Genotypic Characterization of Extracellular Enzyme Producing Thermophilic Bacteria in Balçova Geothermal Region

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ABSTRACT

Thermophiles are the organisms which are adapted to live at high temperatures. The enzymes from thermophiles find a number of commercial applications because of their thermostability and thermoactivity. Therefore, the isolation of thermophilic bacteria from natural sources and their identification are very important in terms of discovering new industrial enzymes.

In keeping with this view, Balçova Geothermal Region could serve as a good source for new thermophilic microorganisms with novel industrially important properties.

The aim of this research was therefore the isolation of industrially important extracellular enzyme producing thermophilic bacteria from Balçova Geothermal Region and their identification by genetical means. 16S-ITS rDNA RFLP, plasmid profiling and pulsed field gel electrophoresis studies were performed for this purpose.

112 thermophilic strains were isolated from various environmental samples collected within Balçova Geothermal Region. These strains were screened for the existence of 6 extracellular enzyme activities. These were, lipases, amylases, proteases, xylanases, cellulases and pectinases. In total, 110 lipase (tween 20 as substrate), 106 amylase, 55 protease, 28 xylanase, 10 cellulase and 3 pectinase activities were detected. Some other phenotypic tests were also performed for these isolated strains. Since all the isolated strains were Gram (+), endospore forming rods, they were identified as *Bacillus* sp.

16S-ITS rDNA RFLP and plasmid RFLP profiles were produced by using two restriction endonucleases *Taq* I and *Hae* III . The isolated strains were clustered into eleven groups by *Taq* I restriction profiles of 16S-ITS rDNA while nine groups were obtained by *Hae* III digestion profiles. When these groups were compared, it was concluded that 17 genotypically different strains existed in total 112 isolates. Two of the isolated strains yielded similar RFLP profiles to those of *Bacillus stearothermophilus* (CECT 43) reference strain.

Plasmid profiling was also performed. It was found that 23 of the isolated strains contained plasmid DNA. *Hae* III restriction profiles indicated the existence of three different types of plasmids.

PFGE optimization studies by *Sma* I restriction endonuclease for thermophilic Bacilli were also performed. A new method for preparation of agarose plugs was developed.

ÖZ

Termofiller, yüksek sıcaklıklarda yaşamaya adapte olmuş organizmalardır. Termofillerden elde edilen enzimler, yüksek sıcaklıklarda gösterdikleri aktivite ve stabilite nedeniyle birçok ticari uygulamalarda kullanılmaktadırlar. Bu sebeple, termofilik bakterilerin doğal kaynaklardan izole edilmeleri ve tanımlanmaları yeni endüstriyel enzimlerin keşfedilmesi açısından önemlidir.

Bu açıdan bakıldığında, Balçova Jeotermal Bölgesi endüstriyel olarak önemli özellikleri olan termofilik mikroorganizmalar için iyi bir kaynak oluşturmaktadır.

Bu çalışmada Balçova Jeotermal Bölgesinden endüstriyel olarak önemli ekstraselüler enzim üreten termofilik bakterilerin izolasyonu ve genetik yöntemlerle tanımlanması amaçlanmıştır. Bu kapsamda, 16S-ITS rDNA RFLP, plazmid profillerinin belirlenmesi ve 'pulsed field' jel elektroforezi (PFGE) çalışmaları yapılmıştır.

Balçova Jeotermal Bölgesinden sağlanan çeşitli örneklerden 112 adet termofilik suş izole edilmiştir. İzolatlar 6 adet ekstraselüler enzim aktivitesi yönünden incelenmiştir. Bu enzimler; lipazlar, amilazlar, proteazlar, ksilanazlar, selülazlar ve pektinazlardır. Toplam 110 adet lipaz (tween 20 substratı), 106 adet amilaz, 55 adet proteaz, 28 adet ksilanaz, 10 adet selülaz ve 3 adet pektinaz aktivitesi saptanmıştır. Ayrıca bu suşların diğer bazı fenotipik özellikleri de incelenmiştir. Tüm izolatlar, Gram (+), çubuk şeklinde olduklarından ve endospor oluşturduklarından dolayı *Bacillus* türü olarak tanımlanmışlardır.

16S-ITS r DNA ve plasmid RFLP profilleri, *Taq* I ve *Hae* III restriksiyon enzimleri kullanılarak oluşturulmuştur. İzole edilen suşlar, *Taq* I restriksiyon enzimi profillerine göre 11 farklı gruba ayrılırken, *Hae* III kesim profillerine göre 9 farklı grup elde edilmiştir. Elde edilen bu gruplar karşılaştırıldığında, 112 adet izolat içinde genotipik olarak 17 farklı suşun bulunduğu sonucuna varılmıştır. İzolatlardan 2 tanesi *Bacillus stearothermophilus* CECT 43 referans suşunun RFLP profilleri ile aynı paternleri vermiştir.

Ayrıca 'plazmid profillerinin belirlenmesi ' metodu da uygulanmıştır. 23 adet suşun plazmid DNA içerdiği belirlenmiştir. *Hae* III restriksiyon profilleri 3 farklı tip plazmidin varlığını ortaya koymuştur.

Termofilik basiller için, *Sma* I restriksiyon enzimi kullanılarak PFGE optimizasyon çalışmaları gerçekleştirilmiştir. Agaroz kalıpların hazırlanması için yeni bir metot geliştirilmiştir.

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ABBREVIATIONS

bp	Base pair
CHEF	Clamped Homogeneous Electrical Field
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetra acetic acid
ITS	Internal Transcribed Spacer
kb	Kilo base
m	minute
Mb	Mega base
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methyl Sulfonyl Floride
PFGE	Pulsed Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
subsp.	Subspecies
sp.	Species
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

1.1. Thermophiles

Thermophiles are the organisms which are adapted to grow optimally at high temperatures (Bertoldo and Antranikian, 2002). They have been isolated from high temperature terrestrial and marine habitats. The most common habitats are volcanically and geothermally heated hydrothermal vent systems such as hotsprings and submarine hot vents (Bertoldo and Antranikian, 2002).

Thermophiles are separated into three categories based on their cardinal growth temperatures:

- Moderate thermophiles have adapted a very large optimum growth temperatures ranging from 35 to 70 ºC
- Extreme thermophiles, having an optimum growth temperature between 55-85 ºC
- Hyperthermophiles, having very high optimum growth temperatures, between 75-113 ºC (Baker *et al.,* 2001).

On the other hand they can also be classified as:

- Obligate thermophiles, having optimum growth temperatures between 65-75 ºC, do not grow below 40 ºC
- Facultative thermophiles could grow optimally at temperatures between 50-60 ºC, they are on the other hand, also able to grow at temperatures around 37 ºC
- Thermotolerant thermophiles show the maximum growth at temperatures around 45-50 ºC, and they can also grow below 30 ºC (Hughes and Williams 1977).

1.1.1. Thermophilic Microorganisms

Ecological studies have showed that both aerobic and anaerobic species and many morphological and physiological types of microorganisms can exist in thermophilic environments (Brock, 2001, Madigan *et al.,* 1997).

Extreme thermophiles are mostly distributed among the genera of *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Thermotoga*, *Aquifex.* Most hyperthermophiles, on the other hand, include the two kingdoms of *Archaea*, *Crenarchacota* (*Sulfolobus*, *Pyrodictium*, *Pyrolobus*.), *Euryarchaeaota* (*Thermococcus*, *Pyrococcus*), methanogenes (*Methanococcus*, *Methanobacterium*), sulfate reducers and halophiles (Bertoldo *et al.,* 2002). Below, the major groups of thermophiles and their optimum growth temperatures were shown (Table 1.1).

Table 1.1 Major groups of thermophilic and hyperthermophilic prokaryotes (Madigan *et al.,*1997).

Genus	Temperature range (°C)
Bacteria	
Phototrophic Bacteria	
Cyanobacteria	55–70 (One strain, 74)
Purple bacteria	45–60
Green bacteria	40–73
Gram-positive Bacteria	
Bacillus	50–70
Clostridium	50–75
Lactic acid bacteria	50–65
Actinomycetes	55–75
Other Bacteria	
Thiobacillus	50–60
Spirochete	54
Desulfotomaculum	37–55
Gram-negative aerobes	50–75
Gram-negative anaerobes	50–75
Thermotoga/Aquifex	55–95
Thermus	60–80
Archaea	
Methanogens	45–110
Sulfur-dependent hyperthermophiles	60–113
Thermoplasma	37–60

Archaeabacteria have been first mentioned as a separate group by Carl R. Woese in 1978 after his 16S rRNA based three-kingdom system proposal (Madigan *et al.,*1997). According to the universal tree, a tripartite division of living world consists of phylogenetic domains Bacteria, Archaea and Eukarya (Figure 1.1).

The root of the universal tree (Figure 1.1) represents a point in evolutionary time that all life on earth shared a common ancestor. It clearly indicates that initial evolution from a universal ancestor was first branched in two directions. These were the bacteria line and the Archaea-eukarya line. Archaea and Eucarya are therefore phylogenetically more related to each other than bacteria (Madigan *et al.,* 1997).

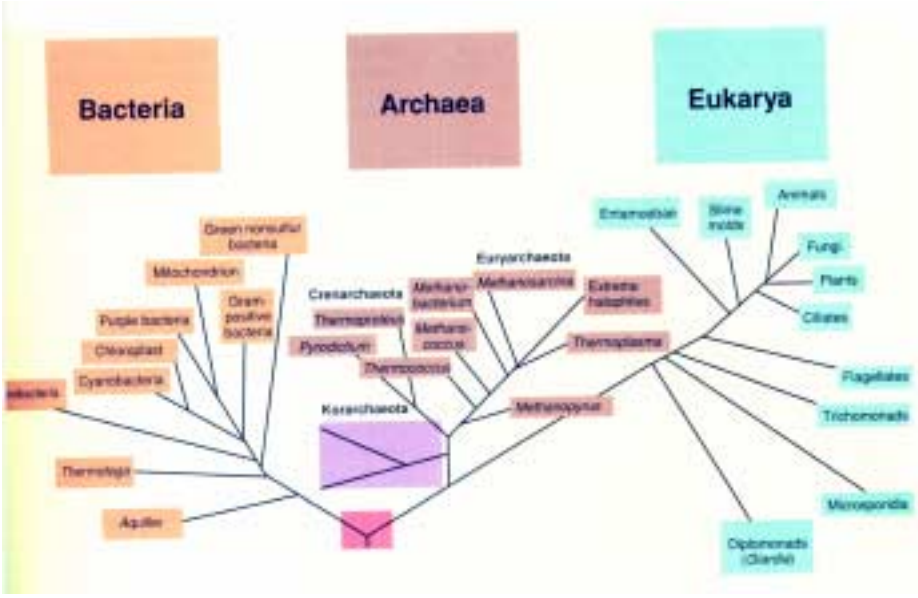

Figure 1.1 Rooted universal phylogenetic tree (Madigan *et al.,* 1997).

