17
2.1.2. Samples used for isolation 17
2.2. Methods 17
2.2.1. Isolation and Preservation of the Isolates 17
2.2.1.2. Isolation of Haloalkalophilic Bacteria 17
2.2.1.3. Preservation and Activation of Isolates 18
2.2.2. Determination of Phenotypic Characteristics 18
2.2.2.1. Gram Staining 18
2.2.2.2. Examination of Endospores 19
2.2.2.3. Catalase Test 19
2.2.2.4. Oxidase Test 19
2.2.2.5. Growth at Different Temperatures 19
2.2.2.6. Growth at Different NaCl Concentrations 19
2.2.2.7. Growth at Different pH Ranges 20
2.2.3. Screening for Extracellular Enzymes 20
2.2.3.1. Screening for Protease Activity 20
2.2.3.2. Screening for Lipase Activity 20
2.2.4. Preparation of Genomic DNA 20
2.2.5. Genotypic Characterization 21
2.2.5.1. Identification of Isolates by 16SrDNA–ITS Region 21
2.2.5.1.1. Amplification of 16S rDNA – ITS Region 21
2.2.5.1.2. Electrophoresis of Amplified 16S-ITS Fragments 22
2.2.5.1.3. Chloroform Extraction of PCR Products 23
2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP) 23

2.2.5.1.5. Electrophoresis of Restriction Fragments 23
2.2.6. Sequencing 24
2.2.6.1. Amplification of 16SrRNA Gene 24
2.2.6.2 Extraction of DNA Fragments from Agarose Gel 25
2.2.6.3 Sequencing Reactions 24
2.2.6.4. Purification of Sequencing Reactions 25
2.2.6.4.1. Removal of unincorporated dye terminators using ethanol presipitation 26
2.2.6.4.2. Assembling the SEQ Sequencer 26

Chapter 3 RESULTS AND DISCUSSION 28
3.1. Isolation of Haloalkalophilic Bacteria 28
3.2. Determination of Phenotypic Characteristics 28
3.2.1. Gram staining 28
3.2.2. Examination of Endospore 28
3.2.3. Catalase and oxidase tests 28
3.2.4. Physiological Tests 29
3.3.Extracellular Enzyme Screening 32
3.3.1. Extracellular Enzyme Profiles of Isolates 33
3.4. Genotypic Characterization 36
3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon 36
3.4.2. 16S-ITS rDNA Based RFLP Profiling 37
3.4.3. Analysis of the Genotypic and Phenotypic Groups 40
3.4.4 Analysis of Sequencing Reactions 41

Chapter 4 CONCLUSION AND FUTURE PERSPECTIVE 42
REFERENCES 43
APENDICES AA1
APENDIX A. CHEMICHALS USED AA1
APENDIX B. RECIPIES MEDIA USED FOR EXPERIMENTS AB1
APPENDIX C. BUFFERS AND STOCK SOLUTIONS AC1
APPENDIX D. STAINS AND INDICATORS AD1

APPENDIX E. PCR RECIPIES AE1
APPENDIX F. OLIGONUCLEOTIDE PRIMERS.. AF1
APPENDIX G. RECOGNITION SITES OF RESTRICTION ENZYMES................AG1
APPENDIX H. ISOLATE NAMES AH1

LIST OF FIGURES

Figure 1.1. Archaea kingdom 4

Figure1.2. Rybotyping 14

Figure3.1. Apperance of extracellular protease 36

Figure 3.2. Apperance of extracellular lipase 36

Figure 3.3. 16S rDNA-ITS Amplification products of some isolates 36

Figure 3.4. *Hae* III and *Taq* I Digests of 16S-ITS rDNA Fragments of Representative Isolates 37

Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles of Isolates 38

Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles of Isolates 38

LIST OF TABLES

Table 1.1. Tolerance for pH, NaCl of selected species of the genus Bacillus 2

Table 1.2. "Natro group" of Halobacteria 5

Table 1.3. Detergent Proteases 7

Table.1.4. Lipolytic activity 9

Table 2.1. Samples used for isolation 17

Table 3.1. Results of the Physiologic test 29

Table.3.2. Tolerance for pH, NaCl of isolated strains 30

Table 3.3 Extracellular Enzyme Tests 33

Table 3.4 Extracellular Enzyme Profiles of isolates 34

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA 38

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups 39

Table 3.7. Analysis of the Genotypic and Phenotypic Groups 40

Table 3.8. Sequencing Results 41

ABBREVIATIONS

A	Adenine
bp	Base pair
C	Cytosine
CGT	Cyclodextrin Glucosyltransferase
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
G	Guanine
EDTA	Ethylenediamine Tetra Acetic Acid
ITS	Internal Transcribed Spacer
ISR	Internal Spacer Region
kb	Kilo base
PAGE	Polyacrylamide Gel Electrophoresis
P.E.	Pectinolytic Enzyme
PGA	Polygalacturonic Acid
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
T	Thymine
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA

CHAPTER 1

INTRODUCTION

1.1. Definition of Haloalkalophiles

Haloalkalophiles are one of the main physiological groups of microorganisms classified in alkaliphiles. Lake Magadii in Kenya, Wadi Natrun in Egypt have been defined as the most stable alkaline environments on earth. Therefore the first ecological studies related to haloalkalophilic microorganisms have been performed in these places. The conditions of high mineralization and/or alkalinity in such environments regulate their prokaryotic communities (Trotsenko and Khmelenia, 2001). Both alkalinity (pH 9) and salinity up to saturation (33% (wt/vol)) are needed for the survival of these microorganisms (Koki Horikoshi, 1999).

Alkaliphiles can be classified as obligately and facultatively alkaliphilic organisms. Obligately alkaliphilic microorganisms cannot grow below pH 9. Facultatively alkaliphilic organisms show optimal growth at pH 10.0 or above but they also have the ability to grow at neutral pH.

Halophilicity divided the organisms into three categories: slightly halophilic, moderately halophilic and extremely halophilic. Halophilic microorganisms require certain concentrations of NaCI for their growth, while the halotolerant ones have the ability to grow in the absence or presence of higher concentrations of NaCl (Mimura and Nagata, 2000).

1.2. Haloalkalophilic Microorganisms

Haloalkalophilic microorganisms inhabiting extremely alkaline and saline environments are found in both Archaea and Eubacteria. Most of the extremely salt tolerant haloalkaliphiles belong to *Halobacteriaceae*.

1.2.1. Eubacterial Haloalkalophilic

1.1.2.1 Haloalkalophilic *Bacillus*

Genus *Bacillus* can be characterized by unique physiological characteristics such as Gram staining, spore examination, catalase and oxidase tests. *Bacillus* is a Gram positive usually catalase and oxidase positive and spore producing organism that classified in Firmiculates. It is well known that *Bacillus* strains do not form endospores under many environmental conditions, and the ability to form spores is often difficult to demonstrate. This is very important for their taxonomy. There have been organisms described as non-spore-formers, which had to be reclassified after the detection of spores (Fritze and Claus, 1992). Recent taxonomic studies have separated some of the *Bacillus* members and grouped into new genera: *Alicyclobacillus, Aneurinibacillus, Brevibacillus, Gracilibacillus, Halobacillus, Paenibacillus, Virgibacillus* (Baumgart, 2003)

Table1.1. Tolerance for pH and NaCl of selected species of the genus *Bacillus.*

Species	Source	pH Tolerance	NaCl Tolerance
B. cohnii	Horse meadow soil	obligate alkalophilic	5%
B. horikoshii	Soil	obligate alkalophilic	17%
B. marismortui	Dead sea water	6.0-9.0, pH optimum 7.5	5-25%
B. agaradhaerens	Soil	obligate alkalophilic	16%
B. aclarkii	Soil	obligate alkalophilic	16%
B. horti	Soil, Japan	alkali tolerant	10%
B. vedderi	Bauxite waste	obligate alkalophilic	7.5%
B. alkalophilus	Soil and faeces	obligate alkalophilic	8%
B. clausii	Garden soil	alkali tolerant	10%
B. haloalkaliphilus	Brine/mud, Wadi Natrun	obligate alkalophilic	25%
B. halodurans	Soil	obligate alkalophilic	12%
B. pseudoalkaliphilus	Soil	obligate alkalophilic	10%
B. pseudofirmus	Lake bank soil	obligate alkalophilic	17%
Bacillus sp. DSM8714	River bank soil	alkalitolerant	10%
Bacillus sp. DSM8717	Horse and elephant manure	alkalitolerant	10%
Halobacillus halophilus	Salt marsh soil and solar salterns	7.0-9.0	15%

1.2.3. Gram Negative Haloalkalophilic Microorganisms

Halomonadaceae members are slightly and moderately halophilic bacteria with a broad pH tolerance. They are isolated from saline water, soil samples, leather processing, seawater and estuarine water. Species belong to the Halomonadaceae are Gram negative, rod shaped, non-spore forming, aerobic and also facultatively anaerobic in the presence of nitrate. (Dobzon and Franzman 1996; Franzman et al., 1988). By 16S rDNA sequence analysis, chemotaxonomic and physiological characteristics *Halomonas* have been defined as the only genus in Halomonadaceae (Dobson et al., 1996; Franzman and Tindall 1990). However, recently it has been proposed that Halomonadaceae has four different genera; *Halomonas*, *Alkanivorax, Carnivoras, Chromohalobacter* and *Zymobacte* (Bromguarte, 2003).

1.2.4. Haloalkalophilic Archea

Bacteria and archaea have been first recognized as two distinct groups of Prokarya by Woese et al. (1997). The original classification based on 16S ribosomal RNA gene sequence has been confirmed by extensive phylogenetic studies (Rosa et al., 2002). Extreme halophiles or Euryarchaeota require high salt concentrations, up to 20% NaCl for growth.

Archaea possess genes with recognizable counterparts in the Bacteria, showing that the two groups have functional similarities. Archaea also possess genes that also found in Eukarya (Rosa et al., 2002).

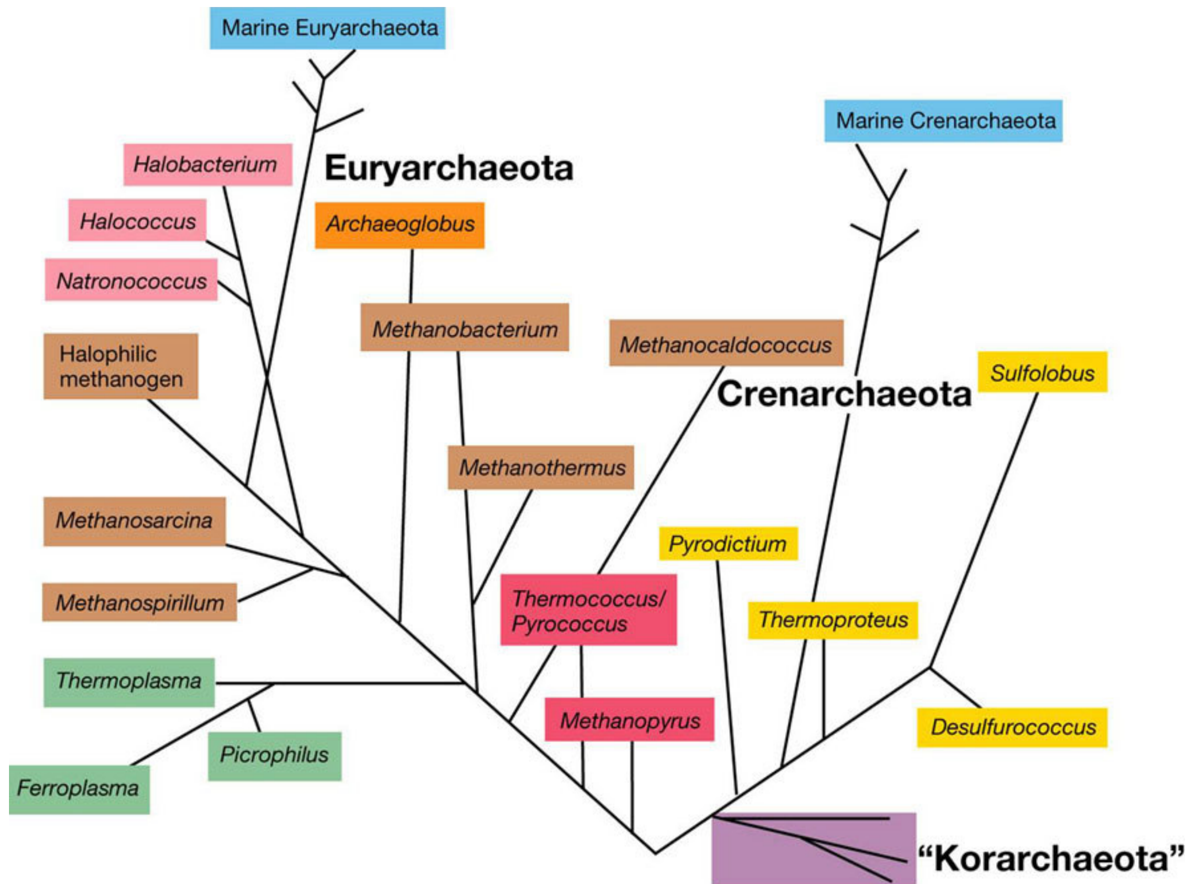


Figure1.1 Archaea kingdom

Euryarchaeaota (*Natronobacterium, Natronococcus, Natronomonas, Natrialba*, *Natronorubrum* genera are classified in "Natro group" of *Halobacteria*. These microorganism are extremely halophilic and alkaliphilic.

Table1.2. “Natro group” of Halobacteria.

Genus	Morphology	Number of species	Habitat
Natronobacterium	Rods	1	Highly saline soda lakes
Natrinema	Rods	2	Salted fish;hides
Natrialba	Rods	2	Soda lakes; beach sand
Natronomonas	Rods	1	Soda lakes
Natronococcus	Cocci	2	Soda lakes
Natronorubrum	Flattened cells	2	Soda lakes

1.2.5. Distribution and Isolation

Isolation of haloalkalophilic microorganisms is performed using different medium ingredients with respect to the group of target organism and the target environment. Actually the salt tolerance range plays an important role because the extremely halophilic organisms can not grow in low concentrations of salt although they have the ability to grow in minimal media. These microorganisms can live at high concentrations of different salts therefore they are populated in salt lakes. Limnological studies focus on the life conditions for extremely halophilic members during seasonal cycles. Anaerobic conditions are suitable in the presence of nitrate for facultatively anaerobes of eucaryotic members.

1.2.6. Biotechnological and Industrial Applications

Extremophilic microorganisms produce cell-associated products that can be used in different industrial applications. First of all their stability and activity in harsh conditions such as alkaline, acidic, high temperature, high concentration of salinity enables broad applications. Extracellular enzymes produced by these organisms are of great interest in biotechnology. Many *Bacillus* species are known to produce alkaline and saline stable proteases, amylases, cellulases, lipases, pectinases and xylanases (Martins et al., 2001)

Enzyme characterization studies with amylase from *Halobacillus* sp. strain MA-2, (Amoozegar et al., 2003) and *Halomonas mediteridiana* (J.Nieto et al., 1999) have

been known. Also the extracellular enzymes from archaeal members have unique features in their molecular structures.

Halophilic microorganisms also used for biological treatment (Kargı and Uygur, 1995).

Halophilic exopolysaccharides have been used in food manufacturing. Furthermore, there is a mereaning demand for non-toxic, biodegradable, environmentally friendly substances. *Halomonas maura* have been studied with novel composition exopolysaccharide (Arias et al., 2003).

1.2.7. Extracellular Enzymes and Their Industrial Application

Extracellular enzymes produced by haloalkalophilic microorganisms have unique properties. They have activity and stability at broad pH ranges and high concentrations of salts in that many other proteins are denaturated. Enzymes from archeal and eubacterial haloalkalophilic organisms have different characteristics. Archaeal enzymes need high concentrations of salt for their activity and stability (Mevarech et al., 2000). This is explained by the comparison of molecular structures from halophilic enzymes with those of nonhalophilic enzymes, this feature is related with adaptation mechanisms used for the protecting of cell from the denaturing effects of salt molecules (Ventosa et al., 1998)

1.2.8. Proteases from Haloalkalophilic Bacteria

Proteases are the enzymes that cleave protein molecules by hydrolysis. The first classification is made by using their cleavage mechanism. Exopeptidases work on the peptide bonds near the amino or carboxy-termini but endopeptidases are active on the peptide bonds distant from the termini of the substrate. The subclassification is based on their catalytic mechanism. Chemically different groups are found on active sites. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao et al., 1998). Subtilisins were grouped in serine type proteases. Catalytic triad of subtilisins consists of aspartic acid, histidine and serine. Although the size of subtilisins varies from 18 kDa to 90 kDa, all the subtilisins used in detergents have a size of

approximately 27 kDa. Most of the alkaline proteases were subtilisins. At present, less than 15 different enzyme molecules are used in detergents worldwide. These enzymes originate from *B. amyloliquefaciens, B. licheniformis, Bacillus clausii, Bacillus lentus, Bacillus alkaloophilus,* and *Bacillus halodurans*

The success of subtilisins is based on several factors, including their high stability and relatively low substrate specificity, features common in extracellular proteases. Their production as extracellular enzymes is of course an important factor in itself, as this greatly simplifies the separation of the enzyme from the biomass and facilitates other downstream processing steps. Another important point is the ability of *Bacillus* strains to secrete enzymes over a very short period of time into the fermentation broth.

Detergent industry prefers the enzymes, which are stable at high pH and compatible with chelating and oxidizing agents (Rao et al., 1998). Table represents the detergent proteases available (Maurer, 2004).

Table1.3. Detergent proteases (Maurer, 2004).

Trade mark	Origin	WT/PE	Production strain	Synonym
Genencor	*B. amyloliquefacies*	PE	*B. subtilis*	
Novozymes	*B. clausii*	WT	*B. clausii*	Subtilisin 309
Genencor	*B. lentus*	WT	*B. subtilis*	
Kao	*B. alkalophilus*	WT	*B. alkalophilus*	
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Novozymes	*B. halodurans*	WT	*B. halodurans*	Subtilisin 147
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. alkalophilus* PB92	PE	*B. alkaliphilus*	

PE, protein engineered; WT, wild type.

Since 1997, several gene-shuffling approaches have been performed with subtilisins. Interesting results from the shuffling of 26 protease genes have been described for properties such as activity in organic solvents, temperature stability, and activity at high or low pH (Minshull et al., 1999). Little, however, has been published on stain removal. Owing to the large number of variant molecules generated by

shuffling and other random techniques, screening methods with high-throughput and increased relevance have had to be developed. Unfortunately, these methods are still not entirely satisfactory, which might explain why no outstanding new subtilisin variant created by one of the gene shuffling technologies is yet present in detergents. Subtilisins for detergents are produced in *Bacillus*, because these species are able to secrete large a mounts of extracellular enzymes. The control mechanisms involved in the production of proteases in Bacillus are extremely complex and still not fully understood. An example is the two-component regulatory system that acts as a quorum sensing mechanism in *B. Subtilis* and which has been found to control the expression of the alkaline protease. This regulatory system is encoded on the chromosome and on endogenous plasmids. (Maurer, 2004)

Arhaea members have proteolytic activity in high salt concentrations (Eichler, 2001). Proteases from *Natronococcus occultus* and *Natrialba magadi* have been characterized (De Castro et al., 2001).

Leather industry uses proteases at dehairing and baiting steps (Kumar and Takagi, 1999). Stable proteases under alkaline and saline conditions would be most effective enzymes for such purposes.

It has been reported that Halophilic bacteria destroied brine-cured hides with their high proteoliytic activity (Bailey et al., 1998).

The studies about using proteases and gelatinases for the pretreatment of leather waste including cromium are important for the convertion of waste material to animal feed (Taylor, 2002).

Silver recovery has been achieved with different types of alkaline proteases at high alkaline conditions (Singh et al., 1999, Kumar and Takagi, 1999).

1.2.9. Lipases from Haloalkalophililes

Lipases, (triacylglycerol acylhydrolases, EC 3.1.1.3), are able to catalyse both the hydrolysis and the synthesis of esters bonds in lipid molecules. Lipid molecules are composed of glycerol and long-chain fatty acid molecules. Esterification and hydrolysis reactions that can occur in organic solvents are very important (Ishıkawa and Ogino,

2000). Most of the lipolytic enzymes used commercially are produced by alkalophilic microorganisms and they are used as detergent addiditives (Sharma et al. 2001).

Table.1.4. Lipolytic activity

Active site	Catalytic triad consisting of the amino acids serine, aspartic (or glutamic acid) and histidine; homologue to the triad of serine proteases.
Substrates	Oils (olive, soybean, mineral oils),Triglycerides (triolein, tributyrin), Synthetic surfactants (Tweens 20 and 40).
Other Activities	Phospholipase, lysophospholipase, cholesterol esterase, cutinase, amidase.

Lipases from *Halomonas* strains have been characterized (Mattiasson et al., 2004).

Alkaline lipases are mainly used in detergent industry. Approximately 1000 tons of lipases are added to the 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Alkaline lipases to be used in detergents should meet certain requirements. They should hydrolyze fats of various compositions, be stable in harsh washing conditions (pH 10-11, 30-60 °C) and in the presence of surfactants (linear alkyl benzene sulfonates (LAS) and proteases) (Sharma *et al.,* 2001).

1.3. Identification of the Haloalkalophilic Organisms

Identification of the haloalkalophilic microorganisms are performed by using the phenotypic and genotypic characteristics.