The universal tree shows that the Archaea has branched off the tree at a point closest to the root. This supports the idea that Archaea include the most primitive organism among the three domains. Placement of the Archaea nearest to the universal ancestor is also supported by the fact that many Archaea tolerate extreme environmental conditions such as high temperature, high salinity, low pH under which the earliest life forms have been thought to be originated. Thus, members of Archaea may be the earth's earliest life forms (Madigan *et al.,* 1997). General characteristics of the three major groups are compared in Table 1.2.

1.1.1.1. Thermophilic *Bacillus*

The genus *Bacillus* is described as a large and heterogeneous collection of aerobic and facultatively anaerobic, rod-shaped, Gram (+) (to Gram variable), endospore forming bacteria (Nazina *et al*., 2001; Goto *et al*., 1999; Slepecky and Hemphill, 1991). Many kinds of species which have thermophilic, psychrophilic, acidophilic, alkalophilic and halophilic properties are included in the genus (Nazina *et al*., 2001). Although the

members of *Bacillus* have been extensively studied, it is generally known that the taxonomy of the genus is unsatisfactory (Ash *et al*., 1991).

Thermophilic bacteria belonging to genus *Bacillus* show optimal growth temperatures in the range 45-70 ˚C (Maugeri *et al*., 2001; Rainey *et al*., 1993). The first publication on the characterization of an aerobic, spore forming, thermophilic, *Bacillus thermophilus*, was achieved in 1888 (Marteinsson *et al*., 1996).

Thermophilic *Bacillus* strains can be isolated from both thermophilic and mesophilic environments (Marteinsson *et al*., 1996). Identification of these strains is imprecise by traditional biochemical techniques (Flint *et al*., 2001). Using 16S rRNA sequences, Ash *et al*., (1991), have proposed the classification of the genus *Bacillus* in five phylogenetically different clusters. According to this division, three thermophilic species *B. stearothermophilus*, *B. kaustophilus* and *B. thermoglucosidasius* have been included in group 5. Rainey *et al*., (1994), have investigated the 16 thermophilic *Bacillus* strains according to their 16S rDNA sequences. Seven strains have been found to cluster with *B. stearothermophilus*, *B. kaustophilus* and *B. thermoglucosidasius* in the rRNA group 5. A recent taxonomic study (Nazina *et al*., 2001) on the other hand, have proposed that many thermophilic bacilli, formerly belonging to *Bacillus* species, form a new species *Geobacillus*.

The importance of thermophilic *Bacillus* have increased because of their biotechnological importance as sources of thermostable enzymes (proteases, amylases, pullulanases, glucose-isomerases, lipases, xylanases, cellulases and DNA restriction endonucleases) (Maugeri *et al*., 2001; Rainey *et al*., 1993).

Table 1.2 Summary of the major features among Bacteria, Archaea and Eukarya (Stetter , 1998; Madigan *et al.,*1997).

Characteristic	Bacteria	Archaea	Eukarya
Prokaryotic cell structure	Yes	Yes	No
Membrane enclosed nucleus	No	No	Yes
Cell wall	Ester-linked	Ether-linked	Ester-linked
Membrane lipids	Muramic acid present	Muramic acid absent	Muramic acid absent
Ribosomes	70S	70S	80S
Organelles (mitochondria,chloroplast)	No	No	Yes
Initiator tRNA	Formylmethionine	Methionine	Methionine
Introns in most genes	No	No	Yes
Operons	Yes	No	No
Capping and pol-A tailing of mRNA	No	No	Yes
Plasmids	Yes	Yes	Rare
RNA polymerases	One (4 subunits) Binds directly to promoter	Several (8-12 subunits each) Basal transcription factors are required for promoter recognition	Three (12-14 subunits each) Basal transcription factors are required for promoter recognition
DNA topology	Negatively supercoiled	Relaxed or positively supercoiled	Negatively supercoiled
Promoter structure	Two conserved boxes at –10(TATAAT) and –35 (TTGACA) from transcription start site	TATA box and/or initiator element	TATA box and/or initiator element.
Methanogenesis	No	Yes	No
Reduction of S to H2S	Yes	Yes	No
Nitrification	Yes	No	No
Denitrification	Yes	Yes	No
Nitrogen fixation	Yes	Yes	No
Chemolithotrophy	Yes	Yes	No
Gas vesicles	Yes	Yes	No
Sensitivity to chloramphenicol, streptomycin and kanamycin	Yes	No	No

1.1.2. Biotechnological and Industrial Applications of Thermophiles

One of the most attractive attributes of thermophiles is that they produce enzymes capable of catalyzing biochemical reactions at temperatures higher than those of mesophilic organisms (Demirjian *et al.,* 2001).

The property of higher thermal stability and tolerance to most of the chemical denaturants e.g. organic solvents, enables them to resist harsh process conditions. They also show high catalytic activity at high temperatures and longer shelf-life as commercial products (Aguilar *et al.,* 1998; Kristjansson, 1989).

The increase of temperature in biotechnological processes has an influence on the bioavailability and solubility of organic compounds such as polyaromatic, aliphatic hydrocarbons and polymeric substances. The elevation of temperature is accompanied by a decrease in viscosity and an increase in the diffusion coefficient of organic compounds. Consequently, higher reaction rates due to smaller boundary layers are expected (Niehaus *et al.,* 1999).

Biological processes where high operational temperatures above 60 ºC employed, the risk of contamination by other organisms is also become substantially reduced (Adams and Kelly, 1998).

Furthermore**,** in large scale fermentations with heat sensitive microorganisms, extensive efforts must be given for cooling the fermentation process and as much as ten percent of the energy cost of a microbial fermentation may be for heat transfer. Thermophilic fermentations, on the other hand, need not to be cooled (Niehaus *et al.,* 1999).

Table 1.3 Main advantages of high temperature and thermostable enzymes (Kristjansson, 1989; Niehaus *et al.,* 1999).

Property	Advantage in process
-Thermostability	Tolerate high temperatures
- High optimum temperature	Little activity at low temperatures, long shelf life
-Resistance to denaturing agents	Tolerate organic solvents, high and low pH
-Solubility	High concentrations of poorly soluble compounds
-Viscosity	Decreases, mixing and pumping can be accelerated, mass transfer rate increases
-Microbial contamination	Growth of pathogens and undesired contaminants are prevented
-Reaction rates	Diffusion and chemical reaction rates are accelerated

Industrially important thermostable extracellular enzymes including proteases, lipases, xylanases, amylases, cellulases, pectinases will be discussed in section 2.1. Another practical example of heat stable enzymes is the DNA polymerase isolated from *Thermus aquaticus*. The enzyme is widely known as *Taq* DNA polymerase. It has found diverse areas of application including research laboratories and hospitals (Brock, 2001). DNA polymerases from two hyperthermophilic Archaea, *Thermococcus littoralis* and *Pyrococcus furiousus,* are also suitable for molecular genetic studies.

Other thermostable enzymes have also been used in the synthesis of amino acids, for the elimination of sulphur containing pollutants like dibenzothiophene and in the production of 1,3-propanediol from glycerol (Haki and Rakshit, 2003).

One important product, which can be obtained from thermophiles, is ethanol. There are advantages of thermophilic ethanol production: (i) The elevated incubation temperature makes distillation process more efficient. (ii) The cooling requirement is obviated. (iii) Some thermophilic bacteria can also carry out a direct fermentation of polysaccharides to ethanol. These properties enable thermophiles to be a better alternative to yeast (Gough *et al.,* 1996).

1.1.3. Thermophilic Enzymes

Taxonomically thermophilic microorganisms are related to the mesophilic species. The thermophile's anatomy, ultrastructure, respiration, and metabolic processes are very similar when compared with those of mesophilic organisms. These similarities indicate that thermophiles and mesophiles have evolved from common ancestors. It has been concluded that an enzyme from a thermophilic organism, in general, is remarkably similar to that of a mesophilic organism in physical properties (Ljungdahl and Sherod, 1960).

Thermophiles, on the other hand have been reported to produce proteins called chaperonins that help to refold to their native form after denaturation. The cell membrane of thermophiles is composed of saturated fatty acids that provide hydrophobic environment for the cell. Archaea, which compose most of the hyperthermophiles, have lipids linked with ether on the cell wall. The DNA of thermophiles have also been reported to have a reverse DNA gyrase producing positive

supercoils in the DNA. Since this difference increases the melting point of DNA, the stability at high temperatures are achieved (Haki and Rakshit, 2003).

No general strategy of stabilization has yet been established (Jaenicke and Böhm, 1998). Thermostability appears to be achieved by proteins using the same forces involved in folding acquired by small structural modifications (Scandurra *et al.,* 1998). Several parameters may be effective for the stability of thermophilic proteins (Table 1.4).

Alterations in the amino acid composition of proteins bring about additional electrostatic interactions, formation of hydrogen and disulfide bonds, enhancement of hydrophobic interactions or compaction of the structure. There are only a few cysteine residues in thermophilic enzymes or they are completely absent. Since the fact that inactivation is often caused by oxidation of SH- groups, lower cysteine content could enable the protein protected against the oxidation type of inactivation. In some cases localization of cystein residues is also important. For example although *Bacillus sterarothermophilus* alcohol dehydrogenase has the same number of cysteine residues as its mesophilic analogue, its SH-groups are localized inside the protein globule (Scandurra *et al.,* 1998; Mozhaev and Martinek, 1984). Many significant substitutions in thermophilic enzymes as Lys to Arg, Ser to Ala, Ser to Thr and Val to Ile have been reported (Scandurra *et al.,* 1998). These substitutions cause an increase in the internal hydrophobicity. Thermophilic proteins generally show a decreased flexibility and increased hydrophobicity within the α-helical regions. The amino acids responsible for decreased flexibility are located in the helices at non-buried or surface positions so that

Table 1.4 Strategies of thermal stabilization of some thermophilic proteins (Jaenicke and Böhm, 1998).

Protein	**Major causes of thermostability**
IPMDH *Thermus thermophilus*	Increased number of ion pairs and hydrogen bonds, extended hydrophobic subunit interactions and improved packing of hydrophobic core, shortened chain termini.
LDH *Thermotoga maritima*	Increased number of ion pairs, decreased hydrophobic area, increased helicity, less cavity volume.
GAPDH *Thermotoga maritima*	Large number of additional salt bridges
Ferredoxin *Thermotoga maritima*	Stabilisation of α-helices, replacement of conformationally strained residues by glycins, strong docking of N-terminal methionine, increase in the number of hydrogen bonds.

they are tightly packaged by means of increased hydrophobic contacts. (Scandurra *et al.,* 1998). Furthermore a helix-favouring residue, arginine, occurs more frequently whereas helix-disfavouring residues cystein, histidine and proline have lower frequencies in thermophilic proteins (Kumar *et al.,* 2000).

Some thermophilic enzymes having proteolytic or amylolytic action are stabilized by Ca, Mg, Zn and other ions. The mechanism of stabilization is through the binding of cations to the labile parts of the globule. It is also known that some mesophilic enzymes are also stabilized by metal ions (Mozhaev and Martinek, 1984).

Hydrophobic interactions are considered the main driving forces in protein folding. In thermophilic proteins, buried apolar surface areas are larger than in mesophilic proteins (Scandurra *et al.,* 1998). However it has been suggested that hydrophobicity show little quantitative differences between thermophiles and mesophiles (Kumar *et al.,* 2000).

Differing the numbers of hydrogen bonds and salt bridges may also be another factor for stability. For example, 19 additional hydrogen bonds have been detected in a thermophilic protease which has not been present in its mesophilic counterpart (Mozhaev and Martinek 1984). Changes in the number of hydrogen bonds change the secondary structure of a protein. It has been observed that the main players of thermal stability were salt bridges and hydrogen bonds. The salt bridges around the active site may help to keep the active site region together by opposing disorder due to greater atomic mobility at high temperatures (Kumar *et al.,* 2000).

It has also been suggested that deletion or shortening of loops may increase the thermal stability and that oligomerisation can be another contributing factor (Kumar *et al.,* 2000).

1.2. Industrially Important Extracellular Enzymes

1.2.1. Proteases

Proteases are the enzymes that are capable of cleaving proteins in short peptides or free amino acids (Sookkheo *et al.,* 2000). Proteases are mainly divided into two major groups depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases

cleave the peptide bonds distant from the termini of the substrate. They can also be further divided into four groups based on the functional group present at the active site. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao *et al.,* 1998).

There is a good correlation between growth temperature of the organism and the stability of its extracellular proteases. Thermophilic bacteria which exist in thermal environments, are often good sources of thermostable proteases, such as thermolysin from *B. thermoproteolyticus*, and aqualysin-I from *T.aquaticus* (Sookkheo *et al.,* 2000). Microorganisms producing thermostable proteases are listed in Table 1.5.

Table 1.5 Source microorganisms and properties of thermostable proteolytic enzymes (Haki and Rakshit, 2003).

Organism	Enzyme properties	
	Optimal temperature (˚C)	Optimal pH
Bacillus brevis	60	10.5
Bacillus licheniformis	70	9.0
Bacillus stearothermophilus	60	
Bacillus stearothermophilus	85	
Bacillus sp. JB – 99	80	6 12
Bacillus stearothermophilus TP26	75	
Bacillus sp. no. AH – 101	80	12.0 13.0
Bacillus thermoruber	45	9
Pyrococcus sp. KODI	100	7
Staphylothermus marinus		9
Thermoacidophiles (archeal&bacterial origin)	60 70	7.0 8.5
Thermococcus aggreganes	90	7.0
Thermococcus celer	95	7.5
Thermococcus litoratis	85	8.5
Thermotoga maritema	95	9.5

1.2.1.1. Industrial Applications of Thermostable Proteases

Proteases represent one of the largest groups of industrial enzymes and account for about 60 % of the world enzyme market (Rao *et al.,* 1998). Approximately 40 % of these enzymes are of microbial origin since they posses almost all the characteristics desired for biotechnological applications.

They are widely used in detergents, silver recovery, medical purposes, food processing, animal feed, pharmaceutical industries, and also in leather treatment and bioremediation processes (Rao *et al.,* 1998; Kumar and Takagi, 1999; Anvar and Saleemuddin*,* 1998).

A crude alkaline protease from a thermophile has been used for the acceleration of the gelatin layer hydrolysis of X-ray films (Fujiwara *et al.,* 1991). In food industry especially in meat tenderization, thermophilic proteases have been preferred as they posses the ability to hydrolyze connective tissue proteins as well as muscle fibre proteins. An alcalase which had optimum activity at pH 8.5 and temperature of 55-60 °C, has been found to be the most appropriate enzyme for solubilisation for meat hydrolysates (Kumar and Takagi, 1999). Waste streams are desirably carried out with above 70 °C thermostable proteases would allow extended hydrolysis periods with low risk of contamination (Kumar and Takagi, 1999). In detergent industry alkaline proteases added to laundry detergents enable the release of proteinaceous materials from stains. Ideally the enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. However, not all the processes require the same thermal stability and lower wash temperatures are also sometimes preferred (Kumar and Takagi, 1999).

1.2.2. Starch Degrading Enzymes

Starch is composed of α-glucose units that are linked by α-1,4- or α-1,6-glycosidic bonds. The two high molecular weight components of starch are amylose, a linear polymer consisting of α-1,4-linked glucopyranose residues, and amylopectin, a branched polymer containing α-1,6-glycosidic linkages at the branching points. (Bertoldo and Antranikian, 2002)**.**

Enzymes involved in the hydrolysis of starch can simply be classified into two groups endo-acting and exo-acting enzymes. Endo-acting enzymes such as α-amylase hydrolyze linkages in the interior of the starch in a random fashion and this leads the formation of linear and branched oligosaccharides. Exo-acting enzymes include β-amylase, glucoamylase and α-glucosidase. These enzymes attack the substrate from the non-reducing end, producing oligo and/or monosaccharides. Enzymes capable of hydrolysing α-1,6-bonds in pullulan and amylopectin are defined as debranching

enzymes. Cyclodextrin glycosyltransferases, produce a series of non-reducing cyclic dextrins from starch, amylose and other polysaccharides (Bertoldo and Antranikian, 2002; Niehaus *et al.,* 1999).

Such enzymes can be derived from mesophilic enzymes by chemical modifications or mutagenesis but natural thermophilic amylases exist. *B. stearothermophilus* and *B. Licheniformis, Thermus* spp. α-amylases are well characterized and widely used in starch processing. Thermophilic amylases are also found in thermophilic and hyperthermophilic Archaea (Bertoldo and Antranikian, 2002; Leveque *et al.,* 2000; Eichler , 2001). Source microorganisms and properties of starch hydrolyzing enzymes are listed in Table 1.6.

Table 1.6 Source microorganisms and properties of thermostable starch hydrolyzing enzymes (Haki and Rakshit, 2003).

Enzymes	Organisms	Enzyme properties	
		Optimal temperature (˚C)	Optimal pH
α-Amylase	*Bacillus amyloliquefaciens*	70	7.0
	Bacillus licheniformis	100	6.0 6.5
	Bacillus stearothermophilus	70 80	5.0 6.0
	Bacillus stearothermophilus	70	
	Bacillus subtilis	70	7.0
	Lactobacillus manihotivorans	55	5.5
	Myceliophthora thermophila	100	5.6
	Pyrococcus furiosus	100	5.5
	Pyrococcus woesei	100	6.5 7.5
	Staphylothermus marinus	65	5.0
	Sulfolobus solfataricus		
	Thermococcus aggreganes	100	5.5
	Thermococcus celer	90	5.5
	Thermococcus fumicolans	95	4.0 6.3
	Thermococcus hydrothermalis	85	4.8 7.8
	Thermomyces lanuginosus	60	5.6
	Thermococcus profoundus	80	4.0 5.0
β-Amylase	*Bacillus circulans*	60	
	Bacillus cereus var. *mycoides*	50	
	Bacillus sp.	50	7.5
	Clostridium thermosulphurogenes	75	5.5
	Clostridium thermosulfurogenes	60	5.8 6.0
Pullulanase	*Bacillus sp.*	60	
	Pyrococcus furiosus	98	5.5
	Pyrococcus woesi	100	5.5 6.0
	Thermococcus aggregans	100	6.5
	Thermus caldophilus GK24	75	5.5

Amylase from the hyperthermophile *Pyrococcus woesei* which is important for starch industry was purified and characterized and its gene was cloned in mesophilic hosts *Bacillus subtilis* and *Escherichia coli* (Aguilar *et al.,* 1998).

1.2.2.1. Industrial Applications of Thermostable Starch Degrading Enzymes

Amylases constitute a class of industrial enzymes having approximately a 25 % share in the world enzyme market (Sidhu *et al.,* 1997). Amylases find potential applications in a number of industrial processes such as food (baking, brewing, dairy industries), fermentation, textile, detergent and paper industries. Microbial amylases have replaced the chemical hydrolysis of starch in starch processing industries. They would also be useful in the pharmaceutical and fine chemical industries (Pandey *et al.,* 2000).

Starch bioprocessing usually involves two steps, liquefaction and saccharification, both require at high temperatures. During liquefaction, starch granules are gelatinized in a jet cooker at 105° C to 110 °C for 5 min in aqueous solution (pH 5.8 to 6.5), and are then partially hydrolyzed at α-1,4 linkages with a thermostable α-amylase at 95 °C for 2 to 3h. Temperature and pH controls are critical at this stage. Alpha-amylases from *B.licheniformis* and *B.stearothermophilus* have been typically used in this step (Crabb, and Mitchinson, 1997; Crabb and Shetty, 1999; Vielle and Zeikus, 2001).

1.2.3. Xylanases

Xylan, the major hemicellulose component of the plant cell wall in most plant species, is hydrolyzed by a group of enzymes. It is a complex polysaccharide comprising a backbone of xylose residues linked by β-1,4-glycosidic bonds (Figure1.2). The main chain of xylan is composed of β-xylopyranose residues. Most xylans contain different substituent groups in the backbone chain and in the side chains. Acetyl, arabinosyl and glucuronysyl residues are the common substituents found on the backbone of xylan (Beg *et al.,* 2001).