1.3.1. Phenotypic Methods

Morphological characteristics can be examined by direct observation, which give simple information on the organism for example colony formation that includes colony shape, diameter and color. Furthermore, microscopic observations after different chemical treatments give detailed information on the cell morphology. These are cell shape, Gram behavior and sporulation type. Phase contrast microscopy gives

information on the cell shape and sporulation type without any chemical treatment. Some tests can provide information about the adaptive mechanisms and relation between interior of the cell and the medium. These tests conditions are: growth at different pH ranges, temperatures, pressure and salt concentrations. Bergey's Manual of Systematic Bacteriology a major taxonomic compilation of prokaryotes gives information in all recognized species of prokaryotes and contains number of keys to evaluate the properties given above (Madigan *et al.,* 1997).

Phenotypic methods also include biotyping, antibiogram, phage typing, serotyping, PAGE/immunoblotting and multilocus enzyme electrophoresis (Busch and Nitschko, 1999).

Although molecular characterization methods provide clear differentiation and precise identification, using of phenotypic characterization methods combined with molecular techniques are required for taxonomic studies and functional purposes.

1.3.2. Molecular Characterization Methods

1.3.2.1. Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is used for the analysis of microbial genomes and molecular characterization of the microorganisms below species level. It enables to the seperation of large DNA molecules and therefore whole genomic DNA can be analysed. Analysis of chromosomal macrorestriction patterns by PFGE is a powerful method to produce fingerprints of closely related strains (Farber, 1996). Application of the method can differ for different type of organism. Different organism can require different cell disruption treatments for the releasing the genomic DNA. Also the size and content of genomic DNA can differ among the organisms. Thus, optimization parameters such as lysis of cells, electrophoresis conditions (pulse time, voltage) are the main points for obtaining fine discriminative PFGE results.

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)

DNA fragments are amplified by the polymerase chain reaction (PCR) using short (generally 10 bp) synthetic primers of random sequence. These oligonucleotides serve as both forward and reverse primer at the same time, and are usually able to amplify fragments at 3-10 genomic sites simultaneously. Amplified fragments (within the 0.5-5 kb range) are separated by gel-electrophoresis and polymorphisms are detected as the presence or absence of bands of particular size. These polymorphisms are considered to be primarily due to variation in the primer annealing sites. Some advantages of RAPD are as follows: low quantities of template DNA are required (5-50 ng per reaction), no sequence data for primer construction are required, easy and quick to assay, low costs involved, generation of multiple bands per reaction, and it suitable for automation.

1.3.2.3 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP (Restriction Fragment Length Polymorphism)

In most prokaryotes the ribosomal RNA genes reside sequentially in the chromosome (16S-23S-5S) and they are transcribed as a single polycistronic RNA molecule (Luz et al., 1998). These ordered genes have long been served as a genetic marker to study microbial diversity. The 16S rRNA gene sequence has been used for assessing bacterial phylogenies (Abd-El-Haleem et al., 2002). However, it has only provided information useful for identifications at genus level (Shaver et al., 2002).

Below the genus level, additional rDNA sequences have been necessary. The use of 23S rDNA has also been limited by its larger size and insufficient sequence data (Abd- El-Haleem et al., 2002). The intergenic spacer region (ISR) or also known as intergenic/internal transcribed spacer (ITS) (Abd-El-Haleem et al., 2002; Daffonchio et al., 2000; Shaver et al., 2002; Fisher and Triplett, 1999; Toth et al., 2001) between 16S and 23S rDNA genes, includes both conserved and highly variable sequence motifs, such as tRNA genes, and anti-terminators (boxA) (Abd-El Haleem et al., 2002). ISRs are generally found in multiple copies in most bacteria genomes. Since they are

hypervariable with respect to adjacent genes, they can differentiate between multiple operons in the cell (Daffonchio et al., 1998). The size of the spacer has been found to differ considerably among different species, and even among the different operons within a single cell (Garcia-Martinez et al., 1999).

Figure 1.8 Schematic representation of a 16S-23S spacer and organization of ITS functional regions (Garcia-Martinez et al., 1999).

The method is based on the amplification of 16S rDNA and ISR regions as singleamplicon. The resulting amplicon is then digested with frequent cutting enzymes. Finally restriction fragments obtained are separated by agarose gel electrophoresis and restriction patterns are then compared. The method could discriminate different groups of bacteria at species level.

1.3.2.4. Plasmid Profiling

Plasmids are the genetic materials of bacteria carried as extracromosomal elements. They are self-replicative and they can be transferred naturally between the microorganisms. Genes coded in plasmid DNA structures give different characteristics to microorganisms.

Plasmids isolated from the microorganisms give variable profiles with respect to the organism type and the habitats. Therefore the gel profiles of plasmids for closely related organism would be useful in taxonomic studies (Farber, 1996).

1.3.2.5. Ribotyping

Nucleic acid probes that recognize ribosomal genes are used for ribotying. Ribosomal RNA (rRNA) genes are composed of 23S, 16S and 5S regions. They are known to be highly conserved. Whereas most of the bacterial genes present in one copy, rRNA operons can be present in anywhere more than one copy (2-11 copies per bacterial cell). Thus the more copies of rRNA operon enables more discriminatory riboyping. In practice, first bacterial DNA is isolated and digested by restriction endonucleases. After agarose gel electrophoresing, DNA is transferred onto nylon or nitrocellulose membrane by capillary system or electrophoresis. The nylon membranes

are used as a solid support for probing. Probes specific to 23S, 16S, 5S rRNA genes are labeled using radioactive or non radioactive labeling techniques (non isotopic cold-labelling systems). Hence fingerprint patterns of bacterial DNA containing ribosomal genes are created. These patterns are varied from 1 to 15 bands and compared among the isolates. For another ribotying technique called as chemiluminescence ribotyping, digoxigenin-labeled cDNA (DNA which has been made by reverse transcription of rRNA) can be seen in figure 2. One of the major advantages of ribotyping is that because of the similarity of ribosomal genes, universal probes can be used. Reproducibility is also another advantage of this genotypic method.

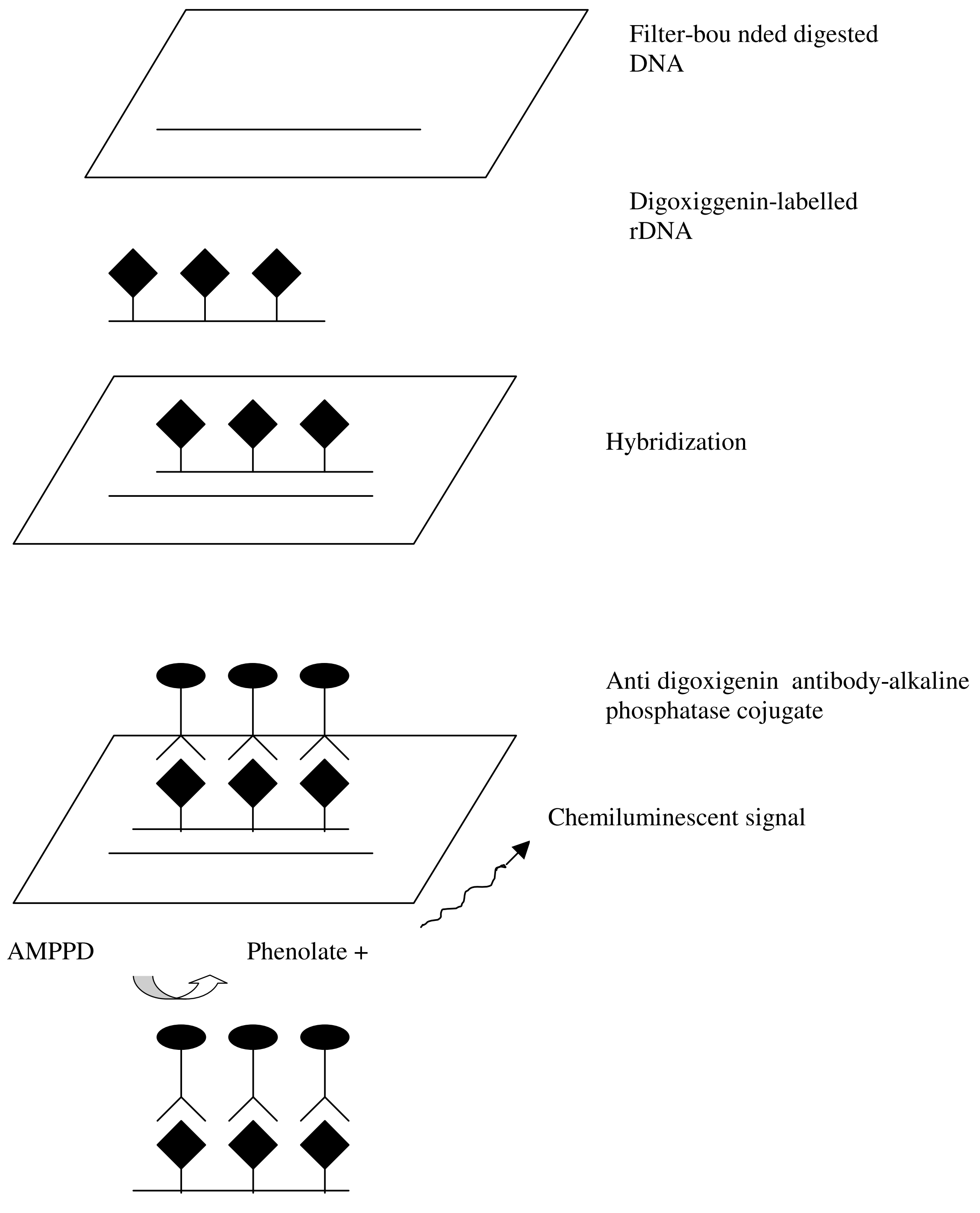


Figure1.2. Schematic representation of chemiluminescence ribotyping. AMPPD, adamantly-1, 2-dioxetane phosphate (Bingen et al., 1994)

1.3.2.6. DNA Sequencing

DNA sequencing is used for investigation of the phylogenetic relationship of organisms. This tecnique is based on the exact order of nucleotides in a region of the DNA molecule Automated DNA sequencing generally includes PCR amplification of a DNA region of interest, computer-aided sequence analysis and interpretation. For DNA sequencing the region of the chromosome to be analysed must be short consisting of a variable sequence flanked by highly conserved region. The variability within the selected sequence must be sufficient to differentiate microorganisms. And also the selected sequence should not be horizontally transmissible to other strains of species (Olive and Bean, 1999).

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir)

Leather factories of Ege Region are populated in Menemen – İzmir. twenty different companies have leather factories in Menemen region. It is known that industrial activities bring the most important pollution problems. For this reason leather factories were placed far from the centre of the city. Leather processing steps are as fallows:

- Curing
- Beamhouse operations which wash and soak the hides or skins and (at most tanneries) remove the attached hair.
- Tanyard processes in which the tanning agent (primarily chromium) reacts with and stabilizes the proteinaceous matter in the hides or skins.
- Finishing or post-tanning processes.

Waste Water of the leather factories are removed from the other factories at Menemen region and a simple waste water treatment is applied. The classical scheme is given below (UNEP/IEO, 1994).

- Pre-treatment: mechanical screening to remove coarse material.

- Primary treatment: sulphide removal from beamhouse effluents; chrome removal from tanning effluents; flow equalization; physical-chemical treatment for

-BOD removal and neutralization.

- Secondary treatment, usually biological.

- Tertiary treatment, including nitrification and denitrification.

- Sedimentation and sludge handling

1.5Thesis Objectives

The purpose of this study was,

- Isolation of haloalkalophilic strains
- Screening for their extracellular enzymes: protease and lipase
- Characterization of these isolates by phenotypic and genotypic methods.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used were listed in Appendix A.

2.1.2. Samples used for isolation

Table 2.1. Samples used for isolation

Sample Type	Location
Polluted Soil	Leather Factories waste (İzmir – Menemen)
Leather processing	Leather Factories (Izmir-Menemen)

2.2. Methods

2.2.1. Isolation and Preservation of the Isolates

2.2.1.2. Isolation of Haloalkalophilic Bacteria

Dilution plate method was used for the isolation of haloalkalophilic bacteria (Mora et al., 1998). Ten g soil, leather or 10 ml of water samples were transferred in 90 ml of 0.85% saline water. In order to homogenize the samples 6h incubation was performed in orbital shaker at room temperature. The samples were pasteurized for 10 m. at 80°C in a water bath in order to kill most of the vegetative cells and eliminate non-

spore forming bacteria (Mora et al., 1998). After the heat treatment, for dilution, 1ml aliquots from each sample was transferred in 9 ml of 0.85 % saline water and 6 fold dilutions were prepared. One ml of the dilutions was plated on appropriate solid media (Appendix B.1) and incubated for 5 days at 37 °C. Single colonies were picked and they were purified using streak plate method.

2.2.1.3. Preservation and Activation of Isolates

Cultures were grown in Horikoshi-I (Apendix B.1) agar plates containing 12% NaCl and incubated for 2 days at 37°C they were then transferred into the isolation broth. After turbidity formation in isolation broth, 0.5ml of each culture were transferred into cryotubes and 0.5ml of isolation broth containing 20% glycerol was added. These steps were all performed on ice. Then tubes were mixed gently by pipetting. Cultures were stored at -20°C for an hour they were then stored at -80°C. For the phenotypic test culture activation was applied from 20% glycerol stocks. For phenotypic and genotypic identification of the isolates, cultures were prepared from these stocks.

2.2.2. Determination of Phenotypic Characteristics

2.2.2.1. Gram Staining

Isolates were stained by modified Gram method for halophilic bacteria. Eighty µl of sterile 0.85% saline water were pipetted onto slides the organisms were then suspended and mixed thoroughly. Overnight culture was spread onto the microscope slides until a thin film formed by using tooth stick. After being air dried, the slides were fixed and desalted simultaneously by immersing in 2% acetic acid for 5 min. Then they were dried again. The smears were covered with 0.25% aqueous solution of crystal violet (Apendix D.2.1) for 1 min. After rinsing under the tap water, the slides were transferred into iodine (Apendix D.2.2) solution and were kept for 1 min. Following this step, slides were washed in alcohol for 6 s. They were then stained with safranin (Apendix D.2.3) for 30 s. After staining, the slides were dried on paper towels and the

cells were examined under light microscope. Gram (+) cells assumed purple color while Gram (-) cells appeared pink or red.

2.2.2.2. Examination of Endospores

Isolates were grown on Horikoshi-I (Apendix B.1) agar plates and 0.0005% $MnCl_2$ (Travers, 1987) were added in to the medium. Observations for spore formation were monitored at 3-4- 5-6th day.

2.2.2.3. Catalase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. Catalase activity was observed after dropping 3% hydrogen peroxide solution onto the target colony. Formation of the air bubbles indicated the presence of catalase activity.

2.2.2.4. Oxidase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. 1% solution of tetramethyl-p-phenylenediamine was poured onto a filter paper placed in a petri dish. Then the target colony was taken from the agar plate and spotted onto wet filter paper. Observation of the blue-purple color indicated the oxsidase activity.

2.2.2.5. Growth at Different Temperatures

The isolates were grown on nutrient agar (Apendix B.2) 45°C and 50°C. Observations for growth were noted during the 5-day incubation.

2.2.2.6. Growth at Different NaCl Concentrations

Cultures were tested for their salt tolerance on Horikoshi-I (Apendix B.1) agar plates containing 0%, 20%, 25 % NaCl during the 5-day incubation.

2.2.2.7. Growth at Different pH Ranges

Cultures were tested for pH tolerance in nutrient agar plates at different pH points (7, 10, and 12). Growth behaviors were observed during the 5-day incubation

2.2.3. Screening for Extracellular Enzymes

2.2.3.1. Screening for Protease Activity

Proteteolytic activity of the isolates observed by adding casein into Horikoshi–I (Apendix B.1) medium adding sikimmed milk. Different concentrations of NaCl (12% and 20%) were added. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete proteases extracellularly, give clear zones around the colony (Horikoshi, 1999).

2.2.3.2. Screening for Lipase Activity

Lipolytic activity of the isolates was observed by using the medium described in Appendix B.5. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete lipase extracellularly, gave opaque zones around the colony (Haba et al., 2000).

2.2.4. Preparation of Genomic DNA

Cultures were grown on solid Horikoshi – I (Apendix B.1) medium. Cells were suspended in 1.5 ml of sterile water in petri dishes. Then cell suspension was transferred into eppendorf tubes. In order to prepare cell pellets, tubes were centrifugated for 5 min at 5000 rpm. Pellets were resuspended in 567 µl 1Xte (Apendix C.4). Thirty µl 10% SDS and 3µl proteinase K solution (20 mg/ml) were added and the solution was mixed thoroughly. Cell lysis was performed with 1h incubation at 37°C. Afterwards 0.1 ml of 5 M NaCl were added and mixed well. Then 80µl of CTAB/NaCl (Appendix C.8) were added, the tubes were then mixed and incubated for 10 min at 65°C. Following the incubation, 0.7 ml of chloroform/isoamyl alcohol (Appendix C.2) were added, mixed thoroughly and centrifuged for 5 min at 10000 rpm. Aqueous, viscous supernatant was

transferred into a fresh eppendorf tube. An equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged for 5 min. The supernatant was again transferred into a fresh eppendorf tube and 0.6 volume of isopropanol were added to precipitate the nucleic acids. The tube was then shaken back and forth until a white DNA precipitate became visible. DNA wool were taken with a pipet tip and placed into a new tube, contain 300µl 70% ethanol. The tubes were stored at -20°C for 1h. Afterwards tubes were centrifuged for 15 min at 6000 rpm. Then the supernatant was discarded and DNA pellet was washed with 500 µl 70% ethanol. It was centrifuged for 10 min at 8000 rpm. After this, ethanol was discarded. The pellet was dried and dissolved in 100 µl 1xTE containing 100 µg/ml RNAase. Then tubes were incubated for 1h at 37°C. After the incubation the sample volume was adjusted to 400 µl with 1xTE. DNA was dissolved by alternating cold-heat shocks (for 10 min at 80°C and 20 min at -20°C, repeated twice). To further purify DNA an equal volume of phenol was added and mixed well. An equal volume of chloroform was added and mixed well. Then the tubes were centrifuged for 5 min at 6000 rpm. The aqueous phase was transferred into a fresh eppendorf tube. Equal volume of chloroform /isoamyl alcohol was added and mixed well. Samples were centrifuged for 5 min at 6000 rpm. Chloroform /isoamyl alcohol step was repeated once again and DNA was precipitated by adding 1/10 sample volume of 5 M NaCl and 2 volumes of 99% ethanol. Pellet was washed in 500 µl 70% ethanol by centrifugation for 5 min at 6000 rpm. After that, ethanol was removed and samples were dried for 10 min at 37°C. DNA was dissolved in 20µl, 50µl, 100µl or 200µl 1xTE according to the pellet size. Then the samples were preserved at -20 °C.

2.2.5. Genotypic Characterization

2.2.5.1. Identification of isolates by 16S rDNA - ITS rDNA - RFLP

2.2.5.1.1. Amplification of 16S ITS rDNA

PCR amplification of 16S-ITS rDNA of the isolates was performed by using two DNA primers (Apendix F). The forward primer was complementary to the upstream of 16S rRNA gene and the reverse was complementary to the upstream of 23S rRNA gene.

Forward: 5. -AGAGTTTGATCCTGGCTCAG-3 (Mora et al., 1998).

Reverse: 5. -CAAGGCATCCACCGT-3 (Jensen et al., 1993).

Amplifications were performed in a Mini Cycler System. PCR conditions were as follows:

Step 1: 94°C for 5 min (initial denaturation)
Step 2: 94°C for 1 min (denaturation)
Step 3: 42°C for 1min (annealing)
Step 4: 72 °C for 1min (elongation)
Step 5: 72 °C for 10 min (final extension)

40cvles

The final reaction volume was 50µl. PCR mix (Appendix E.1) was prepared after 2 µl -4µl of genomic DNA (approx. 500 µg) was placed into 1.5ml eppendorf tubes. During the preperation of the mix, tubes were kept on icebox. Taq DNA polymerase (1.25U) was added and mixed gently and then tubes were centrifuged for 2-3 s. The mix were added to each of the tube. Then 60 µl of mineral oil was added into the tubes while they were on ice. Tubes were centrifuged for 2-3 s then they were placed into the wells of Mini Cycler System (MJ Research INC, USA).

2.2.5.1.2. Electrophoresis of Amplified 16S-ITS rDNA Fragments

Amplified fragments were separated in 0.8 % agarose gel. First, 0.96 g of agarose was dissolved in 120 ml of 1×TAE buffer (Appendix C.4) by boiling (15 µl of ethidium bromide solution (10 mg/ml) were added after cooling the solution to 40°C). The gel was then poured into the gel-casting stand and the combs were placed. 1×TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. Five µl of PCR products were taken and mixed with 1 µl, 6 × gel-loading buffers. The samples were then loaded into the wells of agarose gel starting from the second well. Finally, 5 µl of DNA molecular weight marker were loaded into the first well and they

were electrophoresed at 40 mA until bromophenol blue have reached 2/3 of the gel. DNA fragments were visualized in a photodocumentation system (Vilber-Lourmat, France).

2.2.5.1.3. Chloroform Extraction of PCR Products

Final volumes of the PCR products were adjusted to 100 µl with 1×TE buffer. Then two volume of chloroform were added. After centrifugation for 2 min at 6000 rpm, organic phase was discarded and chloroform extraction was repeated. Then the tubes were centrifuged for 2 min at 6000 rpm. The aqueous phase was transferred into new eppendorf tubes containing 10 µl of 3 M sodium acetate (pH 5.2) and mixed well. After that, 450 µl of 99% ethanol were added and mixed. The samples were centrifuged for 15 min at 7000 rpm. Pellet was washed with 500 µl 70% ethanol. After centrifugation for 5 min at 6000 rpm, alcohol was removed. Pellet was dried for 10 min at 37°C, and they were dissolved in 25 µl 1xTE, and stored at 20°C.