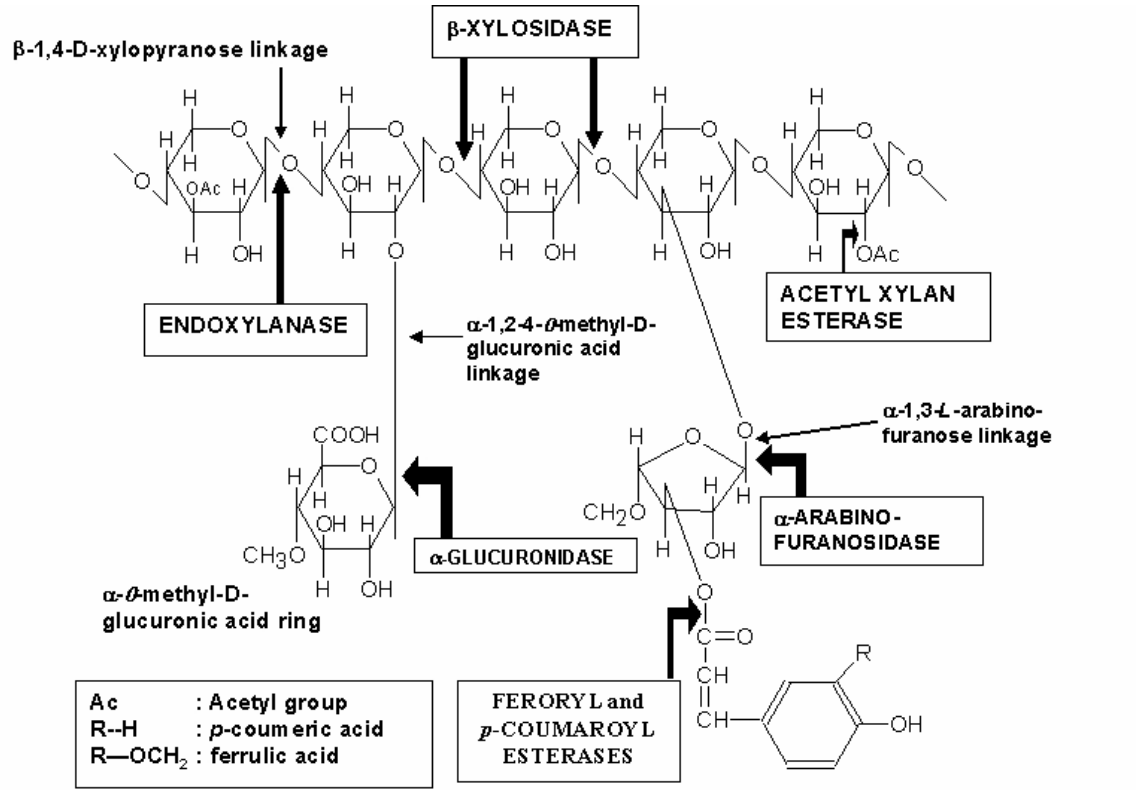

Figure 1.2 A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases (Beg *et al.,* 2001).

A typical xylan degrading enzyme system is composed of

- β-1,4-endoxylanase,
- β-xylosidase,
- α-L-arabinofuranosidase,
- α-glucuronidase,
- acetyl xylan esterase,
- and phenolic acid (ferulic and p-coumaric acid) esterase (Coughlan and Hazlewood, 1993).

So far members of Thermotogales and *Dictyoglomus thermophilum* (Table 1.7) have been described to produce xylanases active and stable at high temperatures (Vielle and Zeikus, 2001).

There have been many reports on thermostable xylan digesting thermophilic *Bacillus* species. *Bacillus thermoleovorans* (Sunna *et al.,* 1997), *Bacillus flavothermus* (Sunna *et al.,* 1997), *Bacillus amyloliquefaciens* (Breccia *et al.,* 1998), *Bacillus*

stearothermophilus (Khasin *et al.,* 1993) , *Bacillus circulans* (Dhillon *et.al.* 2000) (Table 1.7) .

Table 1.7 Source microorganisms and properties of thermostable xylanases (Haki and Rakshit, 2003).

Organism	Enzyme properties			
	Optimal temperature (˚C)		Optimal pH	
Bacillus amyloliquefaciens	80		6.8	7.0
Bacillus circulans	80		6.0	7.0
Bacillus sp.	60	75	8.0	9.0
Basillus sp. *strain* SPS - 0	75		6.0	
Basillus subtilis	50		6.0	
Clostridium abosum	75		8.5	
Dictyoglomus sp. *strain* B_1	90		6.0	7.0
Fusarium proliferatum	55		5.0	5.5
Pyrococcus furiosus	100		6.0	
Pyrococcus furiosus	102			
Scytalidium thermophilum	65		6.0	
Streptomyces sp. strain S38	60		6.0	
Sulfolobus solfataricus	105		5.3	
Teheromyces lanuginosus (wild & mutant)	60	70	7.0	6.7
Teheromyces lanuginosus - SSBP	70	75	6.5	
Thermoascus aurantiacus	50		5.0	
Thermotoga maritima MSB8	92		6.2	
Thermotoga maritima MSB8	95		6.0	7.5
Thermotoga maritima MSB8	75		6.2	
Thermotoga neapolitana	95		6.0	
Thermotoga neapolitana	85		5.5	
Thermotoga neapolitana	102		5.5	
Thermotoga sp. strain FjSS3 - B1	80		7.0	
Thermotoga sp. strain FjSS3 - B1	105		6.8	7.8
Thermotoga sp. strain FjSS3 - B1	115		5.3	
Thermotoga thermarum	80		6.6	

1.2.3.1. Industrial Applications of Thermostable Xylanases

Xylanolytic enzymes have attracted much attention in the last decade. Some major applications of xylanases are listed in Table 1.8.

Table 1.8 Applications of xylanolytic enzymes (Gilbert and Hazlewood, 1993).

Applications of Xylanolytic Enzymes
Extraction of juices, flavours, spices, oils and pigments
Clarification of juices and wines
Production of modified xylans as bulking agents for food processing
Conversion of xylans into monomeric products for conversion into sweetener or flavours
Modification of cereal flours so as to enhance the volume, textural and staling properties of bread.
Improvement of nutritional qualities of silage and of wheat- and rye- based feedstuffs
For cattle, pigs and poultry
Retting of flax, hemp, jute, sisal and bast
Refining of dissolving pulps for production of viscose rayon, cellulose esters, cellulose ethers.
Saccharification of agricultural and forestry wastes and residues for fermentation to fuels and chemical feedstocks
Prebleaching of pulps for paper manufacture

In recent years thermostable xylanases have been mostly used in enzyme-aided bleaching of paper (Viikari *et al.,* 1994). Bleaching is the process of lignin removal from pulp to provide brightness and whiteness (Beg *et al.,* 2001). Organic chlorine by-products formed during this process are toxic mutagenic, bioaccumulative and persistent. The use of xylanases reduces the amount of chlorine in bleaching process (Gilbert and Hazlewood, 1993). Several criteria have been found to be important for candidate xylanases. These are;

- They must not have cellulolytic activity to avoid hydrolysis of cellulose fibres.
- Their molecular mass should be low for facilitating their diffusion in the pulp fibres
- They must be stable and active at high temperatures and at alkaline pH
- They are preferred to be obtained at high yields and very low cost (Niehaus *et al.,* 1999).

1.2.4. Cellulases

Cellulose is the most abundant and renewable natural polymer on earth. Cellulose compounds are structurally heterogeneous and have both amorphous and highly ordered crystalline forms. Thus, they require a multitude of endo- and exo-glucanases that must act synergistically to achieve the desired hydrolysis (Niehaus *et al.,* 1999). The major enzymes in cellulase complex were outlined in Figure 1.3.

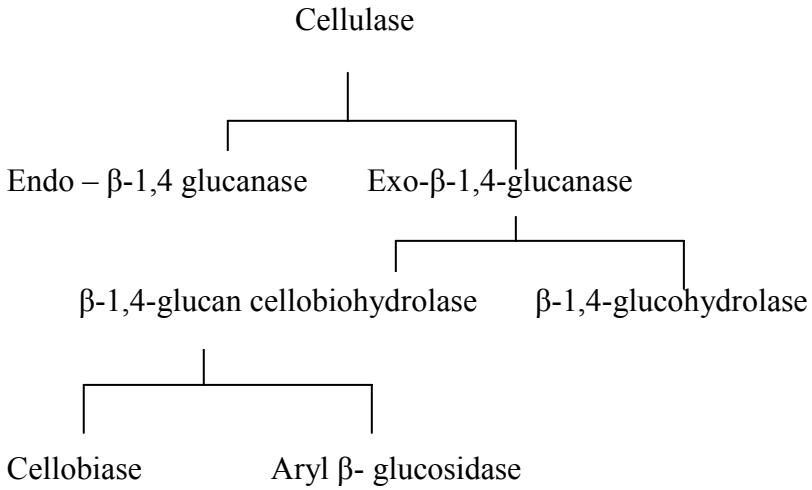

Figure1.3 Enzymes of cellulase complex.

The best characterized cellulases are of fungal origin. Among bacteria *Bacillus* species has been known to produce cellulases. But it has been observed that bacilli lack the complete cellulase system, the main activity is endoglucanase activity (Mavadza *et al.,* 2000). Endoglucanases of two *Bacillus* species isolated from hot springs has been reported with optimum temperature between 65-70 °C, and at pH between 6-10 (Mavadza *et al.,* 2000). The production of cellulases by thermophiles is rare however endoglucanases and cellobiohydrolases have been characterized in Thermotogales (Vielle and Zeikus 2001). A thermostable cellulase from *T. maritima* has been characterized with molecular mass of 27 kDa, optimum temperature at 95°C, and pH between 6.0 and 7.0 (Niehaus *et al.,* 1999), (Table 1.9.).

1.2.4.1. Industrial Applications of Thermostable Cellulases

The major applications of cellulases are in textile industry for bio-polishing of fabrics and in house-hold laundry detergents for improving fabric softness and brightness. They are also used in animal feed, processing of fruit juice, and in baking and deinking of recycled paper (Mavadza *et al.,* 2000).