2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP)

Purified PCR products were separated in 1% agarose gel in order to estimate DNA concentrations for each sample. Then 12.5µl of PCR products were aliquoted into new tubes for digestion. *Taq* I and *Hae* III were the enzymes chosen for this purpose. Reaction mix (Appendix F2) was added on PCR products while the tubes were stored on ice. 5 U of enzyme was used for each reaction. The final volume of the reaction was adjusted to 50 µl. Incubation period was 5 h for both of the enzymes. But the optimum reaction temperatures were 65°C for *Taq I* and 37°C for *Hae* III. Mineral oil was added over the reaction mixtures to avoid evaporations at 65°C. After the incubation DNA was extracted as described in section 2.2.6.1.3. and dissolved in 15 µl of 1xTE.

2.2.5.1.5. Electrophoresis of Restriction Fragments

Restriction fragments were first separated in 2 % agarose gel containing 20 µl of ethidium bromide solution (10mg/ml). Electrophoresis was carried out at 60 mA for the

first 20 min, then the current was adjusted to 80 mA and the electrophoresis was continued further for 2.5 h. Some isolates produced similar restriction fragment patterns. These were contained within distinct groups. One digested sample from each group was picked and re-electrophoresed in order to produce representative restriction patterns for further restriction profile analyses.

2.2.6 Sequencing

2.2.6.1. Amplification of 16SrRNA Gene

16SrDNA region of rRNA genes of the isolates were amplified using the following DNA oligoprimers for sequencing reactions

EGE 1. 5. - AGAGTTTGATCCTGGCTCAG –3 (Jensen *et al.,* 1993)

EGE 2. 5. - CTACGGCTACCTTGTTACGA –3 (Jensen *et al.,* 1993)

Two µl of genomic DNA (approx. 500 µg) were transferred into 0.5 ml eppendorf tubes, as the template. PCR mix was prepared and after the addition of *Taq* DNA polymerase (1.25 U), it was mixed gently and centrifuged for 2-3 s. Forty-eight ml of the mix were distributed into the tubes. All the manipulations were performed on ice. The tubes were then overlaid with 60µl mineral oil, centrifuged for 2-3 s at 6000 rpm. Finally, they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) using the following program:

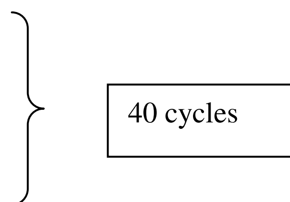
Step1: 95°C for 5 min

Step2: 95°C for 1 min

Step3: 56°C for 1 min

Step4: 72°C for 1 min

Step4: 72°C for 10 min



2.2.6.2 Extraction of DNA Fragments from Agarose Gel

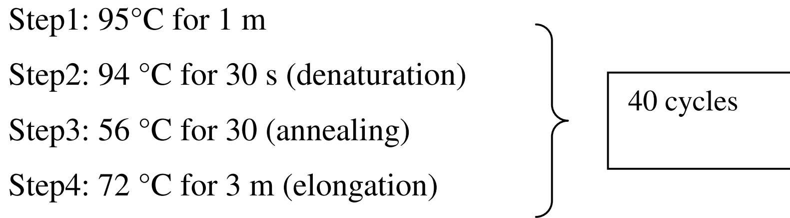
DNA isolation kit (Applichem A3421) was used for extraction of amplification products from agarose gel.

DNA bands (approximately 1600 bp) were excised from the agarose gel by a rasor blade. Three gel slice volume of 6 M NaI (for TAE gels) was added. The samples were incubated for 5 min at 55°C, mixed and continued for incubation for 2 min. Ten µl of well mixed glass powder suspension was then added, mixed well and incubated for 5 min at 55°C by occasional mixing. The samples were then centrifuged for 10 s at 10.000 rpm and the supernatant was discharged. The glass pellet was rinsed with 500 µl wash buffer. And resusupended by pipetting or gently flicking the tube. The samples were then centrifuged for 10 s at 10,000 rpm. The washing step was repeated twice. After the last wash, the supernatant was removed as much as possible. The pellet was suspended in 25 µl of 1xTE buffer. The samples were eluted for 5 min at 55°C with occasional mixing. Centrifugation was performed for 45 s at 10,000 rpm. The supernatant was finally transferred carefully into a new eppendorf tube. The eluted DNA samples were stored at -20°C until use.

2.2.6.3 Sequencing Reactions

The sequencing reactions were performed by using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit.

Four eppendorf tubes were firstly labeled as “A”, “C”, “G”, and “T” for respective termination reactions. Two µl of corresdonding Cy5.5 ddNTP termination mix were then dispensed into the appropriately labelled tubes. The tubes were then capped to prevent evaporation. The master mix (Appendix I) was prepared in an eppendorf tube for each template to be sequenced. The content of master mix was mixed thoroughly and 7µl of the mix was dispensed into each tube labelled “A”, “C”, “G”, and “T”. Each sequencing reaction was mixed thoroughly and overlaid with one drop of mineral oil. Each tube was capped and placed into the thermal cycler. The sequencing condition reastions were:



40 amplification cycles of Step 2, 3 and 4 were performed

The primer used was ;

EGE 1; 5. - AGAGTTTGATCCTGGCTCAG -3. (Jensen *et al.,* 1993)

2.2.6.4 Purification of Sequencing Reactions

2.2.6.4.1 Removal of unincorporated dye terminators using ethanol precipitation

The volume of the sample was adjusted to 50 µl by adding 1xTE. The samples under the mineral oil were then transferred into fresh eppendorf tubes. Twenty µl of 7.5 M ammonium acetate and 200 µl (3 times the reaction volume) of chilled 100% ethanol were added to each tube. The tubes were then mixed thoroughly by vortexing and placed on ice for 20 min to precipitate the DNA. Centrifugation was then performed at 12, 000 rpm for 30 min at 4°C. Supernatants were removed carefully and the pellets were washed with 200 µl of chilled 70% ethanol. After the centrifugation for 5 min, the supernatants were removed and the pellets were dried. Each pellet was then resuspended in 6 µl of formamide loading dye and vortexed vigorously (2-3 min) to ensure complete resuspension. Just prior to loading the samples onto gel, each sample was heated at 70 °C for 2-3 min for denaturation and then placed on ice. One and a half or 2µl of each sample were then loaded into the lanes of sequencing gel.

2.2.6.4.2 Assembling the SEQ Sequencer

The sequencing reactions were performed with SEQ personal sequencing system (Amercham Pharmacia Biotech). The system consists of SEQ personal sequencer, SEQ

Rapidset gel polymerizer, RapidGel QuickFill cassettes, RapidGel-XL-6% cartridge and filling gun and filling fixture.

Preparing a gel involves attaching the RapidGel-XL cartridge to the casting gun, filling the RapidGel cassettes, polymerizing the acyrlamide in the gel polymerizer. After the polymerization the comb was removed from the cassette. After cleaning the cassette, it was placed properly into the buffer chamber. At this step pre-run was performed to measure the backgraound redding. Once the gel cassette was aligned and the pre-run was complete, 1xTE buffer was loaded into the upper and lower buffer chambers until it reached the fill lines. The prepared samples (1-2 µl) were then loaded into the gel with a micropipette. Then the analysis was started. After the run was finished, data were analysed by SEQ software.

The sequences obteined were evaluated in and submitted to GenBank.

Chapter 3

RESULTS AND DISCUSSION

3.1. Isolation of Haloalkalophilic Bacteria

Haloalkalophilic bacteria were isolated from Menemen Region. Target places were leather factories and the wastes of this industry. Therefore the samples were taken from both the processing steps and waste.

3.2. Determination of Phenotypic Characteristics

3.2.1. Gram staining

The modified Gram staining method specific for halophilic bacteria gave desired results with these isolates. All the isolates were gram positive, rod shaped except one cocci (C1).

3.2.2. Examination of Endospore

Isolates were grown on agar plates (Appendix) for 5 days. Isolates were non-sporforming.

3.2.3. Catalase and oxidase tests

All the isolates were catalase and oxsidase positive.

3.2.4. Physiological Tests

Growth at pH 7 was observed to determine whether the isolates were obligate alkalophilic (Table 3.2). Four isolates could not grow at this pH value. Twenty-five of the isolates were alkali tolerant and could not grow at pH 12. Isolates were not thermotolerant since they could not grow at 45ºC and 50ºC on alkaline nutrient agar plates.

Table 3.1. Results of the Physiological Test

Physiologic test	Isolate number
No Growth at 20%NaCl	D2, D10, D20. D26, E4, E16, E24, F22
No Growth at PH 7	D1, D20, F9, F21
No Growth at 0%NaCl pH 10	D2, D6, D18, D23, E4, E43, F5, F7, F29
No Growth at pH 12	D1, D6, D10, D18, D20, D23, D24, D30, D32, E2, E4, E10, E12, E14, E16, E23, E24, E36, F7, F10, F13, F16, F20, F22, F29,

Growth of the isolates was also observed at 0% and 20% NaCl concentrations. Nine of the isolates were oblitely halophilic. Eight of the isolates were not extremely halophilic.

Table3.2. Differentiation of Isolates according to growth at different NaCl concentrations and pH ranges.

İsolate Name	20% NaCl	PH 7	0%NaCl pH 10	pH 12
D1	+	-	+	-
D2	-	+	-	+
D5	+	+	+	+
D6	+	+	-	-
D7	+	+	+	+
D10	-	+	+	-
D11	+	+	+	+
D12	+	+	+	+
D14	+	+	+	+
D18	+	+	-	-
D20	-	-	+	-
D23	+	+	-	-
D24	+	+	+	-
D25	+	+	+	+
D26	-	+	+	+
D29	+	+	+	+
D30	+	+	+	-
D31	+	+	+	+
D32	+	+	+	-
E1	+	+	+	+
E2	+	+	+	-
E3	+	+	+	+
E4	-	+	-	-
E6	+	+	+	+
E7	+	+	+	+
E9	+	+	+	+
E10	+	+	+	-
E12	+	+	+	-
E13	+	+	+	+
E14	+	+	+	-
E15	+	+	+	+

E16	-	+	+	-
E17	+	+	+	+
E20	+	+	+	+
E23	+	+	+	-
E24	-	+	+	-
E25	+	+	+	+
E26	+	+	+	+
E27	+	+	+	+
E28	+	+	+	+
E29	+	+	+	+
E30	+	+	+	+
E33	+	+	+	+
E34	+	+	+	+
E35	+	+	+	+
E36	+	+	+	-
E39	+	+	+	+
E42	+	+	+	+
E43	+	+	-	+
E47	+	+	+	+
F1	+	+	+	+
F2	+	+	+	+
F3	+	+	+	+
F4	+	+	+	+
F5	+	+	-	+
F7	+	+	-	-
F8	+	+	+	+
F9	+	-	+	+
F10	+	+	+	-
F11	+	+	+	+
F12	+	+	+	+
F13	+	+	+	-
F14	+	+	+	+
F15	+	+	+	+
F16	+	+	+	-
F17	+	+	+	+
F18	+	+	+	+

F19	+	+	+	+
F20	+	+	+	-
F21	+	-	+	+
F22	-	+	+	-
F23	+	+	+	+
F24	+	+	+	+
F25	+	+	+	+
F26	+	+	+	+
F27	+	+	+	+
F28	+	+	+	+
F29	+	+	-	-
F30	+	+	+	+
F32	+	+	+	+
F34	+	+	+	+
F35	+	+	+	+

3.3. Extracellular Enzyme Screening

Extracellular enzyme screening was performed on solid agar plates by using different substrates for each enzyme activity. Protease screening based on the observation of clear zones around the colonies (Figure). Lipase activity gave opaque zones around the colonies (Figure). Different NaCI concentratios were used for lipase screening. The enzyme screening studies were repeated performed twice.

3.3.1 Extracellular Enzyme Profiles of the Isolates

Table 3.3 Extracellular Enzyme Tests

Extracellular Enzyme Type	Isolate Name
Did not produce lipase	D5, D10, D20, D26 D31, E23, E43, F11, F29 F33
Utilized Tween80 at 0% NaCl	D1, D2, D30, F25, F15
Utilized Tween 20 at 12% NaCl	D23, D24, D25, D30, E1, E2, E3, E42, F5, F12, F13, F14, F15, F16, F18, F23, F25
Did not Utilized Tween 20	D7, E7, E9, E10, E13, E14, E15, E16, E23, E24, E25, E26, E27, E28, E29, E30, E39, E47, F26, F27, F31
Did not utilase Tween80	D12, E12, E34, F1, F20, F30, F32,
Did not produce lipase without NaCI	D12, D14, E12, E34, F1, F30, F32
Utilase casein	F17, D5, F29, D32, E34, F1, F15, E36, D24, F10, F13, F12, E20, F32, E1, E47, E33, E14, E25, E42, F22

Table 3.4 Extracellular Enzyme Profiles of isolates

Isolate Name	Protease	0%NaCl Tween20	12% NaCl Tween20	20% NaCl Tween20	0% NaCl Tween80	12% NaCl Tween80	20% NaCl Tween 80
D1	-	-	-	-	+	+	-
D2	-	-	-	+	+	-	-
D5	+	-	-	-	-	-	-
D6	-	+	-	-	-	+	-
D7	-	-	-	-	-	+	-
D10	-	-	-	-	-	-	-
D11	-	+	-	+	-	+	+
D12	-	+	-	-	-	-	-
D14	-	+	-	-	-	-	-
D18	-	+	-	+	-	-	+
D20	-	-	-	-	-	-	-
D23	-	+	+	-	-	+	+
D24	+	+	+	-	-	+	+
D25	-	+	+	-	-	-	+
D26	-	-	-	-	-	-	-
D29	-	+	-	-	-	+	+
D30	-	+	+	+	+	+	+
D31	-	-	-	-	-	-	-
D32	+	-	-	-	-	+	+
E1	+	+	+	-	-	+	+
E2	-	+	+	-	-	+	
E3	-	+	+	-	-	+	+
E4	-	+	-	-	-	+	-
E6	-	+	-	-	-	+	+
E7	-	-	-	-	-	+	-
E9	-	-	-	-	-	+	-
E10	-	-	-	-	-	-	+
E12	-	+	-	-	-	-	-
E13	-	-	-	-	-	+	-
E14	+	-	-	-	-	+	-
E15	-	-	-	-	-	+	-
E16	-	-	-	-	-	+	-
E17	-	-	-	-	-	+	-
E20	+	+	-	-	-	+	+
E23	-	-	-	-	-	+	-
E24	-	-	-	-	-	+	-
E25	+	-	-	-	-	+	-
E26	-	-	-	-	-	+	-
E27	-	-	-	-	-	+	-
E28	-	-	-	-	-	+	-
E29	-	-	-	-	-	+	-
E30	-	-	-	-	-	-	+
E33	+	-	-	+	-	+	+
E34	+	+	-	-	-	-	-
E35	-	-	-	+	-	-	+

E36	+	+	-	-	-	+	+
E39	-	-	-	-	-	+	-
E42	+	+	+	-	-	-	+
E43	-	-	-	-	-	-	-
E47	+	-	-	-	-	-	+
F1	+	+	-	-	-	-	-
F2	-	+	-	+	-	+	+
F3	-	+	-	+	-	+	-
F4	-	+	-	-	-	+	+
F5	-	+	+	+	-	+	+
F7	-	+	-	+	-	+	+
F8	-	+	-	-	-	+	+
F9	-	+	-	-	-	-	+
F10	+	+	-	+	-	+	+
F11	-	-	-	-	-	-	-
F12	+	+	+	+	-	+	+
F13	+	+	+	-	-	+	+
F14	-	-	+	-	-	+	+
F15	+	+	+	+	+	-	+
F16	-	+	+	-	-	+	+
F17	+	+	-	-	-	+	+
F18	-	-	+	-	-	+	+
F19	-	+	-	-	-	+	+
F20	-	+	-	+	-	-	-
F21	-	+	-	-	-	+	-
F22	+	+	-	-	-	+	-
F23	-	+	+	+	-	+	+
F24	-	-	-	-	-	-	+
F25	-	+	+	+	+	+	+
F26	-	-	-	-	-	+	+
F27	-	-	-	-	-	-	+
F28	-	+	-	-	-	-	+
F29	+	-	-	-	-	-	-
F30	-	+	-	-	-	-	-
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F35	-	-	-	-	-	-	-
F34	-		-	-	-	-	-

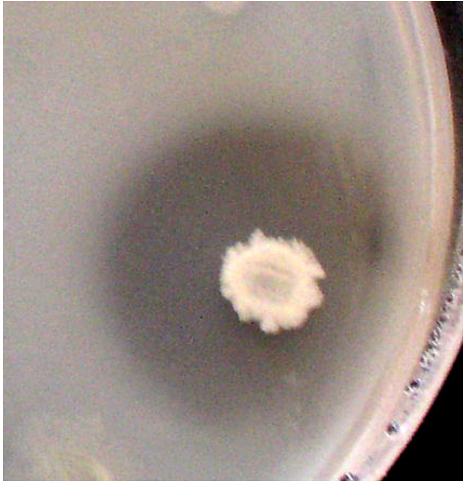

Figure 3.1. Apperance of extracellular protease

Figure 3.2. Apperance of extracellular lipase

3.4. Genotypic Characterization

3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon

The amplification product size differed between 1800 and 2000 bp (Figure)

.

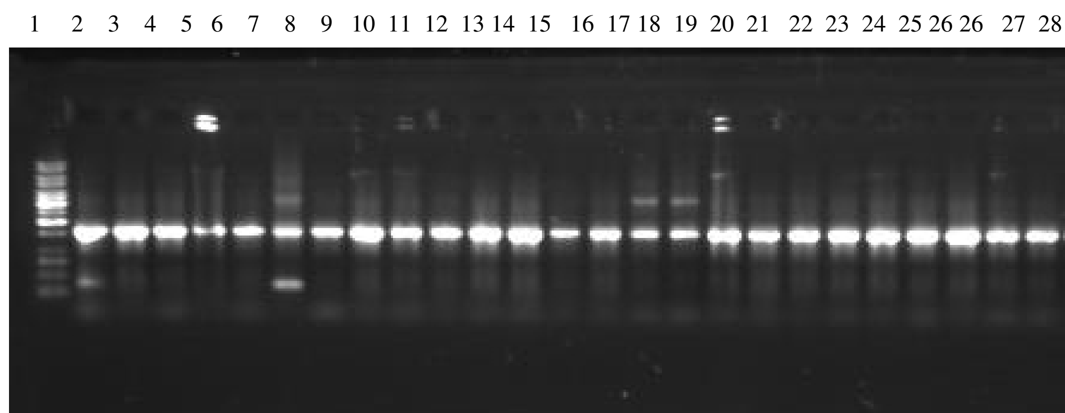

Figure 3.3. 16S rDNA-ITS Amplification products of some isolates. Lanes 1. 1 kb DNA Lac D1, 3. Isolate F2, 4. Isolate F1 5. Isolate D8, 6. Isolate D10, 7. Isolate 12,8. Is Isolate E15, 10. Isolate E17, 11. Isolate E19, 12. Isolate D23 lane 13. Isolate D24

3.4.2. 16S-ITS rDNA - RFLP Profiling

In order to identify the isolates amplification products of 16S-ITS rDNA region was digested with restriction endonucleases. *Taq* I and *Hae* III endonucleases were chosen for digestion. Digestion products were first run in 2% agarose gel to see all different profiles and to make groups within the digestion products. For a specific RFLP group, one representative digestion product was chosen and electrophoresed together in 2.5% agarose gel (Figure 3.3).

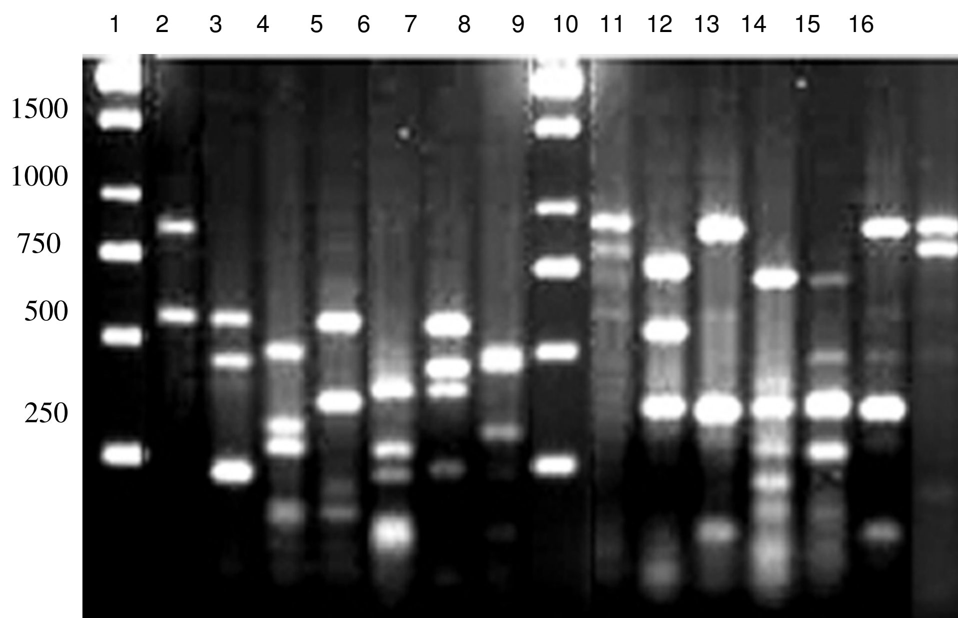

Figure 3.4 16S-ITS rDNA RFLP profiles of *Hae* III groups. Lanes 1. 1 kb DNA ladder, 2. Isolate D5, 3. Isolate D7, 4. Isolate D30, 5. Isolate D12, 6. Isolate D23, 7. Isolate F28, 8. Isolate, F21 16S-ITS rDNA RFLP profiles of *Taq*I groups. Lanes 9. 1 kb DNA ladder,10. Isolate D2, 11. Isolate D18, 12. Isolate D12, 13. Isolate F6, 14. Isolate F20, 15. Isolate F28, 16. IsolateE30,

Resulting gel profiles were analyzed using the program BIO-1D++. Dendrogram of representative strains were carried out using 15% homology coefficient. (Figure 3.3 and 3.4). Fragment sizes were given in Table 3.6.