Industrial ethanol production is based on corn-starch which is liquefied and saccharified. The oligosaccharide syrup is then used for ethanol fermentation. It has been described that the use of cellulases during starch saccharification and liquefaction increase the yields (Vielle and Zeikus, 2001). Since these steps are performed at high temperatures, thermophilic endoglucanases are important for these steps.

Table 1.9 Source microorganisms and properties of thermostable cellulases (Haki and Rakshit, 2003).

Organism	Enzyme properties	
	Optimal temperature (˚C)	Optimal pH
Anaerocellu thermophilum	85 90	5.0 6.6
Bacillus subtilis	65 70	5.0 6.5
Pyrococcus furiosus	102 105	
Pyrococcus horicoshi	97	
Rhodothermus marinus	95	6.5 8.0
Thermotoga maritema MSB8	95	6.0 7.0
Thermotoga neapoltana (EndocellulaseA)	95	6.0
Thermotoga neapoltana (EndocellulaseB)	106	6.0 6.6

1.2.5. Pectinases

Pectin and other pectic substances are complex plant polysaccharides. They form the major components of the middle lamella, a thin layer of adhesive extracellular material found between the primary walls of adjacent young plant cells (Naidu and Panda, 1998). The pectin molecule consists of a chain structure of axial-axial α-(1-4)-linked D-galacturonic acid units, containing blocks of L-rhamnose rich regions, with mainly arabinose, galactose and xylose side chains (Alkorta *et al.,* 1998) (Figure 1.4).

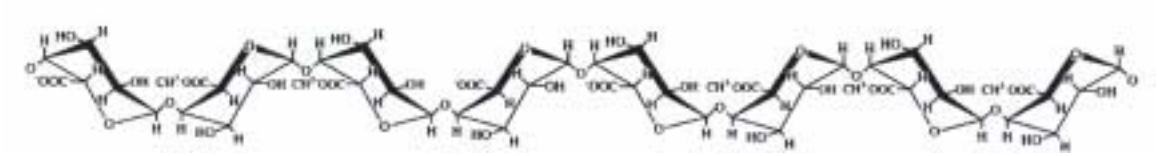

Figure 1.4 Structure of pectin molecule (Alkorta *et al.,* 1998)

Based on the type of modification of the backbone chain, pectic substances are classified into protopectin, pectic acid, pectinic acid and pectin (Kashyap *et al.,* 2001).

Basically three types of pectic enzymes exist; de-esterifying enzymes (pectinesterases), depolymerizing enzymes (hydrolases and lyases) and protopectinases (Table 1.10) (Alkorta *et al.,* 1998).

Table 1.10 Classification of pectic enzymes acting on pectins or pectic acids (Alkorta *et al.,* 1998).

EC suggested name	Common name	EC number	Substrate	Action pattern
De-esterifying enzymes				
Polymethylgalacturonate esterase (PMGE)	Pectinesterase	3.1.1.11	Pectin	Random
Depolymerizing enzymes				
Hydrolases				
Endopolygalacturonase (Endo-PG)	Polygalacturonase	3.2.1.15	Pectate	Random
Exopolygalacturonase 1 (Exo-PG1)	Polygalacturonase	3.2.1.67	Pectate	Terminal
Exopolygalacturonase 2 (Exo-PG2)	Polygalacturonase	3.2.1.82	Pectate	Penultimate bonds
Endopolymethylgalacturonase (Endo-PMG)	Pectin hydrolase		Pectin	Random
Exopolymethylgalacturonase (Exo-PMG)	Pectin hydrolase		Pectin	Terminal
Lyases				
Endopolygalacturonate lyase (Endo-PGL)	Pectate lyase	4.2.2.2	Pectate	Random
Exopolygalacturonate lyase (Exo-PGL)	Pectate lyase	4.2.2.9	Pectate	Penultimate bonds
Endopolymethylgalacturonate lyase (Endo-PMGL)	Pectin lyase	4.2.2.10	Pectin	Random
Exopolymethylgalacturonate lyase (Exo-PMGL)	Pectin lyase		Pectin	Terminal

These enzymes are mainly synthesized by plants and microorganisms. *Aspergillus niger* is used for the industrial production of pectinolytic enzymes (Naidu and Panda, 1998). Several microbial pectinases were listed in Table 1.11.

Table 1.11 Some examples for microbial thermostable alkaline pectinases (Kashyap *et al.,* 2001).

Microorganism	Type of pectinase	Optimum pH	Optimum Temperature
Bacillus sp RK-9	PGL	10.0	75
*Bacillus sp.*NT-33	PG	10.5	75
Bacillus polymyxa	PG	8.4-9.4	45
Bacillus pumilus	PATE	8.0-8.5	60
Bacillus sp. DT 7	Pectate lyase	10.25	70
Bacillus subtilis	PAL	8.5	60-65
Pseudomonas syrigae pv. glycinea	PAL	8.0	40
Bacillus P-4-N	PG	10-10.5	65
Bacillus stearothermophilus	PATE	9.0	70

1.2.5.1. Industrial Applications of Thermostable Pectinases

Pectinases were some of the first enzymes to be used in homes. Their first commercial application was in 1930 for the preparation of wines and fruit juices. The

estimated market value of all industrial enzymes was 1 billion US Dollar in 1995, of which 75 million US Dollar was assesed for pectinases (Kashyap *et al.,* 2001).

In the production of fruit juices, extracts and concentrates, pectinases are very important in maceration and solubilization of fruit pulps and in clarification (Naidu and Panda, 1998). These enzymes have been used in several areas including textile processing and bioscouring of cotton fibers, degumming of plant bast fibers, retting of plant fibers, pre-treatment of pectic wastewaters, coffee and tea fermentations, paper and pulp industry, poultry feed, purification of plant viruses, and oil extraction. (Hoondal *et al.,* 2002).

Sugar beet, the main source of sugar production is very rich in pectin. Sugar beet is extracted at temperatures of 70 °C. A strain of *Bacillus licheniformis* producing thermophilic exopolygalacturonate lyase with a temperature optimum 69 °C and pH optimum 11, has been isolated from extracts of sugar beet (Singh *et al.,* 1999).

1.2.6. Lipases

Lipases, (triacylglycerol acylhydrolases, EC 3.1.1.3), catalyse the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acid (Figure 1.5). Lipases occur widely in nature but only microbial lipases are commercially significant (Sharma *et al.,* 2001).

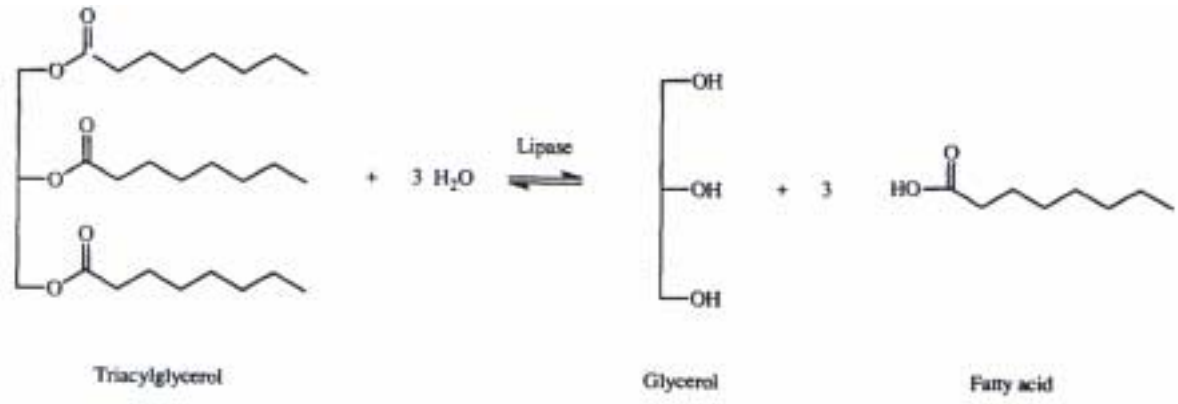

Figure 1.5 Enzymatic reaction of a lipase catalyzing hydrolysis or synthesis of a triacylglycerol substrate (Jaeger *et al.,* 1994).

Lipases are a versatile group of enzymes and often express other activities like phospholipase, isophospholipase, cholesterol esterase, cutinase, amidase and other esterase type of activities (Svendsen, 2000). They have a number of unique characteristics, including substrate specificity, stereospecificity, regiospecificity and ability to catalyse a heterogeneous reaction at the interface of water soluble and water insoluble systems (Sharma *et al.,* 2001).

Recently several thermophilic lipases have been purified and characterized from thermophilic *Bacillus sp* (Sharma *et al.,* 2002; Sidhu *et al.,* 1998), *Bacillus thermoleovorans* (Lee *et al.,* 1999; Lee *et al.,* 2001; Markossian *et al.,* 2000), *Bacillus stearothermophilus (*Sinchaikul *et al.,* 2001)*, Bacillus circulans* (Kademi *et al.,* 2000). Thermostable lipases from *Clostridium saccharolyticum*, the archeon *Sulfolobus acidocaldarius*, *Pyrococcus furiosus,* and several *Pseudomonas sp* have also been reported (Niehaus *et al.,* 1999). However, most of the commercialised lipases are of fungal origin (Jaeger *et al.,* 1994).

Table 1.12 Source microorganisms and properties of thermostable lipases (Haki and Rakshit, 2003).

Organism	Enzyme properties	
	Optimal temprerature (˚C)	Optimal pH
Bacillus acidocaldarius (esterase)	70	
Bacillus sp. RSJ - 1	50	8.0 9.0
Bacillus strain J 33	60	8.0
Bacillus stearothermophilus	68	
Bacillus thermocatenletus	60 70	8.0 9.0
Bacillus thermoleovorans ID - 1	70 75	7.5
Geobacillus sp.	70	9.0
Pseudomonas sp.	65	9.6
Pseudomonas sp.	90	11.0
Pyrobaculum calidifontis	90	
Pyrococcus furiosus (esterase)	100	
Pyrococcus horikoshii	97	5.6
Pyrococcus horikoshii	95	7.0

1.2.6.1. Industrial Applications of Thermostable Lipases

Known industrial applications of thermostable lipases include, production of mono- and diacylglycerides, fatty acids and glycerol through hydrolysis of oils and fats, modification of the composition and physical properties of triacylglycerides, synthesis

of chemicals in organic solvents, paper manufacturing, and biochemical catalyzis in supercritical fluids (Markossian *et al.,* 2000).