Hae III and *Taq* I restriction analysis of 16S-ITS rDNA fragments of strains revealed 7 distinct groups (Table 3.3.).

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2000	854	546	465	546	384	546	465	2000	886	673	808	661	361	838	870
1500	555	440	309	354	257	440	432	1473		509	338	354	338	338	778
1000		219	272	125	211	377	294	934		354	78	265	257	78	
750			125		94	219		685				195			
500					70			465				133			
250								227				31			

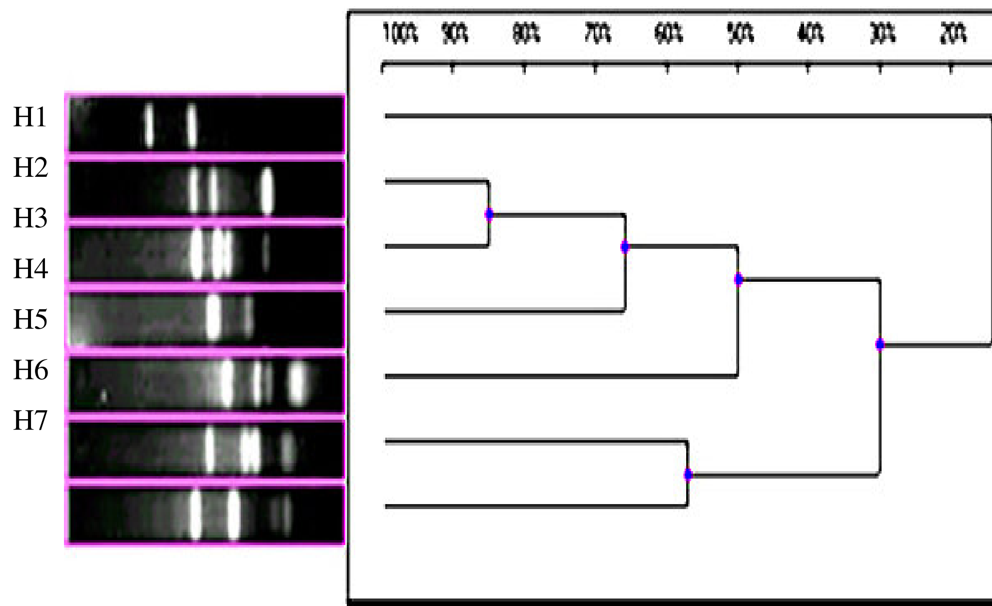

Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles.

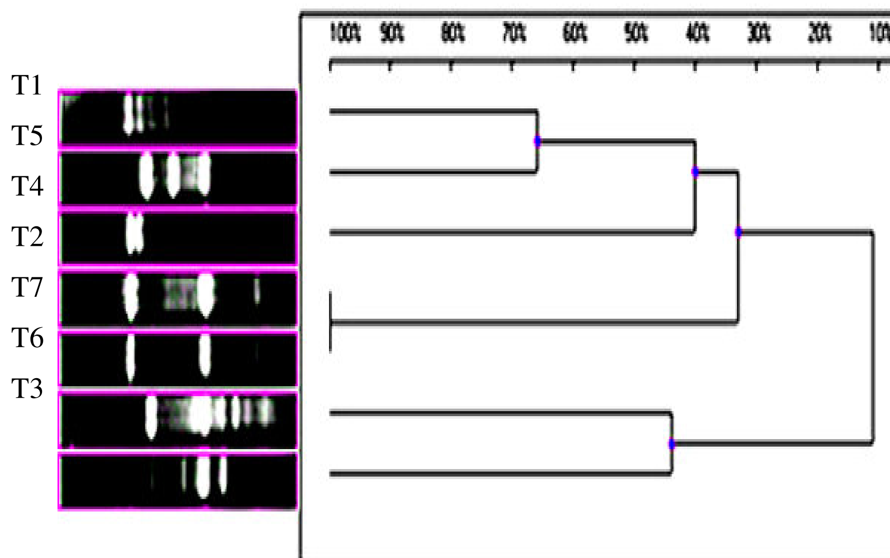

Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles.

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups.

Genotypic groups	Name of the Isolates
HT1	D1, D2, D5, E43,E47,F1
HT2	D6, D7, D10, D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E30, E34, E35
HT3	D12, F32
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E20, E33, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F19, F22, F23, F24, F25, F26, F27
HT5	D30
HT6	F20, F21
HT7	F28

The use of two restriction enzymes enabled the revelation of diversity among the isolates. When the results of both digestion were combined, 7 distinct genotypes were obtained (Table 3.10). The results indicated that isolate group members were differentiated by each restriction enzyme, were the same.

In this study, 16S ITS and rDNA regions were used as a single amplicon for RFLP analysis. No report was found in literature on genotypic characterization of haloalkalophiles using this method. Similar studies have been performed in this institute for genotypic characterization of alkalophilic bacilli (Akbalik, 2004), thermophilic bacilli, (Yavuz, 2004), lactic acid bacteria (Yavuz, 2003, Bulut, 2003.

3.4.3. Analysis of the Genotypic and Phenotypic Groups

Table 3.7. Analysis of the Genotypic and Phenotypic Groups

	Isolate number	20%NaCI	pH 7	0%NaCl	pH 12	Protease	0%NaCl Tween 20	12%NaCl Tween 20	20%NaCl Tween 20	0%NaCl Tween 80
HT1	D1,D2,D5,E34, E43, E47, F1	%80	80%	70%	80%	40%	0%20	0%	20%	%30
HT2	D6,D7,D10,D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E39, F29, F30, F34, F35,	100%	100%	100%	100%	10%	10%	0%	0%	0%
HT3	D12, F32	100%	100%	100%	100%	50%	100%	0%	0%	0%
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E30, E33, E35, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18, F19, F22, F23, F24, F25, F26, F27	90%	100%	90%	70%	30%	90%	40%	30%	20%
HT5	D30	100%	100%	100%	0%	0%	100%	100%	100%	100%
HT6	F20, F21	100%	50%	100%	50%	0%	100%	0%	50%	0%
HT7	F28	100 %	100%	100%	100%	0%	100%	0%	0%	0%

Phenotypic characterization of the isolates indicated that strains of different species had different phenotypic features. When the genotypic results were compared with the results of enzyme screening and phsyical tests, a correlation between a few groups were observed. Most of the isolates produced lipase (Tween 20 and Tween 80) at all NaCI concentrations used grouped in HT4. All members of HT2, HT3, HT5, HT6, HT7 were also able to grow in the presence of 20% NaCI. Most of the isolates that could not grow at pH7 were placed in HT6. Isolates that could not grow at pH12 were placed in HT1, HT5, and HT6.

3.4.4 Analysis of Sequencing Reactions

16S partial sequencing results of six representative isolates are as listed below.

Table 3.8. Sequencing results

Genotypic Group	Isolate Name-Accession Number	Length of bp.-Similarity	Accession Number - Organism	Name of the Family
HT2	D7-AY604872	-89%	*AY345480.1-Bacterium K-29*	Unclassified *Bacillaceae*
HT3	D12-AY601901	716-93%	AF237976.1-*Salinococcus roseus*	Unclassified *Bacillaceae*
HT4	D23-AY601902	323-92%	AJ640133.1-*Halomonas* sp.18bAG	*Halomonadaceae*
HT5	D30-AY601903	-92%	AY383044.1-*Halomonas meridiana*	*Halomonadaceae*
HT6	F20 (C1)-AY604871	-89%	AJ29397-*Nestenkonia* sp.EL-30	*Micrococcaceae*
HT7	F28 (15B)-AY601904	-91%	AY3091336-Bacterium K-2	Unclassified *Bacillaceae*

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, haloalkalophilic bacteria were isolated from samples of waste soil and leather processing. They were first characterized phenotypically (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope and extracellular enzyme screening, physiological tests such as growth at pH 7 and pH 12 , different NaCl concentrations and at different temperatures). Extracellular enzyme screening was performed on solid agar media. Molecular characterization was performed by using 16S-ITS rDNA RFLP.

All the isolated strains were Gram positive but they were not sporeforming. All the strains were catalase positive. Most of the isolates contained oxidase. Four strains could not grow at pH7. They were thus obligately alkalophilic. Twentyfive isolated strain were alkalitolerant because they could not grow at pH 12. Nine isolated strain could not grow with in 0% NaCl they were thus obligately halophilic. Eight of the isolates could not grow at 20% NaCl concentration.

Extracellular enzyme screening resulted in 21 protease and 72 lipase activity. Isolates produced protease were grouped in HT1, HT2, HT3, HT4.Different concentrations of NaCl were used for lipase screening. Seventeen of the isolated strains which could utilize Tween 20 in the presence of 12% NaCI were all grouped in HT4. Isolated strains, which could not utilize T20, were grouped in HT2 except E47, F26, and F27. Seven of the isolated strains were not produce lipase in the presence of NaCl were grouped in HT1, HT2 and HT3.

For restriction analysis of 16S-ITS rDNA fragments of isolated strains, two restriction enzymes, *Taq* I and Hae III, were used. Both enzymes produced similar RFLP groups. Representative group members were subjected to 16S partial sequencing. They had high similarity with *Bacillaceae*, *Halomonadaceae* and *Micrococcaceae* families.

Characterization of the extracellular enzymes from different isolated strains is the essential work for these Haloalkalophilic bacteria because of their unique futures.

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APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Experiments

NO	CHEMICAL	CODE
1	Agar Merck	1.01613
2	Bacteriological pepton Oxoid	LP037
3	D-Glucose AppliChem	A3666
4	Yeast Extract Merck	1.03753
5	Skimmed milk LabM	MC27
6	Glycerol AppliChem	A2926
7	NaCI AppliChem	A2942
8	K2HPO4 AppliChem	A2945
9	MgSO4.7H2O Merck	1.05886
10	Tween80 Applichem	A1390
11	Ammonium sulfate Applichem	A3485
12	Nutrient broth Merck	1.05443
13	Sodium carbonate Merck 1.06392	
14	KH2PO4 Merck	1.04871
15	Disodium hydrogen phosphate Applichem	A2943
16	Immersion oil Applichem	A0699
17	Calcium chloride Applichem	A3652
18	Crystal violet Sigma	C3886
19	Safranin Merck	1.15948
20	N,N,N.,N.- Tetramethyl-p-phenylenediamine Sigma	T3134
21	Congo Red Sigma	C6767
22	Potassium Iodide Sigma	P8256
23	Tris Base Sigma	T6066
24	EDTA AppliChem	A2937
24	Isopropanol AppliChem	A3928
25	Proteinase K AppliChem	A3830
24	Ethidium bromide AppliChem	A1151
25	Ethanol AppliChem	A3678
26	Taq DNA polymerase Promega	M1865
27	Primers: Ege 1 and L1 Promega	
28	dNTP set MBI, Fermentas,	R0181
29	Standard agarose (low electroendoosmosis) AppliChem	A2114
30	Chloroform AppliChem	A3633
31	Isoamyl alcohol AppliChem	A2610
32	Bromophenol blue Merck	1.08122
33	Boric acid AppliChem	A2940

APPENDIX B

RECIPIES FOR MEDIA USED IN EXPERIMENTS

B.1 HORIKOSHI BROTH AND AGAR

HORIKOSHI-I BROTH

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water. Medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

HORIKOSHI-I AGAR (pH=10.2)

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

B.2 NUTRIENT AGAR (pH:10.3) USED FOR GROWTH TESTS AT DIFFERENT TEMPERATURES AND SODIUM CHLORIDE CONCENTRATIONS

Nutrient Broth	8
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 was sterilized separately and added into medium.

B.5 MEDIUM USED FOR LIPASE SCREENING (pH: 10.3)

	g/l
Nutrient Broth	8
$CaCl_2.H_2O$	0.1
Agar	13
Tween20/Tween80	10ml
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 and Tween20/80 was sterilized separately and added into medium.

APPENDIX C

BUFFERS AND STOCK SOLUTIONS

C.1 ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

Ethidium bromide (0.5 g) was dissolved in 50 ml of deionized water.

C.2 CHLOROFORM-ISOAMYL ALCOHOL SOLUTION (24:1)

Forty eight milliliters of chloroform was mixed with 2 ml of isoamyl alcohol.

C.3 50 X TAE

Two hundred and fifty-two grams Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

C.4 1XTAE

Twenty milliliters of 50X TAE buffer was taken and the volume was adjusted to1000 ml with deionized water to obtain 1 liter 1X TAE buffer.

C.5 1X TE BUFFER

10 mM Tris pH 8.0
1 mM EDTA pH 8.0

C.6 10 X TBE

One hundred and eight grams Tris Base and 55 g boric acid were weighed. They

were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was added. The volume was brought to 1000 ml with deionized water.

C.7 1X TBE

Hundred milliliters, 10X TBE was taken and the volume was brought to 1000 ml with deionized water to obtain 1X TBE buffer.

C.8 CTAB/ NaCl (10% CTAB in 0,7 M NaCl)

NaCl (4.1 g) was dissolved in 80 ml water and CTAB was added slowly while heating and stirring. Final volume was adjusted to 100ml.

APPENDIX D

STAINS AND INDICATORS

D.1. BROMTYMOL BLUE SOLUTION

Bromtymol blue	1 g
N/10 NaOH	25 ml
Deionized water	475 ml

D.2. SOLUTIONS FOR GRAM STAINING

D.2.1. CRYSTAL VIOLET STAINING REAGENT

Solution A

Crystal violet	2g
Ethanol (95%)	20ml 83

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

D.2.2. IODINE SOLUTION

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Iodine and potassium iodide were grinded in a mortar, water was added slowly with continuous grinding until the iodine was dissolved. The solution was stored in amber bottle.

D.2.3. SAFRANIN SOLUTION

Safranin (2.5% in 95% alcohol)	10ml
Distilled water	100ml

APPENDIX E

PCR RECIPIES

E.1. PCR MIX

Mg free Taq DNA polymerase buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Sterile deionized water	32 µl
Oligo forward (10 picomole/µl)	1 µl
Oligo reverse (10 picomole/ µl)	1 µl
dNTP (2mM each) 10X	5 µl

E.2. 6X GEL LOADING BUFFER (20 ML)

10x TBE	2 ml
Glycerol	6 ml
Deionized water	12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

E.3. dNTP (10X)

Ten microliters of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at .20 °C.

E.4. RESTRICTION ENZYME MIX

Restriction enzyme buffer	5 µl
DNA	8 µl
Restriction enzyme (10U/ µl)	1.5 µl
Sterile deionized water	35.5 µl

APPENDIX F

OLIGONUCLEOTIDE PRIMERS

L1: 5.- CAAGGCATCCACCGT -3.

Three hundred and fifty micrograms primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliters of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

EGE 1 5.- AGAGTTTGATCCTGGCTCAG -3.

Five hundred and ninty micrograms primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain final concentration of 2 µg / µl stock solutions. Five microliters of stock solution were then taken and mixed with 95 µl sterile deionized water. Therefore, 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

APPENDIX G

RECOGNITION SITES OF RESTRICTION ENZYMES

G1. ***Taq*** **I**

5.- T▼CG A -3.
5.- A GC ▲T -3.

G2. ***Hae*** **III**

5.-GG▼CC-3.
5.-CC▲GG-3.

APPENDIX H

ISOLATE NAMES

DI- C12a
D2- Ş6a
D5- E15
D6-C15a
D7- 20a
D10-19a
D11- B5c
D12- B1a
D14- 23a
D18- C4
D20- B8b0
D22- H2
D23- H1a
D24- P5z
D25- C17
D26- 2b
D29- D3c
D30- B9
D31- A9
D32- D2b
E1- A2
E2- P2y
E3- C6
E4- C2a
E6- G9a
E7- 3a
E9-24a

F26- Ü4z
F27- Ü6a
F28- 15b
F29- F1
F30- 24a
F31- 27b
F32- B4c
F33- T1b
F34- E6z
F35- B8a
E42- C8
E43- C14a
E47- C19y
F1- E11x
F2- F2x
F3- F2y
F4- F2z
F5- G5
F6- G8
F7- H3x
F8- H3y
F9- P1x
F10- P4a
F11- P5y
F12- S1
F13- S2
F14- Ş1

E10- 13b	F15- V3
E12- 29c	F16- A1
E13- 22c	F17- A4
E14- 12c	F18- A6
E15- 27c	F19- A7
E16- 1a	F20- B10
E17- 2d	F21- C1
E20- B2	F22- C2
E23- B8c	F23- C3
E24- 18b	F24- C24
E25- 11a	F25- C10

ISOLATION OF HALOALKALIPHILIC MICROORGANISMS FROM LEATHER INDUSTRY

Hatice Sevgi ÇOBAN

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Isolation of Haloalkaliphilic Microorganisms from Leather Industry

By

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ABSTRACT

Haloalkalophiles are extremophilic microorganisms that are adapted to saline and alkaline conditions. Different species of them have been isolated so far from soda lakes and soil samples. Haloalkalophilic microorganisms have significant adaptive mechanisms to avoid denaturing effect of salts and to balance their interior pH. Extracellular enzymes that are produced by these halophilic and alkalophilic microorganism are applicable for industrial purposes. Therefore isolation of these organisms from their habitats and study on genotypic characterization constitute initial steps for furter biotechnological studies.

In this study, processing steps of leather factories and their wastewater were chosen for sampling. In order to isolate target microorganisms Horikoshi-I medium including 12% NaCl was used. After isolation microorganisms were purified. Phenotypic tests were applied (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope, sensitivity to antibiotics and extracellular enzyme screenings). For genotypic characterization, genomic DNA was isolated and 16S-ITS rDNA region was amplified.

Differentiation was achieved according to Restriction Fragment Length Polymorphism (RFLP) method by using *Hae* III and *Taq* I endonucleases. Isolates, which represented the different RFLP patterns, were chosen for building up the haplotype groups.

As a result of the study seven different RFLP haplotypes were identified. Moreover, 16S ribosomal DNA partial sequencing was also performed on some of the strains in. These haloalkalophilic microorganisms and their enzymes could be used in different biotechnological studies in the future for various industrial applications.

ÖZ

Haloalkalofilik mikroorganizmalar yüksek alkali ve tuzlu koşullara adapte olmuş ekstremofilik canlılardır. Birçok farklı tür toprak ve soda gölü örneklerinden izole edilmiştir. Haloalkalofilik mikroorganizmalar tuzun zararlı etkilerin ve hücre içi pH değerlerini düzenleme yeteneğine sahiptirler. Halofilik ve alkalofilik olan bu organizmaların ürettiği ekstraselüler enzimler endüstride farklı amaçlar için kullanılanılabilir. Haloalkalofilik mikroorganizmaların yaşadıkları habitatlardan izole edilmeleri ve genotipik tanımlamalarının yapılması endüstri alanında kullanım amaçlı olarak genetik potansiyellerinin tanınması ve ileride yapılacak biyoteknolojik çalışmalar adına atılacak ilk adımı oluşturmaktadır.

Deri fabrikaları işlem basamakları ve atık suyu bu çalışma için örnekleme alanı olarak belirlenmiştir. Bu amaç için Horikoshi-I besi yerine %12 tuz ilave edilerek kullanılmıştır. İzolasyon aşamasının ardından organizmaların saflaştırma işlemi tamamlanmıştır. Fenotipik testler uygulanmıştır. Bunlar;Gram boyama, katalaz ve oksidaz testi, faz-kontrast mikroskobu ile spor gözlemi, ekstraselüler enzim taraması, farklı tuz konsantrasyonlarında (%0, %20, %25) gelişme, farklı sıcaklılarda (45-50 °C) gelişme, farklı pH (7-10-12) değerlerinde gelişme özellikleri). Genotipik karakterizasyon için genomik DNA izolasyonunun ardından 16S-ITS rDNA amplifikasyonu yapılmıştır. RFLP için iki farklı restriksiyon enzimi kullanıldı (*Hae* III and *Taq* I). Kesim ürünlerinin analizi yapılmıştır. Farklı jel profillerine sahip olanların oluşturduğu gruplar her iki enzim için de belirlendikten sonra grup temsilcileri kullanılarak dendogramlar oluşturulmuştur. Hae III restiriksiyon enzim kesimi sonucunda elde edilen grup temsilcilerinin 16S rDNA - PCR amplifikasyon ürünleri kısmi dizi analizleri için kullanıldı.