Lipid-rich waste materials produced during processes like oil refining, soap and leather production, contain high concentration of oxidizable substances in addition to low pH values and high temperatures. At temperatures above 50 °C, the lipid aggregates melt and stable emulsions of substrates with large surface area are formed. Under such conditions thermostable lipases are preferred for waste treatment. High temperatures are also desirable because the viscosity of the streams decreases and thus the diffusion and mass transfer are accelerated. Furthermore the sterilisation of waste sludge is achieved at temperatures above 60 °C (Markossian *et al.,* 2000).

1.3. Identification Methods for Bacteria

Microbial classification methods can essentially be divided into two separate groups. These are known as phenotypic and genotypic characterization methods.

1.3.1. Phenotypic Methods

In identifying bacteria, certain general characteristics have primary importance for determining the major groups to which the new isolate is likely to belong. Characteristics which are important and widely used, include morphology (rod, coccus, helical or other), Gram reaction, nutritional classification (phototrophy, chemoorganotrophy, chemolithotrophy), cell wall structure, presence of cell inclusions and storage products, nutritional requirements, ability to use various carbon, nitrogen, sulfur sources, fermentation products, temperature and pH requirements, antibiotic sensitivity, pathogenicity, immunological characteristics, and the habitats (Smibert and Krieg,1994; Madigan *et al.,*1997)

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

Biotyping is based on the classification of microorganisms by their reactions with various biochemical reagents. The method enables differentiation at genus and/or

species level but not at subspecies level. Utilization of different carbon sources is an important example for this technique (Busch and Nitschko, 1999). Antibiogram is the testing of the growth of an organism in the presence of various antibiotics. It is however not discriminatory since antibiotic resistance can change rapidly by the horizontal transformation of plasmids (Busch and Nitschko, 1999). In serotyping, microorganisms are characterized by using special antibodies. Another method, known as immunoblotting is based on separation of whole proteins by polyacrylamide gel electrophoresis to differentiate microorganisms (Busch and Nitschko, 1999).

Since phenotypic markers may not be stably expressed under certain environmental or culture conditions, the phenotypic methods alone can not be satisfactory and reliable for the differentiation of microorganisms (Farber, 1996). The genotypic and phenotypic results therefore must be evaluated together to classify an unknown isolate.

1.3.2. Molecular Characterization Methods

Molecular characterization methods involve DNA based analysis of chromosomal and extrachromosomal genetic material (Farber, 1996).

For evaluating typing systems factors like reproducibility, typeability, discriminatory power, ease of interpretation and ease of performance are important. Genotypic methods have many advantages over phenotyping methods in terms of these factors (Farber, 1996).

Reproducibility, is the ability to repeatedly obtain the same typing profile result with the same bacterial strain. This is especially important for construction of reliable databases. Because the genomic DNA is much more stable, the result is not effected easily by cultural conditions or methods of preparation (Olive and Bean, 1999). Thus, it is always possible to extract DNA from bacteria, all strains can be typed (Farber, 1996).

Discriminatory power is the ability to produce results that can distinguish two closely related strains and demonstrate the relationship of organisms from different sources. Molecular methods have greater discriminatory power than phenotypic methods (Bush and Nitschko, 1999).

The results of genotypic methods can also be analysed statistically and are suitable for automation (Bush and Nitschko, 1999).

The most common molecular characterization methods involve plasmid typing, sequencing, ribosomal RNA genes (*rrn* operon) based methods including 16S rDNA-ITS (Internally Transcribed Spacer) region-RFLP (Restriction Fragment Length Polymorphism), ribotyping, and genomic DNA based methods including pulsed field gel electrophoresis (Farber, 1996).

1.3.2.1. Pulsed Field Gel Electrophoresis

Pulsed Field Gel Electrophoresis **(**PFGE) is a very discriminating and reproducible typing method which allows extremely large DNA molecules to be resolved. It has the discriminatory power at subspecies level.

For PFGE, bacterial isolates grown in both or solid media are combined with molten agarose. The embedded cells are first lysed by a suitable lytic agent, and then the deproteinization by proteinase K digestion is achieved. Several washing steps are applied to avoid the inhibitory affects of the latter compounds. The intact chromosomal DNA was then digested with a low-frequency cutting enzyme. The digested bacterial plugs are inserted into a PFGE grade agarose gel, and are then subjected to electrophoresis. Finally, the restricted genomic DNA fragmentation patterns are visualised with ethidium bromide (Farber, 1996; Olive and Bean, 1999). A schematic illustration of a PFGE experiment was shown in Figure 1.6.

In PFGE separation, when the first electric field (E1) is applied to the gel DNA molecules elongate in the direction of the field and begin to migrate in the porous gel.

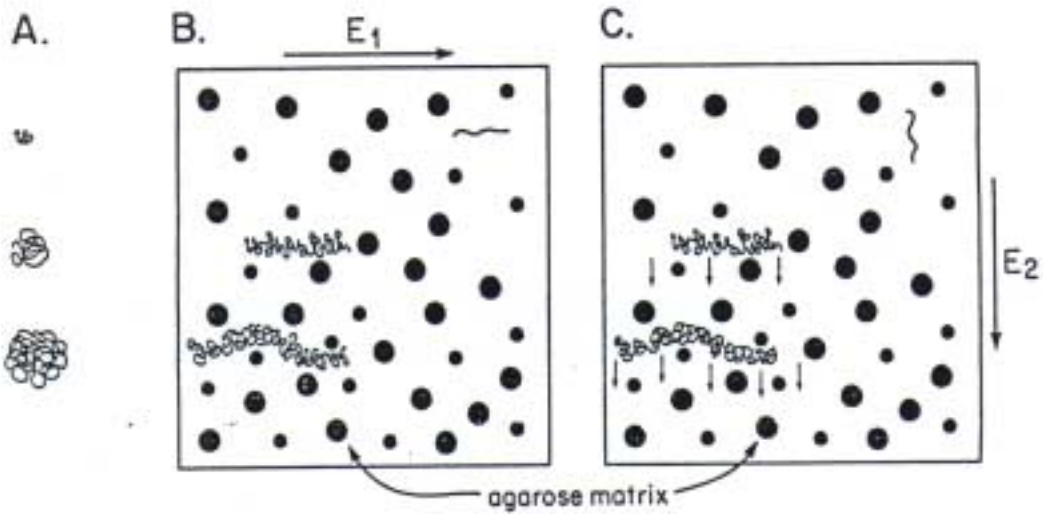

Figure 1.6 Schematic illustration of DNA separation in a typical pulsed field gel electrophoresis (Birren and Lai , 1993)

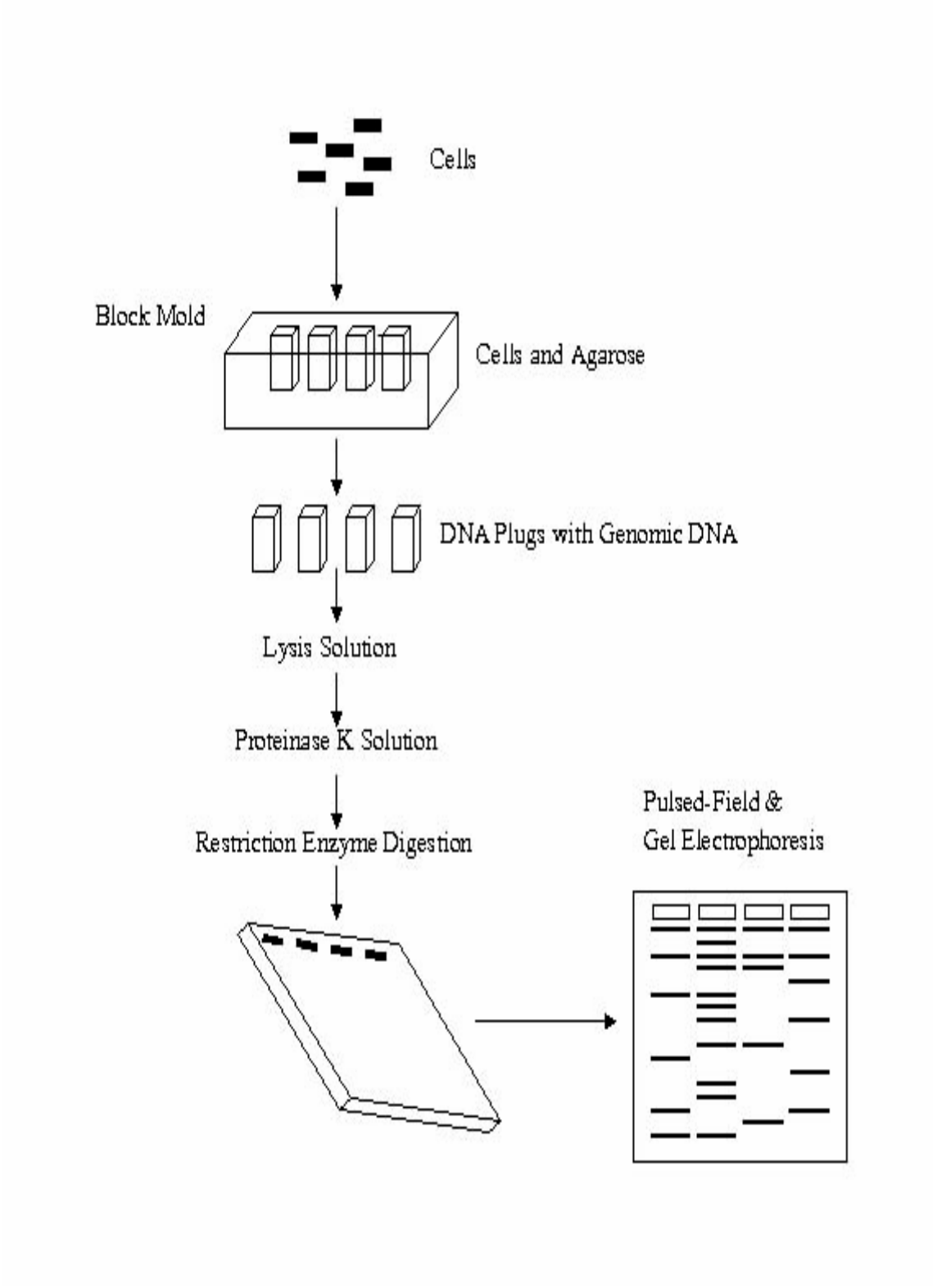

Figure 1.7 Schematic illustration of PFGE (Farber, 1996).

Then the second electric field (E-2) at some angle is applied to force the DNA fragments to change their conformations and reorient themselves to the direction of new electric field. As long as the alternating fields are equal in respect of time and voltage, DNA will migrate in a straight path down the gel. This principle allows the separation of very high molecular weight of DNA (Birren and Lai, 1993).

Clamped homogeneous electric field system (CHEF) is the most commonly used PFGE system (Bio-Rad Manual, 2001). In this system, principles of electrostatics have been adopted to generate homogeneous electric fields through twenty four electrodes, which have been arranged in a hexagonal array and clamped. In the system 120º reorientation angle is generated which is optimal for separating DNA molecules ranging from 100kb to 6 mb in size. (Birren and Lai, 1993; Bio-Rad Manual, 2001).

Pulsed field gels are remarkably sensitive to changes in all electrophoretic parameters. These are:

- switch interval,
- voltage gradient,
- agarose,
- running temperature,
- buffer type and strength,
- and electrophoresis time.

Switch interval is the amount of time during which each of the alternating electric fields is active. It is also named as switch time or pulse time. The size of the DNA to be resolved is directly proportional to the length of switch interval. Therefore, larger DNA molecules require longer times to reorient themselves while small molecules reorient quickly, thus requiring shorter switch intervals (Birren and Lai, 1993).

A voltage gradient is the difference between the electrical potential of the electrodes. DNA migration will increase by increasing the voltage gradient however high voltage can reduce the resolution capacity of the system (Bio-Rad Manual, 2001). Although high voltage increases DNA migration rate for high molecular weight DNA fragments (>2Mb), field strength should be decreased. To obtain comparable resolution, when applying lower voltages longer switch intervals must be chosen (Birren and Lai 1993)

Changes in agarose concentration will also affect both the speed of separation and the size range of fragments resolved. When the agarose concentration is decreased the DNA migration rate increases and larger size range of DNA fragments is separated, (Birren and Lai 1993). Agarose concentration of 1 % is optimum for DNA molecules up

to 3Mb in size. For DNA molecules greater than 3 Mb, however, 0.5-0.9 % agarose concentration must be used (Bio-Rad Manual, 2001).

As the running temperature increases DNA will migrate quickly. However the resolution is diminished. The most suitable temperature for proper speed and resolution is 14 ºC (Birren and Lai 1993).

Buffer concentration affects the mobility and resolution of DNA molecules. When buffers with low ionic strength are used, DNA will migrate much more quickly but the buffering capacity of the diluted buffers is reduced. The most suitable buffer must balance the separation speed and buffering capacity (Bio-Rad Manual, 2001).

The electrophoresis run time is selected by migration rates of the DNA. If the migration rate of DNA molecules decreases, the electrophoresis run time must be increased (Birren and Lai 1993).

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 (Figure 1.8)(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 (*box*A) (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).

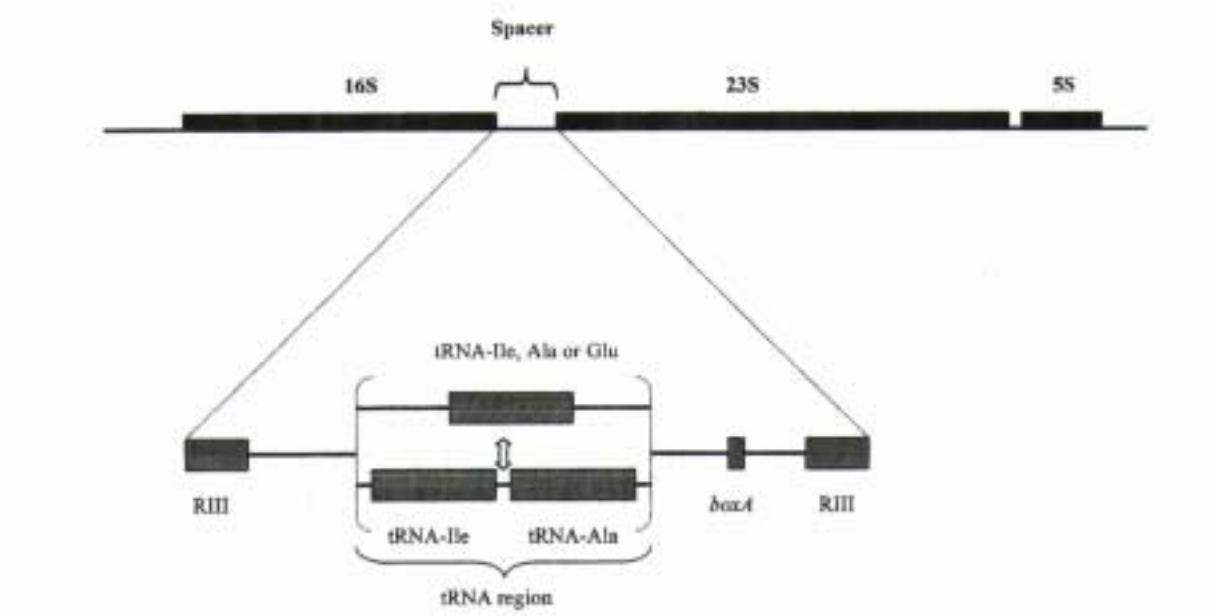

Figure 1.8 Schematic representation of a 16S-23S spacer and organization of ITS functional regions (Garcia-Martinez *et al.,* 1999).

The method is based on the amplification of 16S rDNA and ISR regions as a single amplicon. 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 small, usually double stranded, circular DNA and exist within the cells independent of the chromomal DNA. They are self-replicating (Farber, 1996). Although not essential for the survival of bacteria, plasmids may encode, many different genetic determinants which could be beneficial to the host. Some plasmids include information for their own transfer from one cell to another (F plasmids) and some of them encode resistance to antibiotics (R plasmids). Some of them may carry specific genes for utilization of metobolites which have importance in agriculture and industry. And some of them have no apparent functional coding genes (cryptic plasmids) (Crosa *et al.,* 1994).

The size of the plasmids ranges from 1.5 kb to around 300kb (Farber, 1996). Some plasmids are found in high copy number (10-100) in a host cell. Some are found in a few copies (1-4) and these are named low-copy number plasmids (Crosa *et al.,* 1994).

In plasmid typing, plasmids are isolated from each strain and digested with a specific restriction enzyme. The fragments are then separated electrophoretically in an agarose gel in order to obtain plasmid restriction patterns. As different plasmids give different patterns, plasmid typing is achieved.

Plasmid profiling is the one of the oldest and simplest genotypic characterization method. When compared with other genotypic methods, it has some advantages and disadvantages. The method is relatively quick and easy. The results can also be standardized using known markers. However the facts that plasmids are usually unstable and some organisms may not have plasmids, the use of this method is limited (Farber, 1996).

1.3.2.5. DNA Sequencing

DNA sequencing is the process of determining the exact order of nucleotides in a region of 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 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. A Brief Information on Balçova (Agamemnon) Geothermal District Heating System and on Balçova Physical Therapy and Rehabilitation Centre

The town of Balçova, situated at the west-end of Izmir, include a very rich geothermal area. Balçova is a Persian word and it means "a happy and colourful habitat" (Çanakçı, 2003). The Balçova geothermal site has been studied since 1963 through drilling of various wells. The first well, drilled in 1963, was 40 m in depth, and the water produced from the well had a temperature of 124 ^{o}C. After this, 11 more wells have been drilled to produce hot water for the Balçova geothermal district heating system which is the largest geothermal heating system in Turkey (Çanakçı, 2003).

Balçova is the first known geothermal hospital in the world, dating back as far as the Troian War, B.C. 1200. Some historical evidence, Homeros' Iliada for example, implies that after the Troian War, Agamemnon had been to Izmir, and his soldiers healed their wounds in the Balçova hot springs. And people of Balçova had then named the geothermal springs after Agamemnon.

Today, Agamemnon hot springs serves as the modern geothermal hospital called Balçova Physical Therapy and Rehabilitation Centre, by applying essentially two therapeutic methods: balneotherapy, and mud pack therapy. Majority of the patients are from Northern Europe: Norway, Denmark, the Netherlands, and Germany. Since 1992, for example, approximately 15,000 patients mainly from Norway and Denmark have been treated with 90-95 % curing efficiency.

Balneotherapy is defined as the direct utilization of spa waters in the form of bathing and drinking. This type of therapy has been developed in places where spa water flowed up to the earth's surface (Karagülle, 2000).

In mud pack therapy, however, mud to be used undergoes a maturation process (Bellometti, 2000). The mud is incubated in special tanks for a period of time (1-60 days), where it is in continuous contact with thermal water at temperatures varying between 60-90 ^{o}C. During this process, certain thermophilic microorganisms are grown. These microorganisms can contribute to the ingredients and somewhat modify some of the chemical and physical properties of mud by producing certain organic substances, for example sulpho-gluco-lipids, which is known to have anti-inflammatory properties (Bellometti, 2000). In keeping with this view, thermoaerophilic bacterial flora of Balçova geothermal site was investigated in the scope of the thesis project.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used in this study were shown in Appendix A

2.1.2. Samples

Eight water and mud samples from Balçova Geothermal Region were taken aseptically. The water samples were collected into thermos flasks in order to maintain the temperature during transportation. Two of the samples were from reinjection water ; one from the entrance of the system and one from the exit of the system from Geothermal District Heating Centre**.** The temperature of the samples was between 60°C -65 °C and the pH was approximately 8.0. Two of the samples were taken from uncontrolled thermal leak. The temperature was 70-75 °C and the pH was approximately 8.5. Four of mud and water samples were from thermal treatment centre. The temperature of the the water samples were 60°C -65°C, and the pH between 8.0-8.5. The mud and soil samples were at room temperature. The pH was between 8-9.

2.1.3. Reference strains

Reference strains were obtained from The Spanish Type Culture Collection (CECT), Agricultural Research Service Culture Collection (ARS/NRRL; Peoria, IL., USA)

Bacillus stearothermophilus CECT 49

Bacillus stearothermophilus CECT 47

Bacillus stearothermophilus CECT 43

Bacillus stearothermophilus CECT 48

Bacillus thermoglucosidasius CECT 4038

Bacillus thermoglucosidasius NRRL 1407

Bacillus kaustophilus CECT 4264

2.2. Methods

2.2.1. Isolation of Thermophilic Bacteria

Both dilution plate method and enrichment method were used for isolation of thermophilic bacteria (Holt and Krieg, 1994).