Haloalkalofilik mikroorganizmalar ve enzimleri daha ileride yapılacak biyoteknolojik çalışmalarda kullanılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES.. xi

LIST OF ABREVIATIONS xii

Chapter1 INTRODUCTION.. 1

1.1.Definition of Haloalkalophiles.. 1

1.2.Haloalkalophilic Microorganisms.. 1

1.2.1. Eubacterial Haloalkalophiliphiles.. 2

1.2.2. Haloalkalophilic Bacillus.. 2

1.2.3. Gram NegativeHaloalkalophilicSpecies.. 3

1.2.4. Archaeal Haloalkalophilic Species.. 3

1.2.5. Distribution and Isolation.. 5

1.2.6. Biotechnological and Industrial Applications.. 5

1.2.7. Extracellular Enzymes and Their Industrial Application.. 6

1.2.8. Proteases from Haloalkalophilic Bacteria.. 6

1.2.9. Lipases from Haloalkalophiles.. 8

1.3. Identification of the Haloalkaolphilic Organisms.. 9

1.3.1. Phenotypic Methods.. 9

1.3.2. Molecular Characterization Methods.. 10

1.3.2.1. Pulsed Field Gel Electrophoresis.. 10

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD).. 11

1.3.2.3. 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP.. 11

1.3.2.4. Plasmid Profiling.. 12

1.3.2.5. Ribotyping.. 12

1.3.2.6. DNA Sequencing.. 15

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir) 15
1.5.Thesis Objective 16

Chapter 2 MATERIAL METHODS 17
2.1. Materials 17
2.1.1. Chemicals 17
2.1.2. Samples used for isolation 17
2.2. Methods 17
2.2.1. Isolation and Preservation of the Isolates 17
2.2.1.2. Isolation of Haloalkalophilic Bacteria 17
2.2.1.3. Preservation and Activation of Isolates 18
2.2.2. Determination of Phenotypic Characteristics 18
2.2.2.1. Gram Staining 18
2.2.2.2. Examination of Endospores 19
2.2.2.3. Catalase Test 19
2.2.2.4. Oxidase Test 19
2.2.2.5. Growth at Different Temperatures 19
2.2.2.6. Growth at Different NaCl Concentrations 19
2.2.2.7. Growth at Different pH Ranges 20
2.2.3. Screening for Extracellular Enzymes 20
2.2.3.1. Screening for Protease Activity 20
2.2.3.2. Screening for Lipase Activity 20
2.2.4. Preparation of Genomic DNA 20
2.2.5. Genotypic Characterization 21
2.2.5.1. Identification of Isolates by 16SrDNA–ITS Region 21
2.2.5.1.1. Amplification of 16S rDNA – ITS Region 21
2.2.5.1.2. Electrophoresis of Amplified 16S-ITS Fragments 22
2.2.5.1.3. Chloroform Extraction of PCR Products 23
2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP) 23

2.2.5.1.5. Electrophoresis of Restriction Fragments........... 23
2.2.6. Sequencing... 24
2.2.6.1. Amplification of 16SrRNA Gene 24
2.2.6.2 Extraction of DNA Fragments from Agarose Gel 25
2.2.6.3 Sequencing Reactions 24
2.2.6.4. Purification of Sequencing Reactions.................................. 25
2.2.6.4.1. Removal of unincorporated dye terminators using ethanol presipitation 26
2.2.6.4.2. Assembling the SEQ Sequencer 26

Chapter 3 RESULTS AND DISCUSSION 28
3.1. Isolation of Haloalkalophilic Bacteria 28
3.2. Determination of Phenotypic Characteristics 28
3.2.1. Gram staining... 28
3.2.2. Examination of Endospore.. 28
3.2.3. Catalase and oxidase tests.. 28
3.2.4. Physiological Tests 29
3.3.Extracellular Enzyme Screening.. 32
3.3.1. Extracellular Enzyme Profiles of Isolates.. 33
3.4. Genotypic Characterization... 36
3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon 36
3.4.2. 16S-ITS rDNA Based RFLP Profiling 37
3.4.3. Analysis of the Genotypic and Phenotypic Groups 40
3.4.4 Analysis of Sequencing Reactions.. 41

Chapter 4 CONCLUSION AND FUTURE PERSPECTIVE....................................... 42
REFERENCES 43
APENDICES AA1
APENDIX A. CHEMICHALS USED AA1
APENDIX B. RECIPIES MEDIA USED FOR EXPERIMENTS AB1
APPENDIX C. BUFFERS AND STOCK SOLUTIONS..AC1
APPENDIX D. STAINS AND INDICATORS AD1

APPENDIX E. PCR RECIPIES AE1
APPENDIX F. OLIGONUCLEOTIDE PRIMERS.. AF1
APPENDIX G. RECOGNITION SITES OF RESTRICTION ENZYMES................AG1
APPENDIX H. ISOLATE NAMES AH1

LIST OF FIGURES

Figure 1.1. Archaea kingdom 4

Figure1.2. Rybotyping 14

Figure3.1. Apperance of extracellular protease 36

Figure 3.2. Apperance of extracellular lipase 36

Figure 3.3. 16S rDNA-ITS Amplification products of some isolates 36

Figure 3.4. *Hae* III and *Taq* I Digests of 16S-ITS rDNA Fragments of Representative Isolates 37

Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles of Isolates 38

Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles of Isolates 38

LIST OF TABLES

Table 1.1. Tolerance for pH, NaCl of selected species of the genus Bacillus 2

Table 1.2. "Natro group" of Halobacteria 5

Table 1.3. Detergent Proteases 7

Table.1.4. Lipolytic activity 9

Table 2.1. Samples used for isolation 17

Table 3.1. Results of the Physiologic test 29

Table.3.2. Tolerance for pH, NaCl of isolated strains 30

Table 3.3 Extracellular Enzyme Tests 33

Table 3.4 Extracellular Enzyme Profiles of isolates 34

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA 38

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups 39

Table 3.7. Analysis of the Genotypic and Phenotypic Groups 40

Table 3.8. Sequencing Results 41

ABBREVIATIONS

A	Adenine
bp	Base pair
C	Cytosine
CGT	Cyclodextrin Glucosyltransferase
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
G	Guanine
EDTA	Ethylenediamine Tetra Acetic Acid
ITS	Internal Transcribed Spacer
ISR	Internal Spacer Region
kb	Kilo base
PAGE	Polyacrylamide Gel Electrophoresis
P.E.	Pectinolytic Enzyme
PGA	Polygalacturonic Acid
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
T	Thymine
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA

CHAPTER 1

INTRODUCTION

1.1. Definition of Haloalkalophiles

Haloalkalophiles are one of the main physiological groups of microorganisms classified in alkaliphiles. Lake Magadii in Kenya, Wadi Natrun in Egypt have been defined as the most stable alkaline environments on earth. Therefore the first ecological studies related to haloalkalophilic microorganisms have been performed in these places. The conditions of high mineralization and/or alkalinity in such environments regulate their prokaryotic communities (Trotsenko and Khmelenia, 2001). Both alkalinity (pH 9) and salinity up to saturation (33% (wt/vol)) are needed for the survival of these microorganisms (Koki Horikoshi, 1999).

Alkaliphiles can be classified as obligately and facultatively alkaliphilic organisms. Obligately alkaliphilic microorganisms cannot grow below pH 9. Facultatively alkaliphilic organisms show optimal growth at pH 10.0 or above but they also have the ability to grow at neutral pH.

Halophilicity divided the organisms into three categories: slightly halophilic, moderately halophilic and extremely halophilic. Halophilic microorganisms require certain concentrations of NaCI for their growth, while the halotolerant ones have the ability to grow in the absence or presence of higher concentrations of NaCl (Mimura and Nagata, 2000).

1.2. Haloalkalophilic Microorganisms

Haloalkalophilic microorganisms inhabiting extremely alkaline and saline environments are found in both Archaea and Eubacteria. Most of the extremely salt tolerant haloalkaliphiles belong to *Halobacteriaceae*.

1.2.1. Eubacterial Haloalkalophilic

1.1.2.1 Haloalkalophilic *Bacillus*

Genus *Bacillus* can be characterized by unique physiological characteristics such as Gram staining, spore examination, catalase and oxidase tests. *Bacillus* is a Gram positive usually catalase and oxidase positive and spore producing organism that classified in Firmiculates. It is well known that *Bacillus* strains do not form endospores under many environmental conditions, and the ability to form spores is often difficult to demonstrate. This is very important for their taxonomy. There have been organisms described as non-spore-formers, which had to be reclassified after the detection of spores (Fritze and Claus, 1992). Recent taxonomic studies have separated some of the *Bacillus* members and grouped into new genera: *Alicyclobacillus, Aneurinibacillus, Brevibacillus, Gracilibacillus, Halobacillus, Paenibacillus, Virgibacillus* (Baumgart, 2003)

Table1.1. Tolerance for pH and NaCl of selected species of the genus *Bacillus.*

Species	Source	pH Tolerance	NaCl Tolerance
B. cohnii	Horse meadow soil	obligate alkalophilic	5%
B. horikoshii	Soil	obligate alkalophilic	17%
B. marismortui	Dead sea water	6.0-9.0, pH optimum 7.5	5-25%
B. agaradhaerens	Soil	obligate alkalophilic	16%
B. aclarkii	Soil	obligate alkalophilic	16%
B. horti	Soil, Japan	alkali tolerant	10%
B. vedderi	Bauxite waste	obligate alkalophilic	7.5%
B. alkalophilus	Soil and faeces	obligate alkalophilic	8%
B. clausii	Garden soil	alkali tolerant	10%
B. haloalkaliphilus	Brine/mud, Wadi Natrun	obligate alkalophilic	25%
B. halodurans	Soil	obligate alkalophilic	12%
B. pseudoalkaliphilus	Soil	obligate alkalophilic	10%
B. pseudofirmus	Lake bank soil	obligate alkalophilic	17%
Bacillus sp. DSM8714	River bank soil	alkalitolerant	10%
Bacillus sp. DSM8717	Horse and elephant manure	alkalitolerant	10%
Halobacillus halophilus	Salt marsh soil and solar salterns	7.0-9.0	15%

1.2.3. Gram Negative Haloalkalophilic Microorganisms

Halomonadaceae members are slightly and moderately halophilic bacteria with a broad pH tolerance. They are isolated from saline water, soil samples, leather processing, seawater and estuarine water. Species belong to the Halomonadaceae are Gram negative, rod shaped, non-spore forming, aerobic and also facultatively anaerobic in the presence of nitrate. (Dobzon and Franzman 1996; Franzman et al., 1988). By 16S rDNA sequence analysis, chemotaxonomic and physiological characteristics *Halomonas* have been defined as the only genus in Halomonadaceae (Dobson et al., 1996; Franzman and Tindall 1990). However, recently it has been proposed that Halomonadaceae has four different genera; *Halomonas*, *Alkanivorax, Carnivoras, Chromohalobacter* and *Zymobacte* (Bromguarte, 2003).

1.2.4. Haloalkalophilic Archea

Bacteria and archaea have been first recognized as two distinct groups of Prokarya by Woese et al. (1997). The original classification based on 16S ribosomal RNA gene sequence has been confirmed by extensive phylogenetic studies (Rosa et al., 2002). Extreme halophiles or Euryarchaeota require high salt concentrations, up to 20% NaCl for growth.

Archaea possess genes with recognizable counterparts in the Bacteria, showing that the two groups have functional similarities. Archaea also possess genes that also found in Eukarya (Rosa et al., 2002).

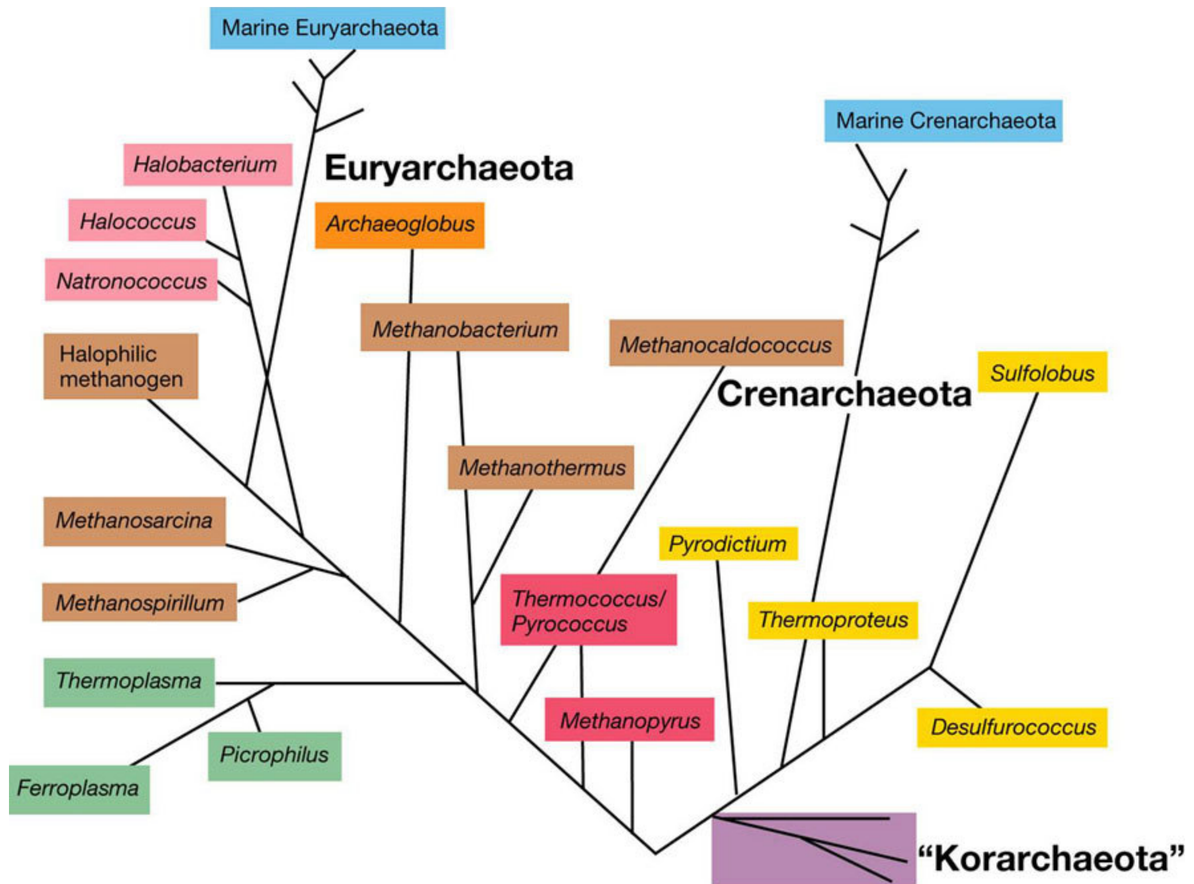


Figure1.1 Archaea kingdom

Euryarchaeaota (*Natronobacterium, Natronococcus, Natronomonas, Natrialba*, *Natronorubrum* genera are classified in "Natro group" of *Halobacteria*. These microorganism are extremely halophilic and alkaliphilic.

Table1.2. "Natro group" of Halobacteria.

Genus	Morphology	Number of species	Habitat
Natronobacterium	Rods	1	Highly saline soda lakes
Natrinema	Rods	2	Salted fish;hides
Natrialba	Rods	2	Soda lakes; beach sand
Natronomonas	Rods	1	Soda lakes
Natronococcus	Cocci	2	Soda lakes
Natronorubrum	Flattened cells	2	Soda lakes

1.2.5. Distribution and Isolation

Isolation of haloalkalophilic microorganisms is performed using different medium ingredients with respect to the group of target organism and the target environment. Actually the salt tolerance range plays an important role because the extremely halophilic organisms can not grow in low concentrations of salt although they have the ability to grow in minimal media. These microorganisms can live at high concentrations of different salts therefore they are populated in salt lakes. Limnological studies focus on the life conditions for extremely halophilic members during seasonal cycles. Anaerobic conditions are suitable in the presence of nitrate for facultatively anaerobes of eucaryotic members.

1.2.6. Biotechnological and Industrial Applications

Extremophilic microorganisms produce cell-associated products that can be used in different industrial applications. First of all their stability and activity in harsh conditions such as alkaline, acidic, high temperature, high concentration of salinity enables broad applications. Extracellular enzymes produced by these organisms are of great interest in biotechnology. Many *Bacillus* species are known to produce alkaline and saline stable proteases, amylases, cellulases, lipases, pectinases and xylanases (Martins et al., 2001)

Enzyme characterization studies with amylase from *Halobacillus* sp. strain MA-2, (Amoozegar et al., 2003) and *Halomonas mediteridiana* (J.Nieto et al., 1999) have

been known. Also the extracellular enzymes from archaeal members have unique features in their molecular structures.

Halophilic microorganisms also used for biological treatment (Kargı and Uygur, 1995).

Halophilic exopolysaccharides have been used in food manufacturing. Furthermore, there is a mereaning demand for non-toxic, biodegradable, environmentally friendly substances. *Halomonas maura* have been studied with novel composition exopolysaccharide (Arias et al., 2003).

1.2.7. Extracellular Enzymes and Their Industrial Application

Extracellular enzymes produced by haloalkalophilic microorganisms have unique properties. They have activity and stability at broad pH ranges and high concentrations of salts in that many other proteins are denaturated. Enzymes from archeal and eubacterial haloalkalophilic organisms have different characteristics. Archaeal enzymes need high concentrations of salt for their activity and stability (Mevarech et al., 2000). This is explained by the comparison of molecular structures from halophilic enzymes with those of nonhalophilic enzymes, this feature is related with adaptation mechanisms used for the protecting of cell from the denaturing effects of salt molecules (Ventosa et al., 1998)

1.2.8. Proteases from Haloalkalophilic Bacteria

Proteases are the enzymes that cleave protein molecules by hydrolysis. The first classification is made by using their cleavage mechanism. Exopeptidases work on the peptide bonds near the amino or carboxy-termini but endopeptidases are active on the peptide bonds distant from the termini of the substrate. The subclassification is based on their catalytic mechanism. Chemically different groups are found on active sites. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao et al., 1998). Subtilisins were grouped in serine type proteases. Catalytic triad of subtilisins consists of aspartic acid, histidine and serine. Although the size of subtilisins varies from 18 kDa to 90 kDa, all the subtilisins used in detergents have a size of

approximately 27 kDa. Most of the alkaline proteases were subtilisins. At present, less than 15 different enzyme molecules are used in detergents worldwide. These enzymes originate from *B. amyloliquefaciens, B. licheniformis, Bacillus clausii, Bacillus lentus, Bacillus alkaloophilus,* and *Bacillus halodurans*

The success of subtilisins is based on several factors, including their high stability and relatively low substrate specificity, features common in extracellular proteases. Their production as extracellular enzymes is of course an important factor in itself, as this greatly simplifies the separation of the enzyme from the biomass and facilitates other downstream processing steps. Another important point is the ability of *Bacillus* strains to secrete enzymes over a very short period of time into the fermentation broth.

Detergent industry prefers the enzymes, which are stable at high pH and compatible with chelating and oxidizing agents (Rao et al., 1998). Table represents the detergent proteases available (Maurer, 2004).

Table1.3. Detergent proteases (Maurer, 2004).

Trade mark	Origin	WT/PE	Production strain	Synonym
Genencor	*B. amyloliquefacies*	PE	*B. subtilis*	
Novozymes	*B. clausii*	WT	*B. clausii*	Subtilisin 309
Genencor	*B. lentus*	WT	*B. subtilis*	
Kao	*B. alkalophilus*	WT	*B. alkalophilus*	
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Genencor	*B. lentus*	PE	*B. subtilis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Henkel	*B. lentus*	PE	*B. licheniformis*	
Novozymes	*B. halodurans*	WT	*B. halodurans*	Subtilisin 147
Novozymes	*B. clausii*	PE	*B. clausii*	
Genencor	*B. alkalophilus* PB92	PE	*B. alkaliphilus*	

PE, protein engineered; WT, wild type.

Since 1997, several gene-shuffling approaches have been performed with subtilisins. Interesting results from the shuffling of 26 protease genes have been described for properties such as activity in organic solvents, temperature stability, and activity at high or low pH (Minshull et al., 1999). Little, however, has been published on stain removal. Owing to the large number of variant molecules generated by

shuffling and other random techniques, screening methods with high-throughput and increased relevance have had to be developed. Unfortunately, these methods are still not entirely satisfactory, which might explain why no outstanding new subtilisin variant created by one of the gene shuffling technologies is yet present in detergents. Subtilisins for detergents are produced in *Bacillus*, because these species are able to secrete large a mounts of extracellular enzymes. The control mechanisms involved in the production of proteases in Bacillus are extremely complex and still not fully understood. An example is the two-component regulatory system that acts as a quorum sensing mechanism in *B. Subtilis* and which has been found to control the expression of the alkaline protease. This regulatory system is encoded on the chromosome and on endogenous plasmids. (Maurer, 2004)

Arhaea members have proteolytic activity in high salt concentrations (Eichler, 2001). Proteases from *Natronococcus occultus* and *Natrialba magadi* have been characterized (De Castro et al., 2001).

Leather industry uses proteases at dehairing and baiting steps (Kumar and Takagi, 1999). Stable proteases under alkaline and saline conditions would be most effective enzymes for such purposes.

It has been reported that Halophilic bacteria destroied brine-cured hides with their high proteoliytic activity (Bailey et al., 1998).

The studies about using proteases and gelatinases for the pretreatment of leather waste including cromium are important for the convertion of waste material to animal feed (Taylor, 2002).

Silver recovery has been achieved with different types of alkaline proteases at high alkaline conditions (Singh et al., 1999, Kumar and Takagi, 1999).

1.2.9. Lipases from Haloalkalophililes

Lipases, (triacylglycerol acylhydrolases, EC 3.1.1.3), are able to catalyse both the hydrolysis and the synthesis of esters bonds in lipid molecules. Lipid molecules are composed of glycerol and long-chain fatty acid molecules. Esterification and hydrolysis reactions that can occur in organic solvents are very important (Ishıkawa and Ogino,

2000). Most of the lipolytic enzymes used commercially are produced by alkalophilic microorganisms and they are used as detergent addiditives (Sharma et al. 2001).

Table.1.4. Lipolytic activity

Active site	Catalytic triad consisting of the amino acids serine, aspartic (or glutamic acid) and histidine; homologue to the triad of serine proteases.
Substrates	Oils (olive, soybean, mineral oils),Triglycerides (triolein, tributyrin), Synthetic surfactants (Tweens 20 and 40).
Other Activities	Phospholipase, lysophospholipase, cholesterol esterase, cutinase, amidase.

Lipases from *Halomonas* strains have been characterized (Mattiasson et al., 2004).

Alkaline lipases are mainly used in detergent industry. Approximately 1000 tons of lipases are added to the 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Alkaline lipases to be used in detergents should meet certain requirements. They should hydrolyze fats of various compositions, be stable in harsh washing conditions (pH 10-11, 30-60 °C) and in the presence of surfactants (linear alkyl benzene sulfonates (LAS) and proteases) (Sharma *et al.,* 2001).

1.3. Identification of the Haloalkalophilic Organisms

Identification of the haloalkalophilic microorganisms are performed by using the phenotypic and genotypic characteristics.

1.3.1. Phenotypic Methods

Morphological characteristics can be examined by direct observation, which give simple information on the organism for example colony formation that includes colony shape, diameter and color. Furthermore, microscopic observations after different chemical treatments give detailed information on the cell morphology. These are cell shape, Gram behavior and sporulation type. Phase contrast microscopy gives

information on the cell shape and sporulation type without any chemical treatment. Some tests can provide information about the adaptive mechanisms and relation between interior of the cell and the medium. These tests conditions are: growth at different pH ranges, temperatures, pressure and salt concentrations. Bergey's Manual of Systematic Bacteriology a major taxonomic compilation of prokaryotes gives information in all recognized species of prokaryotes and contains number of keys to evaluate the properties given above (Madigan *et al.,* 1997).

Phenotypic methods also include biotyping, antibiogram, phage typing, serotyping, PAGE/immunoblotting and multilocus enzyme electrophoresis (Busch and Nitschko, 1999).

Although molecular characterization methods provide clear differentiation and precise identification, using of phenotypic characterization methods combined with molecular techniques are required for taxonomic studies and functional purposes.

1.3.2. Molecular Characterization Methods

1.3.2.1. Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is used for the analysis of microbial genomes and molecular characterization of the microorganisms below species level. It enables to the seperation of large DNA molecules and therefore whole genomic DNA can be analysed. Analysis of chromosomal macrorestriction patterns by PFGE is a powerful method to produce fingerprints of closely related strains (Farber, 1996). Application of the method can differ for different type of organism. Different organism can require different cell disruption treatments for the releasing the genomic DNA. Also the size and content of genomic DNA can differ among the organisms. Thus, optimization parameters such as lysis of cells, electrophoresis conditions (pulse time, voltage) are the main points for obtaining fine discriminative PFGE results.

1.3.2.2. Randomly Amplified Polymorphic DNA (RAPD)

DNA fragments are amplified by the polymerase chain reaction (PCR) using short (generally 10 bp) synthetic primers of random sequence. These oligonucleotides serve as both forward and reverse primer at the same time, and are usually able to amplify fragments at 3-10 genomic sites simultaneously. Amplified fragments (within the 0.5-5 kb range) are separated by gel-electrophoresis and polymorphisms are detected as the presence or absence of bands of particular size. These polymorphisms are considered to be primarily due to variation in the primer annealing sites. Some advantages of RAPD are as follows: low quantities of template DNA are required (5-50 ng per reaction), no sequence data for primer construction are required, easy and quick to assay, low costs involved, generation of multiple bands per reaction, and it suitable for automation.

1.3.2.3 16S rDNA – ITS (Internally Transcribed Spacer) Region RFLP (Restriction Fragment Length Polymorphism)

In most prokaryotes the ribosomal RNA genes reside sequentially in the chromosome (16S-23S-5S) and they are transcribed as a single polycistronic RNA molecule (Luz et al., 1998). These ordered genes have long been served as a genetic marker to study microbial diversity. The 16S rRNA gene sequence has been used for assessing bacterial phylogenies (Abd-El-Haleem et al., 2002). However, it has only provided information useful for identifications at genus level (Shaver et al., 2002).

Below the genus level, additional rDNA sequences have been necessary. The use of 23S rDNA has also been limited by its larger size and insufficient sequence data (Abd- El-Haleem et al., 2002). The intergenic spacer region (ISR) or also known as intergenic/internal transcribed spacer (ITS) (Abd-El-Haleem et al., 2002; Daffonchio et al., 2000; Shaver et al., 2002; Fisher and Triplett, 1999; Toth et al., 2001) between 16S and 23S rDNA genes, includes both conserved and highly variable sequence motifs, such as tRNA genes, and anti-terminators (boxA) (Abd-El Haleem et al., 2002). ISRs are generally found in multiple copies in most bacteria genomes. Since they are

hypervariable with respect to adjacent genes, they can differentiate between multiple operons in the cell (Daffonchio et al., 1998). The size of the spacer has been found to differ considerably among different species, and even among the different operons within a single cell (Garcia-Martinez et al., 1999).

Figure 1.8 Schematic representation of a 16S-23S spacer and organization of ITS functional regions (Garcia-Martinez et al., 1999).

The method is based on the amplification of 16S rDNA and ISR regions as singleamplicon. The resulting amplicon is then digested with frequent cutting enzymes. Finally restriction fragments obtained are separated by agarose gel electrophoresis and restriction patterns are then compared. The method could discriminate different groups of bacteria at species level.

1.3.2.4. Plasmid Profiling

Plasmids are the genetic materials of bacteria carried as extracromosomal elements. They are self-replicative and they can be transferred naturally between the microorganisms. Genes coded in plasmid DNA structures give different characteristics to microorganisms.

Plasmids isolated from the microorganisms give variable profiles with respect to the organism type and the habitats. Therefore the gel profiles of plasmids for closely related organism would be useful in taxonomic studies (Farber, 1996).

1.3.2.5. Ribotyping

Nucleic acid probes that recognize ribosomal genes are used for ribotying. Ribosomal RNA (rRNA) genes are composed of 23S, 16S and 5S regions. They are known to be highly conserved. Whereas most of the bacterial genes present in one copy, rRNA operons can be present in anywhere more than one copy (2-11 copies per bacterial cell). Thus the more copies of rRNA operon enables more discriminatory riboyping. In practice, first bacterial DNA is isolated and digested by restriction endonucleases. After agarose gel electrophoresing, DNA is transferred onto nylon or nitrocellulose membrane by capillary system or electrophoresis. The nylon membranes

are used as a solid support for probing. Probes specific to 23S, 16S, 5S rRNA genes are labeled using radioactive or non radioactive labeling techniques (non isotopic cold-labelling systems). Hence fingerprint patterns of bacterial DNA containing ribosomal genes are created. These patterns are varied from 1 to 15 bands and compared among the isolates. For another ribotying technique called as chemiluminescence ribotyping, digoxigenin-labeled cDNA (DNA which has been made by reverse transcription of rRNA) can be seen in figure 2. One of the major advantages of ribotyping is that because of the similarity of ribosomal genes, universal probes can be used. Reproducibility is also another advantage of this genotypic method.

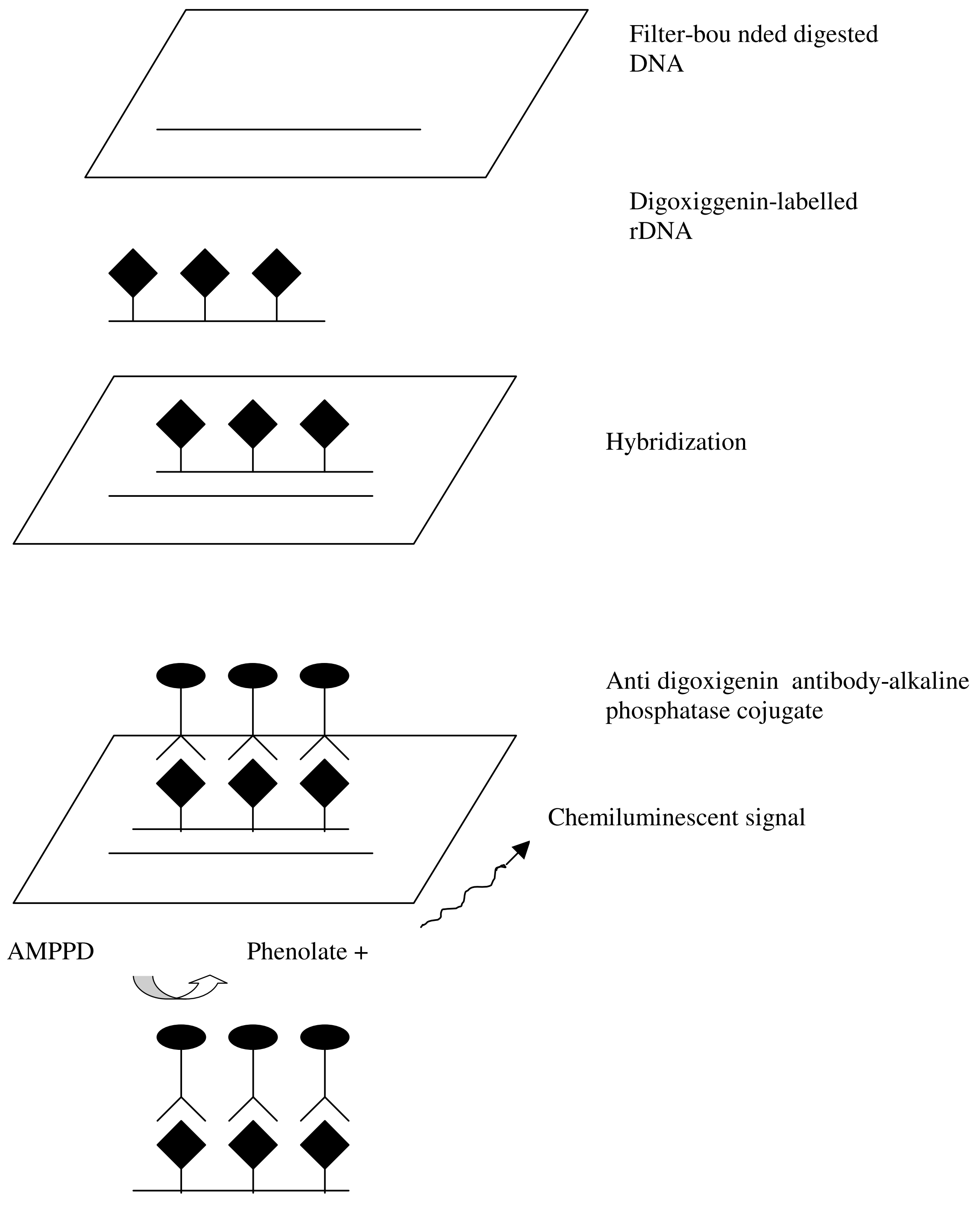


Figure1.2. Schematic representation of chemiluminescence ribotyping. AMPPD, adamantly-1, 2-dioxetane phosphate (Bingen et al., 1994)

1.3.2.6. DNA Sequencing

DNA sequencing is used for investigation of the phylogenetic relationship of organisms. This tecnique is based on the exact order of nucleotides in a region of the DNA molecule Automated DNA sequencing generally includes PCR amplification of a DNA region of interest, computer-aided sequence analysis and interpretation. For DNA sequencing the region of the chromosome to be analysed must be short consisting of a variable sequence flanked by highly conserved region. The variability within the selected sequence must be sufficient to differentiate microorganisms. And also the selected sequence should not be horizontally transmissible to other strains of species (Olive and Bean, 1999).

1.4. Brief Information About the sampling Area (Leather Factories Menemen-İzmir)

Leather factories of Ege Region are populated in Menemen – İzmir. twenty different companies have leather factories in Menemen region. It is known that industrial activities bring the most important pollution problems. For this reason leather factories were placed far from the centre of the city. Leather processing steps are as fallows:

- Curing
- Beamhouse operations which wash and soak the hides or skins and (at most tanneries) remove the attached hair.
- Tanyard processes in which the tanning agent (primarily chromium) reacts with and stabilizes the proteinaceous matter in the hides or skins.
- Finishing or post-tanning processes.

Waste Water of the leather factories are removed from the other factories at Menemen region and a simple waste water treatment is applied. The classical scheme is given below (UNEP/IEO, 1994).

- Pre-treatment: mechanical screening to remove coarse material.

- Primary treatment: sulphide removal from beamhouse effluents; chrome removal from tanning effluents; flow equalization; physical-chemical treatment for

-BOD removal and neutralization.

- Secondary treatment, usually biological.
- Tertiary treatment, including nitrification and denitrification.
- Sedimentation and sludge handling

1.5Thesis Objectives

The purpose of this study was,

- Isolation of haloalkalophilic strains
- Screening for their extracellular enzymes: protease and lipase
- Characterization of these isolates by phenotypic and genotypic methods.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used were listed in Appendix A.

2.1.2. Samples used for isolation

Table 2.1. Samples used for isolation

Sample Type	Location
Polluted Soil	Leather Factories waste (İzmir – Menemen)
Leather processing	Leather Factories (Izmir-Menemen)

2.2. Methods

2.2.1. Isolation and Preservation of the Isolates

2.2.1.2. Isolation of Haloalkalophilic Bacteria

Dilution plate method was used for the isolation of haloalkalophilic bacteria (Mora et al., 1998). Ten g soil, leather or 10 ml of water samples were transferred in 90 ml of 0.85% saline water. In order to homogenize the samples 6h incubation was performed in orbital shaker at room temperature. The samples were pasteurized for 10 m. at 80°C in a water bath in order to kill most of the vegetative cells and eliminate non-

spore forming bacteria (Mora et al., 1998). After the heat treatment, for dilution, 1ml aliquots from each sample was transferred in 9 ml of 0.85 % saline water and 6 fold dilutions were prepared. One ml of the dilutions was plated on appropriate solid media (Appendix B.1) and incubated for 5 days at 37 °C. Single colonies were picked and they were purified using streak plate method.

2.2.1.3. Preservation and Activation of Isolates

Cultures were grown in Horikoshi-I (Apendix B.1) agar plates containing 12% NaCl and incubated for 2 days at 37°C they were then transferred into the isolation broth. After turbidity formation in isolation broth, 0.5ml of each culture were transferred into cryotubes and 0.5ml of isolation broth containing 20% glycerol was added. These steps were all performed on ice. Then tubes were mixed gently by pipetting. Cultures were stored at -20°C for an hour they were then stored at -80°C. For the phenotypic test culture activation was applied from 20% glycerol stocks. For phenotypic and genotypic identification of the isolates, cultures were prepared from these stocks.

2.2.2. Determination of Phenotypic Characteristics

2.2.2.1. Gram Staining

Isolates were stained by modified Gram method for halophilic bacteria. Eighty µl of sterile 0.85% saline water were pipetted onto slides the organisms were then suspended and mixed thoroughly. Overnight culture was spread onto the microscope slides until a thin film formed by using tooth stick. After being air dried, the slides were fixed and desalted simultaneously by immersing in 2% acetic acid for 5 min. Then they were dried again. The smears were covered with 0.25% aqueous solution of crystal violet (Apendix D.2.1) for 1 min. After rinsing under the tap water, the slides were transferred into iodine (Apendix D.2.2) solution and were kept for 1 min. Following this step, slides were washed in alcohol for 6 s. They were then stained with safranin (Apendix D.2.3) for 30 s. After staining, the slides were dried on paper towels and the

cells were examined under light microscope. Gram (+) cells assumed purple color while Gram (-) cells appeared pink or red.

2.2.2.2. Examination of Endospores

Isolates were grown on Horikoshi-I (Apendix B.1) agar plates and 0.0005% $MnCl_2$ (Travers, 1987) were added in to the medium. Observations for spore formation were monitored at 3-4- 5-6th day.

2.2.2.3. Catalase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. Catalase activity was observed after dropping 3% hydrogen peroxide solution onto the target colony. Formation of the air bubbles indicated the presence of catalase activity.

2.2.2.4. Oxidase Test

Isolates were grown on Horikoshi-I (Apendix B.1) agar for 24 h at 37°C. 1% solution of tetramethyl-p-phenylenediamine was poured onto a filter paper placed in a petri dish. Then the target colony was taken from the agar plate and spotted onto wet filter paper. Observation of the blue-purple color indicated the oxsidase activity.

2.2.2.5. Growth at Different Temperatures

The isolates were grown on nutrient agar (Apendix B.2) 45°C and 50°C. Observations for growth were noted during the 5-day incubation.

2.2.2.6. Growth at Different NaCl Concentrations

Cultures were tested for their salt tolerance on Horikoshi-I (Apendix B.1) agar plates containing 0%, 20%, 25 % NaCl during the 5-day incubation.

2.2.2.7. Growth at Different pH Ranges

Cultures were tested for pH tolerance in nutrient agar plates at different pH points (7, 10, and 12). Growth behaviors were observed during the 5-day incubation

2.2.3. Screening for Extracellular Enzymes

2.2.3.1. Screening for Protease Activity

Proteteolytic activity of the isolates observed by adding casein into Horikoshi–I (Apendix B.1) medium adding sikimmed milk. Different concentrations of NaCl (12% and 20%) were added. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete proteases extracellularly, give clear zones around the colony (Horikoshi, 1999).

2.2.3.2. Screening for Lipase Activity

Lipolytic activity of the isolates was observed by using the medium described in Appendix B.5. Agar plates were incubated for 5 day at 37ºC. The isolates, which secrete lipase extracellularly, gave opaque zones around the colony (Haba et al., 2000).

2.2.4. Preparation of Genomic DNA

Cultures were grown on solid Horikoshi – I (Apendix B.1) medium. Cells were suspended in 1.5 ml of sterile water in petri dishes. Then cell suspension was transferred into eppendorf tubes. In order to prepare cell pellets, tubes were centrifugated for 5 min at 5000 rpm. Pellets were resuspended in 567 µl 1Xte (Apendix C.4). Thirty µl 10% SDS and 3µl proteinase K solution (20 mg/ml) were added and the solution was mixed thoroughly. Cell lysis was performed with 1h incubation at 37°C. Afterwards 0.1 ml of 5 M NaCl were added and mixed well. Then 80µl of CTAB/NaCl (Appendix C.8) were added, the tubes were then mixed and incubated for 10 min at 65°C. Following the incubation, 0.7 ml of chloroform/isoamyl alcohol (Appendix C.2) were added, mixed thoroughly and centrifuged for 5 min at 10000 rpm. Aqueous, viscous supernatant was

transferred into a fresh eppendorf tube. An equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged for 5 min. The supernatant was again transferred into a fresh eppendorf tube and 0.6 volume of isopropanol were added to precipitate the nucleic acids. The tube was then shaken back and forth until a white DNA precipitate became visible. DNA wool were taken with a pipet tip and placed into a new tube, contain 300µl 70% ethanol. The tubes were stored at -20°C for 1h. Afterwards tubes were centrifuged for 15 min at 6000 rpm. Then the supernatant was discarded and DNA pellet was washed with 500 µl 70% ethanol. It was centrifuged for 10 min at 8000 rpm. After this, ethanol was discarded. The pellet was dried and dissolved in 100 µl 1xTE containing 100 µg/ml RNAase. Then tubes were incubated for 1h at 37°C. After the incubation the sample volume was adjusted to 400 µl with 1xTE. DNA was dissolved by alternating cold-heat shocks (for 10 min at 80°C and 20 min at -20°C, repeated twice). To further purify DNA an equal volume of phenol was added and mixed well. An equal volume of chloroform was added and mixed well. Then the tubes were centrifuged for 5 min at 6000 rpm. The aqueous phase was transferred into a fresh eppendorf tube. Equal volume of chloroform /isoamyl alcohol was added and mixed well. Samples were centrifuged for 5 min at 6000 rpm. Chloroform /isoamyl alcohol step was repeated once again and DNA was precipitated by adding 1/10 sample volume of 5 M NaCl and 2 volumes of 99% ethanol. Pellet was washed in 500 µl 70% ethanol by centrifugation for 5 min at 6000 rpm. After that, ethanol was removed and samples were dried for 10 min at 37°C. DNA was dissolved in 20µl, 50µl, 100µl or 200µl 1xTE according to the pellet size. Then the samples were preserved at -20 °C.

2.2.5. Genotypic Characterization

2.2.5.1. Identification of isolates by 16S rDNA - ITS rDNA - RFLP

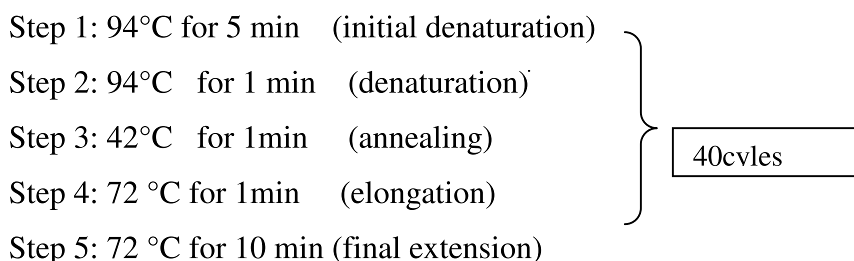
2.2.5.1.1. Amplification of 16S ITS rDNA

PCR amplification of 16S-ITS rDNA of the isolates was performed by using two DNA primers (Apendix F). The forward primer was complementary to the upstream of 16S rRNA gene and the reverse was complementary to the upstream of 23S rRNA gene.

Forward: 5. -AGAGTTTGATCCTGGCTCAG-3 (Mora et al., 1998).

Reverse: 5. -CAAGGCATCCACCGT-3 (Jensen et al., 1993).

Amplifications were performed in a Mini Cycler System. PCR conditions were as follows:



The final reaction volume was 50µl. PCR mix (Appendix E.1) was prepared after 2 µl -4µl of genomic DNA (approx. 500 µg) was placed into 1.5ml eppendorf tubes. During the preperation of the mix, tubes were kept on icebox. Taq DNA polymerase (1.25U) was added and mixed gently and then tubes were centrifuged for 2-3 s. The mix were added to each of the tube. Then 60 µl of mineral oil was added into the tubes while they were on ice. Tubes were centrifuged for 2-3 s then they were placed into the wells of Mini Cycler System (MJ Research INC, USA).

2.2.5.1.2. Electrophoresis of Amplified 16S-ITS rDNA Fragments

Amplified fragments were separated in 0.8 % agarose gel. First, 0.96 g of agarose was dissolved in 120 ml of 1×TAE buffer (Appendix C.4) by boiling (15 µl of ethidium bromide solution (10 mg/ml) were added after cooling the solution to 40°C). The gel was then poured into the gel-casting stand and the combs were placed. 1×TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. Five µl of PCR products were taken and mixed with 1 µl, 6 × gel-loading buffers. The samples were then loaded into the wells of agarose gel starting from the second well. Finally, 5 µl of DNA molecular weight marker were loaded into the first well and they

were electrophoresed at 40 mA until bromophenol blue have reached 2/3 of the gel. DNA fragments were visualized in a photodocumentation system (Vilber-Lourmat, France).

2.2.5.1.3. Chloroform Extraction of PCR Products

Final volumes of the PCR products were adjusted to 100 µl with 1×TE buffer. Then two volume of chloroform were added. After centrifugation for 2 min at 6000 rpm, organic phase was discarded and chloroform extraction was repeated. Then the tubes were centrifuged for 2 min at 6000 rpm. The aqueous phase was transferred into new eppendorf tubes containing 10 µl of 3 M sodium acetate (pH 5.2) and mixed well. After that, 450 µl of 99% ethanol were added and mixed. The samples were centrifuged for 15 min at 7000 rpm. Pellet was washed with 500 µl 70% ethanol. After centrifugation for 5 min at 6000 rpm, alcohol was removed. Pellet was dried for 10 min at 37°C, and they were dissolved in 25 µl 1xTE, and stored at 20°C.

2.2.5.1.4. Restriction Fragment Length Polymorphism (RFLP)

Purified PCR products were separated in 1% agarose gel in order to estimate DNA concentrations for each sample. Then 12.5µl of PCR products were aliquoted into new tubes for digestion. *Taq* I and *Hae* III were the enzymes chosen for this purpose. Reaction mix (Appendix F2) was added on PCR products while the tubes were stored on ice. 5 U of enzyme was used for each reaction. The final volume of the reaction was adjusted to 50 µl. Incubation period was 5 h for both of the enzymes. But the optimum reaction temperatures were 65°C for *Taq I* and 37°C for *Hae* III. Mineral oil was added over the reaction mixtures to avoid evaporations at 65°C. After the incubation DNA was extracted as described in section 2.2.6.1.3. and dissolved in 15 µl of 1xTE.

2.2.5.1.5. Electrophoresis of Restriction Fragments

Restriction fragments were first separated in 2 % agarose gel containing 20 µl of ethidium bromide solution (10mg/ml). Electrophoresis was carried out at 60 mA for the

first 20 min, then the current was adjusted to 80 mA and the electrophoresis was continued further for 2.5 h. Some isolates produced similar restriction fragment patterns. These were contained within distinct groups. One digested sample from each group was picked and re-electrophoresed in order to produce representative restriction patterns for further restriction profile analyses.

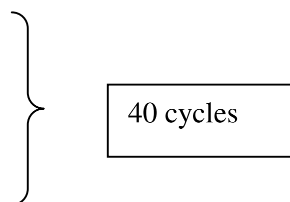
2.2.6 Sequencing

2.2.6.1. Amplification of 16SrRNA Gene

16SrDNA region of rRNA genes of the isolates were amplified using the following DNA oligoprimers for sequencing reactions
EGE 1. 5. - AGAGTTTGATCCTGGCTCAG –3 (Jensen *et al.,* 1993)
EGE 2. 5. - CTACGGCTACCTTGTTACGA –3 (Jensen *et al.,* 1993)

Two µl of genomic DNA (approx. 500 µg) were transferred into 0.5 ml eppendorf tubes, as the template. PCR mix was prepared and after the addition of *Taq* DNA polymerase (1.25 U), it was mixed gently and centrifuged for 2-3 s. Forty-eight ml of the mix were distributed into the tubes. All the manipulations were performed on ice. The tubes were then overlaid with 60µl mineral oil, centrifuged for 2-3 s at 6000 rpm. Finally, they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) using the following program:

Step1: 95°C for 5 min
Step2: 95°C for 1 min
Step3: 56°C for 1 min
Step4: 72°C for 1 min
Step4: 72°C for 10 min



2.2.6.2 Extraction of DNA Fragments from Agarose Gel

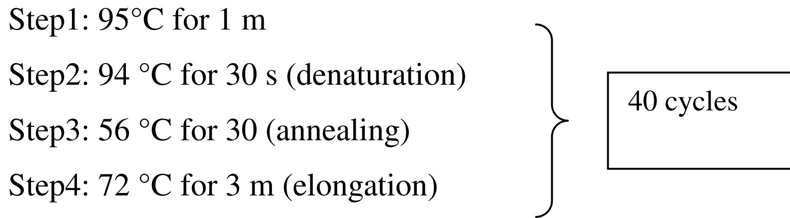
DNA isolation kit (Applichem A3421) was used for extraction of amplification products from agarose gel.

DNA bands (approximately 1600 bp) were excised from the agarose gel by a rasor blade. Three gel slice volume of 6 M NaI (for TAE gels) was added. The samples were incubated for 5 min at 55°C, mixed and continued for incubation for 2 min. Ten µl of well mixed glass powder suspension was then added, mixed well and incubated for 5 min at 55°C by occasional mixing. The samples were then centrifuged for 10 s at 10.000 rpm and the supernatant was discharged. The glass pellet was rinsed with 500 µl wash buffer. And resusupended by pipetting or gently flicking the tube. The samples were then centrifuged for 10 s at 10,000 rpm. The washing step was repeated twice. After the last wash, the supernatant was removed as much as possible. The pellet was suspended in 25 µl of 1xTE buffer. The samples were eluted for 5 min at 55°C with occasional mixing. Centrifugation was performed for 45 s at 10,000 rpm. The supernatant was finally transferred carefully into a new eppendorf tube. The eluted DNA samples were stored at -20°C until use.

2.2.6.3 Sequencing Reactions

The sequencing reactions were performed by using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit.

Four eppendorf tubes were firstly labeled as “A”, “C”, “G”, and “T” for respective termination reactions. Two µl of corresdonding Cy5.5 ddNTP termination mix were then dispensed into the appropriately labelled tubes. The tubes were then capped to prevent evaporation. The master mix (Appendix I) was prepared in an eppendorf tube for each template to be sequenced. The content of master mix was mixed thoroughly and 7µl of the mix was dispensed into each tube labelled “A”, “C”, “G”, and “T”. Each sequencing reaction was mixed thoroughly and overlaid with one drop of mineral oil. Each tube was capped and placed into the thermal cycler. The sequencing condition reastions were:



40 amplification cycles of Step 2, 3 and 4 were performed
The primer used was ;
EGE 1; 5. - AGAGTTTGATCCTGGCTCAG -3. (Jensen *et al.,* 1993)

2.2.6.4 Purification of Sequencing Reactions

2.2.6.4.1 Removal of unincorporated dye terminators using ethanol precipitation

The volume of the sample was adjusted to 50 µl by adding 1xTE. The samples under the mineral oil were then transferred into fresh eppendorf tubes. Twenty µl of 7.5 M ammonium acetate and 200 µl (3 times the reaction volume) of chilled 100% ethanol were added to each tube. The tubes were then mixed thoroughly by vortexing and placed on ice for 20 min to precipitate the DNA. Centrifugation was then performed at 12, 000 rpm for 30 min at 4°C. Supernatants were removed carefully and the pellets were washed with 200 µl of chilled 70% ethanol. After the centrifugation for 5 min, the supernatants were removed and the pellets were dried. Each pellet was then resuspended in 6 µl of formamide loading dye and vortexed vigorously (2-3 min) to ensure complete resuspension. Just prior to loading the samples onto gel, each sample was heated at 70 °C for 2-3 min for denaturation and then placed on ice. One and a half or 2µl of each sample were then loaded into the lanes of sequencing gel.

2.2.6.4.2 Assembling the SEQ Sequencer

The sequencing reactions were performed with SEQ personal sequencing system (Amercham Pharmacia Biotech). The system consists of SEQ personal sequencer, SEQ

Rapidset gel polymerizer, RapidGel QuickFill cassettes, RapidGel-XL-6% cartridge and filling gun and filling fixture.

Preparing a gel involves attaching the RapidGel-XL cartridge to the casting gun, filling the RapidGel cassettes, polymerizing the acyrlamide in the gel polymerizer. After the polymerization the comb was removed from the cassette. After cleaning the cassette, it was placed properly into the buffer chamber. At this step pre-run was performed to measure the backgraound redding. Once the gel cassette was aligned and the pre-run was complete, 1xTE buffer was loaded into the upper and lower buffer chambers until it reached the fill lines. The prepared samples (1-2 μl) were then loaded into the gel with a micropipette. Then the analysis was started. After the run was finished, data were analysed by SEQ software.

The sequences obteined were evaluated in and submitted to GenBank.

Chapter 3

RESULTS AND DISCUSSION

3.1. Isolation of Haloalkalophilic Bacteria

Haloalkalophilic bacteria were isolated from Menemen Region. Target places were leather factories and the wastes of this industry. Therefore the samples were taken from both the processing steps and waste.

3.2. Determination of Phenotypic Characteristics

3.2.1. Gram staining

The modified Gram staining method specific for halophilic bacteria gave desired results with these isolates. All the isolates were gram positive, rod shaped except one cocci (C1).

3.2.2. Examination of Endospore

Isolates were grown on agar plates (Appendix) for 5 days. Isolates were non-sporforming.

3.2.3. Catalase and oxidase tests

All the isolates were catalase and oxsidase positive.

3.2.4. Physiological Tests

Growth at pH 7 was observed to determine whether the isolates were obligate alkalophilic (Table 3.2). Four isolates could not grow at this pH value. Twenty-five of the isolates were alkali tolerant and could not grow at pH 12. Isolates were not thermotolerant since they could not grow at 45ºC and 50ºC on alkaline nutrient agar plates.

Table 3.1. Results of the Physiological Test

Physiologic test	Isolate number
No Growth at 20%NaCl	D2, D10, D20. D26, E4, E16, E24, F22
No Growth at PH 7	D1, D20, F9, F21
No Growth at 0%NaCl pH 10	D2, D6, D18, D23, E4, E43, F5, F7, F29
No Growth at pH 12	D1, D6, D10, D18, D20, D23, D24, D30, D32, E2, E4, E10, E12, E14, E16, E23, E24, E36, F7, F10, F13, F16, F20, F22, F29,

Growth of the isolates was also observed at 0% and 20% NaCl concentrations. Nine of the isolates were oblitely halophilic. Eight of the isolates were not extremely halophilic.

Table3.2. Differentiation of Isolates according to growth at different NaCl concentrations and pH ranges.

İsolate Name	20% NaCl	PH 7	0%NaCl pH 10	pH 12
D1	+	-	+	-
D2	-	+	-	+
D5	+	+	+	+
D6	+	+	-	-
D7	+	+	+	+
D10	-	+	+	-
D11	+	+	+	+
D12	+	+	+	+
D14	+	+	+	+
D18	+	+	-	-
D20	-	-	+	-
D23	+	+	-	-
D24	+	+	+	-
D25	+	+	+	+
D26	-	+	+	+
D29	+	+	+	+
D30	+	+	+	-
D31	+	+	+	+
D32	+	+	+	-
E1	+	+	+	+
E2	+	+	+	-
E3	+	+	+	+
E4	-	+	-	-
E6	+	+	+	+
E7	+	+	+	+
E9	+	+	+	+
E10	+	+	+	-
E12	+	+	+	-
E13	+	+	+	+
E14	+	+	+	-
E15	+	+	+	+

E16	-	+	+	-
E17	+	+	+	+
E20	+	+	+	+
E23	+	+	+	-
E24	-	+	+	-
E25	+	+	+	+
E26	+	+	+	+
E27	+	+	+	+
E28	+	+	+	+
E29	+	+	+	+
E30	+	+	+	+
E33	+	+	+	+
E34	+	+	+	+
E35	+	+	+	+
E36	+	+	+	-
E39	+	+	+	+
E42	+	+	+	+
E43	+	+	-	+
E47	+	+	+	+
F1	+	+	+	+
F2	+	+	+	+
F3	+	+	+	+
F4	+	+	+	+
F5	+	+	-	+
F7	+	+	-	-
F8	+	+	+	+
F9	+	-	+	+
F10	+	+	+	-
F11	+	+	+	+
F12	+	+	+	+
F13	+	+	+	-
F14	+	+	+	+
F15	+	+	+	+
F16	+	+	+	-
F17	+	+	+	+
F18	+	+	+	+

F19	+	+	+	+
F20	+	+	+	-
F21	+	-	+	+
F22	-	+	+	-
F23	+	+	+	+
F24	+	+	+	+
F25	+	+	+	+
F26	+	+	+	+
F27	+	+	+	+
F28	+	+	+	+
F29	+	+	-	-
F30	+	+	+	+
F32	+	+	+	+
F34	+	+	+	+
F35	+	+	+	+

3.3. Extracellular Enzyme Screening

Extracellular enzyme screening was performed on solid agar plates by using different substrates for each enzyme activity. Protease screening based on the observation of clear zones around the colonies (Figure). Lipase activity gave opaque zones around the colonies (Figure). Different NaCI concentratios were used for lipase screening. The enzyme screening studies were repeated performed twice.

3.3.1 Extracellular Enzyme Profiles of the Isolates

Table 3.3 Extracellular Enzyme Tests

Extracellular Enzyme Type	Isolate Name
Did not produce lipase	D5, D10, D20, D26 D31, E23, E43, F11, F29 F33
Utilized Tween80 at 0% NaCl	D1, D2, D30, F25, F15
Utilized Tween 20 at 12% NaCl	D23, D24, D25, D30, E1, E2, E3, E42, F5, F12, F13, F14, F15, F16, F18, F23, F25
Did not Utilized Tween 20	D7, E7, E9, E10, E13, E14, E15, E16, E23, E24, E25, E26, E27, E28, E29, E30, E39, E47, F26, F27, F31
Did not utilase Tween80	D12, E12, E34, F1, F20, F30, F32,
Did not produce lipase without NaCI	D12, D14, E12, E34, F1, F30, F32
Utilase casein	F17, D5, F29, D32, E34, F1, F15, E36, D24, F10, F13, F12, E20, F32, E1, E47, E33, E14, E25, E42, F22

Table 3.4 Extracellular Enzyme Profiles of isolates

Isolate Name	Protease	0%NaCl Tween20	12% NaCl Tween20	20% NaCl Tween20	0% NaCl Tween80	12% NaCl Tween80	20% NaCl Tween 80
D1	-	-	-	-	+	+	-
D2	-	-	-	+	+	-	-
D5	+	-	-	-	-	-	-
D6	-	+	-	-	-	+	-
D7	-	-	-	-	-	+	-
D10	-	-	-	-	-	-	-
D11	-	+	-	+	-	+	+
D12	-	+	-	-	-	-	-
D14	-	+	-	-	-	-	-
D18	-	+	-	+	-	-	+
D20	-	-	-	-	-	-	-
D23	-	+	+	-	-	+	+
D24	+	+	+	-	-	+	+
D25	-	+	+	-	-	-	+
D26	-	-	-	-	-	-	-
D29	-	+	-	-	-	+	+
D30	-	+	+	+	+	+	+
D31	-	-	-	-	-	-	-
D32	+	-	-	-	-	+	+
E1	+	+	+	-	-	+	+
E2	-	+	+	-	-	+	
E3	-	+	+	-	-	+	+
E4	-	+	-	-	-	+	-
E6	-	+	-	-	-	+	+
E7	-	-	-	-	-	+	-
E9	-	-	-	-	-	+	-
E10	-	-	-	-	-	-	+
E12	-	+	-	-	-	-	-
E13	-	-	-	-	-	+	-
E14	+	-	-	-	-	+	-
E15	-	-	-	-	-	+	-
E16	-	-	-	-	-	+	-
E17	-	-	-	-	-	+	-
E20	+	+	-	-	-	+	+
E23	-	-	-	-	-	+	-
E24	-	-	-	-	-	+	-
E25	+	-	-	-	-	+	-
E26	-	-	-	-	-	+	-
E27	-	-	-	-	-	+	-
E28	-	-	-	-	-	+	-
E29	-	-	-	-	-	+	-
E30	-	-	-	-	-	-	+
E33	+	-	-	+	-	+	+
E34	+	+	-	-	-	-	-
E35	-	-	-	+	-	-	+

E36	+	+	-	-	-	+	+
E39	-	-	-	-	-	+	-
E42	+	+	+	-	-	-	+
E43	-	-	-	-	-	-	-
E47	+	-	-	-	-	-	+
F1	+	+	-	-	-	-	-
F2	-	+	-	+	-	+	+
F3	-	+	-	+	-	+	-
F4	-	+	-	-	-	+	+
F5	-	+	+	+	-	+	+
F7	-	+	-	+	-	+	+
F8	-	+	-	-	-	+	+
F9	-	+	-	-	-	-	+
F10	+	+	-	+	-	+	+
F11	-	-	-	-	-	-	-
F12	+	+	+	+	-	+	+
F13	+	+	+	-	-	+	+
F14	-	-	+	-	-	+	+
F15	+	+	+	+	+	-	+
F16	-	+	+	-	-	+	+
F17	+	+	-	-	-	+	+
F18	-	-	+	-	-	+	+
F19	-	+	-	-	-	+	+
F20	-	+	-	+	-	-	-
F21	-	+	-	-	-	+	-
F22	+	+	-	-	-	+	-
F23	-	+	+	+	-	+	+
F24	-	-	-	-	-	-	+
F25	-	+	+	+	+	+	+
F26	-	-	-	-	-	+	+
F27	-	-	-	-	-	-	+
F28	-	+	-	-	-	-	+
F29	+	-	-	-	-	-	-
F30	-	+	-	-	-	-	-
F32	+	+	-	-	-		-
F35	-	-	-	-	-	-	-
F34	-		-	-	-	-	-

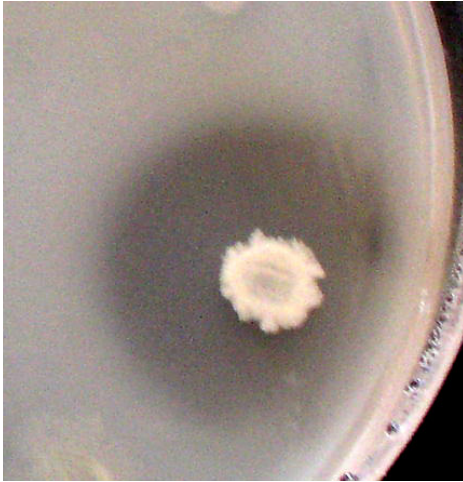


Figure 3.1. Apperance of extracellular protease

Figure 3.2. Apperance of extracellular lipase

3.4. Genotypic Characterization

3.4.1. Amplification of 16S-ITS Region of Ribosomal RNA Operon

The amplification product size differed between 1800 and 2000 bp (Figure)

.

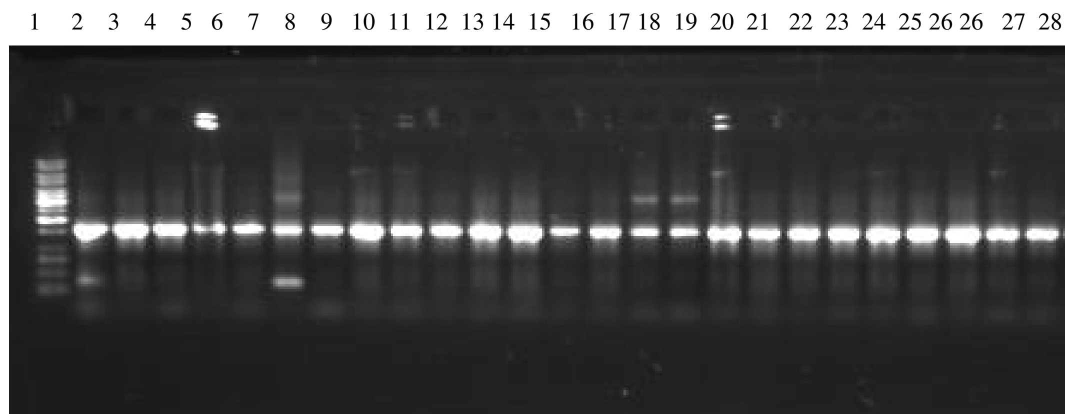


Figure 3.3. 16S rDNA-ITS Amplification products of some isolates. Lanes 1. 1 kb DNA Lac D1, 3. Isolate F2, 4. Isolate F1 5. Isolate D8, 6. Isolate D10, 7. Isolate 12,8. Is Isolate E15, 10. Isolate E17, 11. Isolate E19, 12. Isolate D23 lane 13. Isolate D24

3.4.2. 16S-ITS rDNA - RFLP Profiling

In order to identify the isolates amplification products of 16S-ITS rDNA region was digested with restriction endonucleases. *Taq* I and *Hae* III endonucleases were chosen for digestion. Digestion products were first run in 2% agarose gel to see all different profiles and to make groups within the digestion products. For a specific RFLP group, one representative digestion product was chosen and electrophoresed together in 2.5% agarose gel (Figure 3.3).

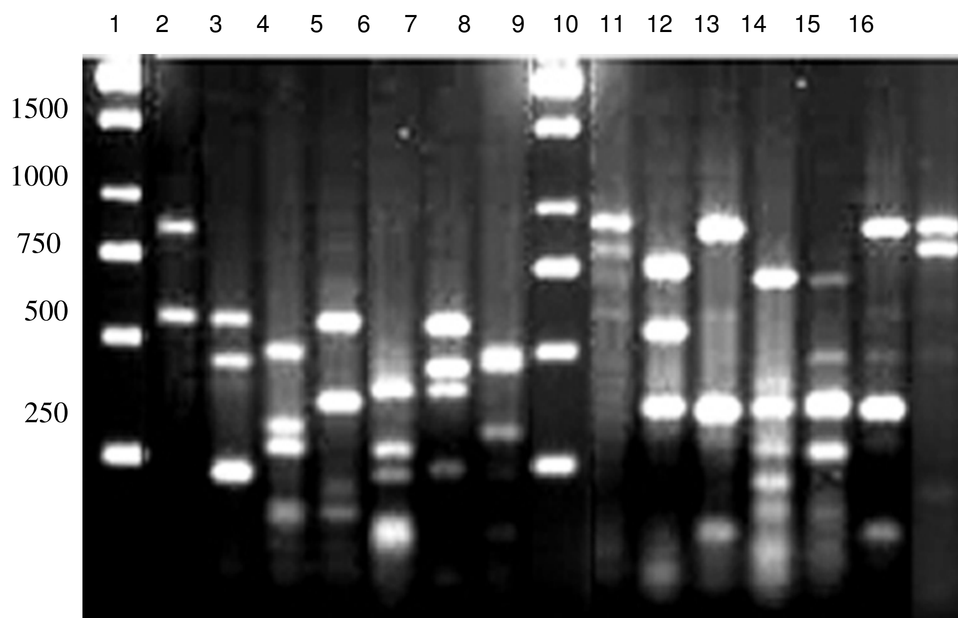


Figure 3.4 16S-ITS rDNA RFLP profiles of *Hae* III groups. Lanes 1. 1 kb DNA ladder, 2. Isolate D5, 3. Isolate D7, 4. Isolate D30, 5. Isolate D12, 6. Isolate D23, 7. Isolate F28, 8. Isolate, F21 16S-ITS rDNA RFLP profiles of *Taq*I groups. Lanes 9. 1 kb DNA ladder,10. Isolate D2, 11. Isolate D18, 12. Isolate D12, 13. Isolate F6, 14. Isolate F20, 15. Isolate F28, 16. IsolateE30,

Resulting gel profiles were analyzed using the program BIO-1D++. Dendrogram of representative strains were carried out using 15% homology coefficient. (Figure 3.3 and 3.4). Fragment sizes were given in Table 3.6.

Hae III and *Taq* I restriction analysis of 16S-ITS rDNA fragments of strains revealed 7 distinct groups (Table 3.3.).

Table 3.5. Fragment Sizes of *Taq* I and *Hae* III Digests of 16S-ITS rDNA

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2000	854	546	465	546	384	546	465	2000	886	673	808	661	361	838	870
1500	555	440	309	354	257	440	432	1473		509	338	354	338	338	778
1000		219	272	125	211	377	294	934		354	78	265	257	78	
750			125		94	219		685				195			
500					70			465				133			
250								227				31			

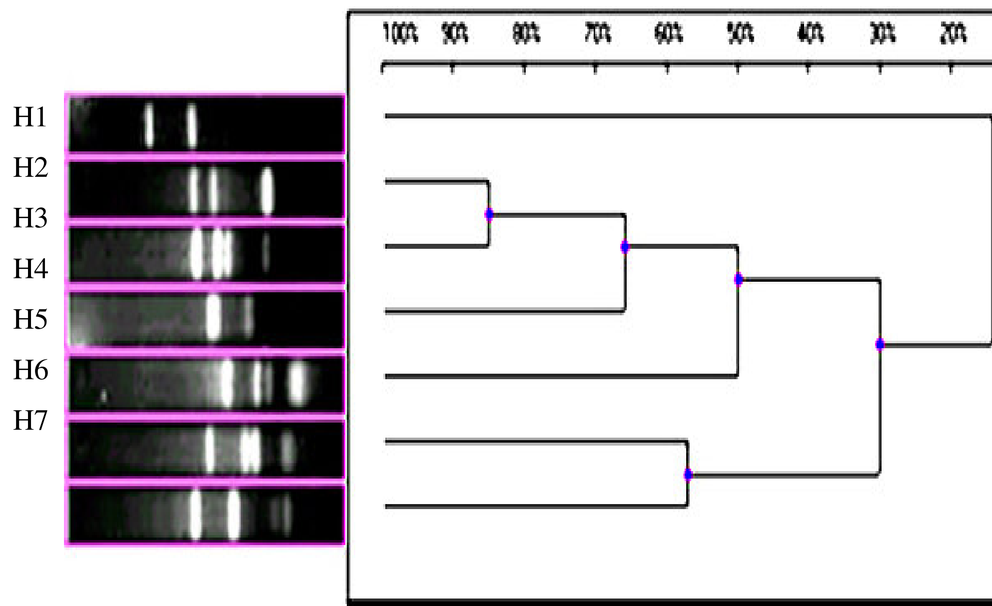


Figure 3.5. Dendrogram of Representative *Hae* III RFLP Profiles.

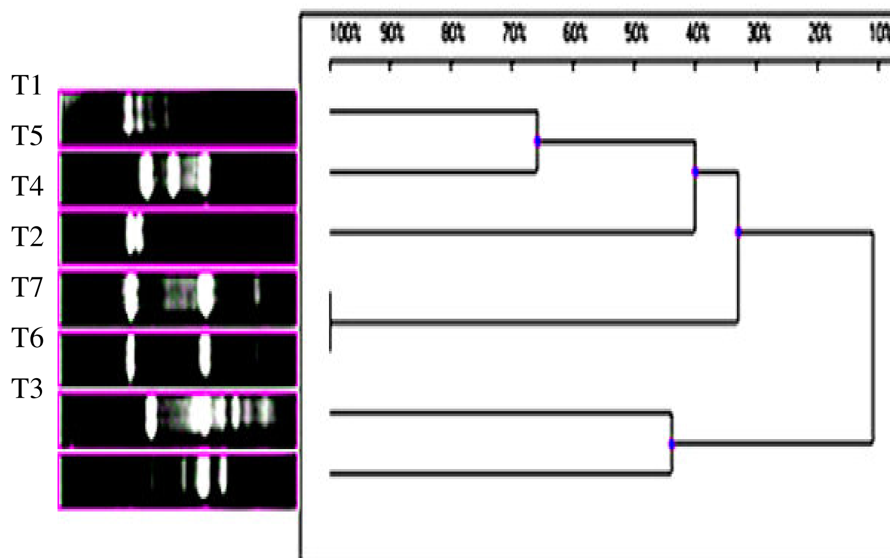


Figure 3.6. Dendrogram of Representative *Taq* I RFLP Profiles.

Table 3.6. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups.

Genotypic groups	Name of the Isolates
HT1	D1, D2, D5, E43,E47,F1
HT2	D6, D7, D10, D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E30, E34, E35
HT3	D12, F32
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E20, E33, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F19, F22, F23, F24, F25, F26, F27
HT5	D30
HT6	F20, F21
HT7	F28

The use of two restriction enzymes enabled the revelation of diversity among the isolates. When the results of both digestion were combined, 7 distinct genotypes were obtained (Table 3.10). The results indicated that isolate group members were differentiated by each restriction enzyme, were the same.

In this study, 16S ITS and rDNA regions were used as a single amplicon for RFLP analysis. No report was found in literature on genotypic characterization of haloalkalophiles using this method. Similar studies have been performed in this institute for genotypic characterization of alkalophilic bacilli (Akbalik, 2004), thermophilic bacilli, (Yavuz, 2004), lactic acid bacteria (Yavuz, 2003, Bulut, 2003.

3.4.3. Analysis of the Genotypic and Phenotypic Groups

Table 3.7. Analysis of the Genotypic and Phenotypic Groups

	Isolate number	20%NaCI	pH 7	0%NaCl	pH 12	Protease	0%NaCl Tween 20	12%NaCl Tween 20	20%NaCl Tween 20	0%NaCl Tween 80
HT1	D1,D2,D5,E34, E43, E47, F1	%80	80%	70%	80%	40%	0%20	0%	20%	%30
HT2	D6,D7,D10,D14, D20, D26, D29, D31, D32, E7, E9, E10, E12, E13, E14, E15, E16, E17, E23, E24, E25, E26, E27, E28, E29, E39, F29, F30, F34, F35,	100%	100%	100%	100%	10%	10%	0%	0%	0%
HT3	D12, F32	100%	100%	100%	100%	50%	100%	0%	0%	0%
HT4	D11, D18, D23, D24, D25, D30, E1, E2, E3, E4, E6, E30, E33, E35, E36, E42, F2, F3, F4, F5, F7, F8, F9, F10, F11, F12, F13, F14, F15, F16, F17, F18, F19, F22, F23, F24, F25, F26, F27	90%	100%	90%	70%	30%	90%	40%	30%	20%
HT5	D30	100%	100%	100%	0%	0%	100%	100%	100%	100%
HT6	F20, F21	100%	50%	100%	50%	0%	100%	0%	50%	0%
HT7	F28	100 %	100%	100%	100%	0%	100%	0%	0%	0%

Phenotypic characterization of the isolates indicated that strains of different species had different phenotypic features. When the genotypic results were compared with the results of enzyme screening and phsyical tests, a correlation between a few groups were observed. Most of the isolates produced lipase (Tween 20 and Tween 80) at all NaCI concentrations used grouped in HT4. All members of HT2, HT3, HT5, HT6, HT7 were also able to grow in the presence of 20% NaCI. Most of the isolates that could not grow at pH7 were placed in HT6. Isolates that could not grow at pH12 were placed in HT1, HT5, and HT6.

3.4.4 Analysis of Sequencing Reactions

16S partial sequencing results of six representative isolates are as listed below.

Table 3.8. Sequencing results

Genotypic Group	Isolate Name-Accession Number	Length of bp.-Similarity	Accession Number - Organism	Name of the Family
HT2	D7-AY604872	-89%	*AY345480.1-Bacterium K-29*	Unclassified *Bacillaceae*
HT3	D12-AY601901	716-93%	AF237976.1-*Salinococcus roseus*	Unclassified *Bacillaceae*
HT4	D23-AY601902	323-92%	AJ640133.1-*Halomonas* sp.18bAG	*Halomonadaceae*
HT5	D30-AY601903	-92%	AY383044.1-*Halomonas meridiana*	*Halomonadaceae*
HT6	F20 (C1)-AY604871	-89%	AJ29397-*Nestenkonia* sp.EL-30	*Micrococcaceae*
HT7	F28 (15B)-AY601904	-91%	AY3091336-Bacterium K-2	Unclassified *Bacillaceae*

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, haloalkalophilic bacteria were isolated from samples of waste soil and leather processing. They were first characterized phenotypically (Gram staining, catalase and oxidase behaviors, spore observation by phase contrast microscope and extracellular enzyme screening, physiological tests such as growth at pH 7 and pH 12 , different NaCl concentrations and at different temperatures). Extracellular enzyme screening was performed on solid agar media. Molecular characterization was performed by using 16S-ITS rDNA RFLP.

All the isolated strains were Gram positive but they were not sporeforming. All the strains were catalase positive. Most of the isolates contained oxidase. Four strains could not grow at pH7. They were thus obligately alkalophilic. Twentyfive isolated strain were alkalitolerant because they could not grow at pH 12. Nine isolated strain could not grow with in 0% NaCl they were thus obligately halophilic. Eight of the isolates could not grow at 20% NaCl concentration.

Extracellular enzyme screening resulted in 21 protease and 72 lipase activity. Isolates produced protease were grouped in HT1, HT2, HT3, HT4.Different concentrations of NaCl were used for lipase screening. Seventeen of the isolated strains which could utilize Tween 20 in the presence of 12% NaCI were all grouped in HT4. Isolated strains, which could not utilize T20, were grouped in HT2 except E47, F26, and F27. Seven of the isolated strains were not produce lipase in the presence of NaCl were grouped in HT1, HT2 and HT3.

For restriction analysis of 16S-ITS rDNA fragments of isolated strains, two restriction enzymes, *Taq* I and Hae III, were used. Both enzymes produced similar RFLP groups. Representative group members were subjected to 16S partial sequencing. They had high similarity with *Bacillaceae*, *Halomonadaceae* and *Micrococcaceae* families.

Characterization of the extracellular enzymes from different isolated strains is the essential work for these Haloalkalophilic bacteria because of their unique futures.

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APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Experiments

NO	CHEMICAL	CODE
1	Agar Merck	1.01613
2	Bacteriological pepton Oxoid	LP037
3	D-Glucose AppliChem	A3666
4	Yeast Extract Merck	1.03753
5	Skimmed milk LabM	MC27
6	Glycerol AppliChem	A2926
7	NaCI AppliChem	A2942
8	K2HPO4 AppliChem	A2945
9	MgSO4.7H2O Merck	1.05886
10	Tween80 Applichem	A1390
11	Ammonium sulfate Applichem	A3485
12	Nutrient broth Merck	1.05443
13	Sodium carbonate Merck 1.06392	
14	KH2PO4 Merck	1.04871
15	Disodium hydrogen phosphate Applichem	A2943
16	Immersion oil Applichem	A0699
17	Calcium chloride Applichem	A3652
18	Crystal violet Sigma	C3886
19	Safranin Merck	1.15948
20	N,N,N.,N.- Tetramethyl-p-phenylenediamine Sigma	T3134
21	Congo Red Sigma	C6767
22	Potassium Iodide Sigma	P8256
23	Tris Base Sigma	T6066
24	EDTA AppliChem	A2937
24	Isopropanol AppliChem	A3928
25	Proteinase K AppliChem	A3830
24	Ethidium bromide AppliChem	A1151
25	Ethanol AppliChem	A3678
26	Taq DNA polymerase Promega	M1865
27	Primers: Ege 1 and L1 Promega	
28	dNTP set MBI, Fermentas,	R0181
29	Standard agarose (low electroendoosmosis) AppliChem	A2114
30	Chloroform AppliChem	A3633
31	Isoamyl alcohol AppliChem	A2610
32	Bromophenol blue Merck	1.08122
33	Boric acid AppliChem	A2940

APPENDIX B

RECIPIES FOR MEDIA USED IN EXPERIMENTS

B.1 HORIKOSHI BROTH AND AGAR

HORIKOSHI-I BROTH

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water. Medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

HORIKOSHI-I AGAR (pH=10.2)

	g/l
Glucose	10.0
Polypepton	5.0
K_2HPO_4	1.0
$MgSO_4.7\ H_20$	0.2
Na_2CO_3	10.0
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and medium was sterilized by autoclaving for 15 m at 121° C. Na_2CO_3 was sterilized separately and added into medium.

B.2 NUTRIENT AGAR (pH:10.3) USED FOR GROWTH TESTS AT DIFFERENT TEMPERATURES AND SODIUM CHLORIDE CONCENTRATIONS

Nutrient Broth	8
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 was sterilized separately and added into medium.

B.5 MEDIUM USED FOR LIPASE SCREENING (pH: 10.3)

	g/l
Nutrient Broth	8
$CaCl_2 . H_2O$	0.1
Agar	13
Tween20/Tween80	10ml
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. Na_2CO_3 and Tween20/80 was sterilized separately and added into medium.

APPENDIX C

BUFFERS AND STOCK SOLUTIONS

C.1 ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

Ethidium bromide (0.5 g) was dissolved in 50 ml of deionized water.

C.2 CHLOROFORM-ISOAMYL ALCOHOL SOLUTION (24:1)

Forty eight milliliters of chloroform was mixed with 2 ml of isoamyl alcohol.

C.3 50 X TAE

Two hundred and fifty-two grams Tris base was dissolved in deionized water, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were added. Volume was adjusted to 1000 ml with deionized water.

C.4 1XTAE

Twenty milliliters of 50X TAE buffer was taken and the volume was adjusted to1000 ml with deionized water to obtain 1 liter 1X TAE buffer.

C.5 1X TE BUFFER

10 mM Tris pH 8.0
1 mM EDTA pH 8.0

C.6 10 X TBE

One hundred and eight grams Tris Base and 55 g boric acid were weighed. They

were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was added. The volume was brought to 1000 ml with deionized water.

C.7 1X TBE

Hundred milliliters, 10X TBE was taken and the volume was brought to 1000 ml with deionized water to obtain 1X TBE buffer.

C.8 CTAB/ NaCl (10% CTAB in 0,7 M NaCl)

NaCl (4.1 g) was dissolved in 80 ml water and CTAB was added slowly while heating and stirring. Final volume was adjusted to 100ml.

APPENDIX D

STAINS AND INDICATORS

D.1. BROMTYMOL BLUE SOLUTION

Bromtymol blue	1 g
N/10 NaOH	25 ml
Deionized water	475 ml

D.2. SOLUTIONS FOR GRAM STAINING

D.2.1. CRYSTAL VIOLET STAINING REAGENT

Solution A

Crystal violet	2g
Ethanol (95%)	20ml 83

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Solution A and B were mixed to obtain crystal violet staining reagent.

D.2.2. IODINE SOLUTION

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Iodine and potassium iodide were grinded in a mortar, water was added slowly with continuous grinding until the iodine was dissolved. The solution was stored in amber bottle.

D.2.3. SAFRANIN SOLUTION

Safranin (2.5% in 95% alcohol)	10ml
Distilled water	100ml

APPENDIX E

PCR RECIPIES

E.1. PCR MIX

Mg free Taq DNA polymerase buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Sterile deionized water	32 µl
Oligo forward (10 picomole/µl)	1 µl
Oligo reverse (10 picomole/ µl)	1 µl
dNTP (2mM each) 10X	5 µl

E.2. 6X GEL LOADING BUFFER (20 ML)

10x TBE	2 ml
Glycerol	6 ml
Deionized water	12 ml

Bromophenol blue was added with toothpick until obtaining sufficient color of the solution.

E.3. dNTP (10X)

Ten microliters of each 100mM dATP, dCTP, dGTP and dTTP in separate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl sterile deionized water was added. They were mixed gently and 2mM concentration of each was obtained and stored at .20 °C.

E.4. RESTRICTION ENZYME MIX

Restriction enzyme buffer	5 µl
DNA	8 µl
Restriction enzyme (10U/ µl)	1.5 µl
Sterile deionized water	35.5 µl

APPENDIX F

OLIGONUCLEOTIDE PRIMERS

L1: 5.- CAAGGCATCCACCGT -3.

Three hundred and fifty micrograms primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliters of stock solution were then taken and mixed with 96 µl sterile deionized water. Therefore 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

EGE 1 5.- AGAGTTTGATCCTGGCTCAG -3.

Five hundred and ninty micrograms primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain final concentration of 2 µg / µl stock solutions. Five microliters of stock solution were then taken and mixed with 95 µl sterile deionized water. Therefore, 100 µl, 10 picomole / µl working solution was obtained. Stock and working solutions were stored at .20 °C.

APPENDIX G

RECOGNITION SITES OF RESTRICTION ENZYMES

G1. ***Taq*** **I**

5.- T▼CG A -3.
5.- A GC ▲T -3.

G2. ***Hae*** **III**

5.-GG▼CC-3.
5.-CC▲GG-3.

APPENDIX H

ISOLATE NAMES

DI- C12a
D2- Ş6a
D5- E15
D6-C15a
D7- 20a
D10-19a
D11- B5c
D12- B1a
D14- 23a
D18- C4
D20- B8b0
D22- H2
D23- H1a
D24- P5z
D25- C17
D26- 2b
D29- D3c
D30- B9
D31- A9
D32- D2b
E1- A2
E2- P2y
E3- C6
E4- C2a
E6- G9a
E7- 3a
E9-24a

F26- Ü4z
F27- Ü6a
F28- 15b
F29- F1
F30- 24a
F31- 27b
F32- B4c
F33- T1b
F34- E6z
F35- B8a
E42- C8
E43- C14a
E47- C19y
F1- E11x
F2- F2x
F3- F2y
F4- F2z
F5- G5
F6- G8
F7- H3x
F8- H3y
F9- P1x
F10- P4a
F11- P5y
F12- S1
F13- S2
F14- Ş1

E10- 13b	F15- V3
E12- 29c	F16- A1
E13- 22c	F17- A4
E14- 12c	F18- A6
E15- 27c	F19- A7
E16- 1a	F20- B10
E17- 2d	F21- C1
E20- B2	F22- C2
E23- B8c	F23- C3
E24- 18b	F24- C24
E25- 11a	F25- C10