For the enrichment method, 10 gr or 10 ml of samples were transferred into 100 ml of appropiate broth media (Appendix B). Incubation was performed in rotary shaker at 60 °C until a turbidty occured. Then 500 µl of the broth was plated on the solid media (Appendix B). For the dilution plate method 10g soil or 10 ml of water samples were transferred in 90 ml of 0.85% saline water. The mud samples only were pasteurized for 10 minutes at 80 °C in a waterbath in order to kill most of the vegetative cells and thus to eliminate non-spore forming bacteria (*Mora et al.*, 1998). Following the heat treatment 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) and incubated for 24h at 60 °C. The plates were covered with cooking bags to prevent drying of agar at this temperature. Single colonies were picked and they were purified using streak plate method (Holt and Krieg, 1994).

2.2.2. Preservation of the Isolates

Reference strains and the isolates were stored in isolation broth containing 20 % glycerol. Cultures were grown overnight in isolation broth. 0.5 ml of each culture were transferred into cryo tubes and 0.5 ml broth containing 40% glycerol was added. These steps were all performed on ice. Then tubes were mixed gently. Cultures were stored at –80 °C.

2.2.3. Determination of Phenotypic Characteristics

2.2.3.1. Morphology and Gram Reaction

Isolates were stained by Gram method. A loopfull of the overnight culture was spread onto the microscope slides until a thin film formed. After drying, they were fixed by passing the slide through flame for 1-3 seconds. The heat fixed smear was first

stained with crystal violet for 1 min. After rinsing under the tap water, the slides were transferred into iodine solution and was kept for 1 min. After this step, slides were washed in 95% alcohol for 6 s. They were then stained with safranin for 30 s. After staining, the slides were dried on paper towels and the cells were examined under light microscope. Gram (+) cells assumed purple colour while Gram (–) cells appeared pink or red.

2.2.3.2. Examination of Endospores

Isolates were grown on sporulation media (Appendix B) for 1-2 days. The cells were suspended in 3-5 µl of sterile distilled water on a glass slide and covered with another slide. Endospores were observed under the phase-contrast microscope. For the determination of cell and spore diameters, a micrometric slide and micrometric ocular disc were used. First, diameter (mm or µm) of one space of ocular disc according to the objective and ocular had to be calculated. For this reason the beginning points of micrometric slide and ocular disc were overlapped for each objective. The data were recorded. The micrometric slide was then taken away and a cell containing slide was examined. The diameters of the cells and spores were noted in terms of spaces on ocular disc. According to previous data, the exact diameters were calculated.

2.2.3.3. Catalase Test

Isolates were grown on nutrient agar for 24 h at 55°C. 3% hydrogen peroxide was poured onto the colonies. Formation of air bubbles indicated the presence of catalase activity.

2.2.3.4. Oxidase Test

Isolates were grown on nutrient agar for 24 h at 55°C. A filter paper was placed into a petri dish and was wetted with 1% solution of tetramethyl-p-phenylenediamine. One large colony was taken with a loop and placed onto the wet filter paper. The presence of oxidase activity was indicated by the formation of blue-purple color.

2.2.3.5. Growth at Different Temperatures

Isolates were grown in nutrient agar plates and incubated for 1-3 days at 37°C, 45 °C, 65°C, 70 °C. Growth was checked periodically.

2.2.3.6. Growth at Different NaCl Concentrations

The ability of the isolates to grow in 0.5 %, 1%, 3%, 6% NaCl was tested in nutrient agar plates containing one of above NaCl concentrations. Isolates were grown for 1-3 days at 55 °C. Growth was checked periodically.

2.2.3.7. Growth at Different pH Ranges

The ability of the isolates to grow at pH 8, pH 9, pH 10 was tested in nutrient agar plates. pH was adjusted with sodium carbonate (separately autoclaved). Isolates were grown on media for 1-3 days at 55 °C. Growth was checked periodically.

2.2.4. Screening for Extracellular Enzymes

2.2.4.1. Screening for Protease Activity

Media used for protease screening were listed in Appendix B (Priest *et. al.* 1988). Cultures were inoculated in the media, after incubation for 24h at 55 ºC, clear zones around colonies indicated proteolytic activity.

2.2.4.2. Screening for Amylase Activity

After inoculation of the cultures (Appendix B) and incubation for 1-2 days at 55 ºC, iodine solution (I_2 =1 g, KI= 2 g/ 300 ml) was poured on the plates, white clear zones against a blue background around the colonies were taken as the indication positive for amylase activity (Bragger *et al*., 1989).

2.2.4.3. Screening for Xylanase Activity

After inoculation of the cultures, (Appendix B) and incubation for 3-4 days at 55 ºC, 0,1 % congo red solution was poured onto the plates. After 30 min incubation at room temperature, the plates were washed with 1 M NaCl solution. Clear zones around the colonies on a red background were taken as the evidence for the xylanase activity (Bragger *et al*., 1989).

2.2.4.4. Screening for Cellulase Activity

After inoculation of the cultures, (Appendix B) and incubation for 3-4 days at 55 ºC, 1 % congo red solution was poured onto the plates. After 30 min incubation at room temperature, the plates were washed with 1 M NaCl solution. Clear zones around the colonies on a red background were taken as the evidence for the cellulase activity (Bragger *et al*., 1989).

2.2.4.5. Screening for Lipase Activity

After inoculation of the cultures in the media (Appendix B) and incubation for 2 days at 55 ºC. Opaque halos occur around the colonies showed the presence of lipase activity (Haba *et al.,* 2000).

2.2.4.6. Screening for Pectinase Activity

Cultures were inoculated in the solid media (Appendix B) and incubated for 3-4 days at 55 ºC. After colonies were formed, 1% cetyltrimethylammoniumbromide solution was poured onto the surface of the plates. After 10 minutes incubation at room temperature, colonies with clear zones indicated pectinase activity (Kobayashi *et al.,* 1999).

2.2.5. Preparation of Genomic DNA

The CTAB/NaCl method (Ausubel *et al*., 1994), with some minor modifications, was used. Twenty four hour cultures grown on solid media were scraped and suspended in 1.5 ml sterile water. Cells were pelleted by centrifugation for 5 min at 5000 rpm. Pelleted cells were resuspended in 567 µl 1xTE. Thirty µl 10% SDS and 3 µl proteinase K solution (20 mg/ml) were added and the solution was mixed throughly. The samples were incubated for 1 h at 37 ^{o}C. After the incubation, 100 µl 5 M NaCl was added and mixed throughly. After this step 80µl CTAB/NaCl solution (10% cetyltrimethylammonium bromide, 0.7 M NaCl) were added, mixed throughly and the samples were then incubated for 10 min at 65 ^{o}C. Chloroform extraction was performed twice (Section 2.2.6.1.3.). DNA wool was obtained by the addition of isopropanol (0.6 volume) and was then washed in 500 µl ethanol (70%). DNA was pelleted, dried and dissolved in 100µl TE using alternating heat shocks (10 min at 80 ^{o}C, 20 min at -20 ^{o}C and 10 min at 80 ^{o}C). The DNA solutions were preserved at -20 C.

Also DNA extraction was performed as described by *Bravo et.al.* (1998) for reference strains of thermophilic *Bacillus.* The strains were grown overnight on nutrient agar plates at 55 ^{o}C. A loopfull of cells were transferred into 0.1 ml of sterile distilled water. The mixture was frozen for 20 minutes at –80 ^{o}C. It was then transferred immediately into a boiling water in waterbath and incubated for 10 minutes. Finally, the cell lysate was centrifuged for 10 s at 10 000 rpm and used as DNA template.

2.2.6. Genotypic Characterization

2.2.6.1. Identification of Isolates by 16S rDNA –ITS (Internally Transcribed Spacer) - RFLP (Restriction Fragment Length Polymorphism)

2.2.6.1.1. Amplification of 16S rDNA – ITS Region

Polymerase chain reaction (PCR) was performed in a final volume of 50µl containing approximately 500ng of genomic DNA template, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of each of the DNA primers, 1x PCR buffer, and 1,25 u *Taq* DNA polymerase (Appendix C).

Two µl of DNA samples were transferred into 0.5 ml PCR tubes. PCR mixture was prepared as described in Appendix C and it was mixed gently and centrifuged for a few seconds. 48µl of the PCR mix were then distributed into each tubes. The tubes were then overlaid with 60µl mineral oil. They were then centrifuged for a few seconds at 6000 rpm. All the steps were performed on ice. 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) } 40 cycles
Step 4: 72 ˚ C for 1min (elongation)

Step 5: 72 ˚ C for 10 min (final extension)

DNA primers used in the experiments were,

Forward, L1 : 5'-AGAGTTTGATCCTGGCTCAG-3' (Mora *et al.,* 1998)

Reverse, EGE1 : 5'-CAAGGCATCCACCGT-3' (Jensen *et al.,* 1993).

The forward primer was complementary to the upstream of 16S rDNA and the reverse was complementary to the downstream sequences of ITS region.

2.2.6.1.2. Electrophoresis of Amplified 16S-ITS Fragments

PCR products fragments were separated in 0.8 % agarose gels. First, 0.80 gr agarose was dissolved in 100ml of 1× TAE buffer by boiling. Ten µl of ethidium bromide solution (10 mg/ml) were added after cooling the agarose solution to 40 ^{o}C. It was then poured into gel casting stand and combs were placed. After the gel was cooled, the combs were removed. The casting tray carrying gel was placed into the tank. 1× TAE buffer was carefully added until the buffer covers the gel. Five µl of PCR products were taken and mixed with 1µl of gel 6x loading buffer (Appendix C) Then the samples and 4µl of DNA molecular weight marker were loaded into the wells of agarose gel. The electrophoresis was performed for approximately 2.5 h at 60 mA. The PCR products were visualised on an UV illuminator. Images of the gels were visualised in a gel documentation system (Vilber Lourmat, France) and further analyses were performed on these gels by using BioRD^{++} computer programme.

2.2.6.1.3. Chloroform Extraction of PCR Products

Samples below the mineral oil was taken gently by using a micropipette and transferred into eppendorf tubes. The volume of the PCR product was adjusted to 100 µl with 1x TE. The solution was then centrifuged for 5 minutes at 10.000 rpm. Two volumes of chloroform isoamyl alcohol solution (chloroform/isoamyl alcohol: 24/1) were added onto the samples and mixed throughly. They were then centrifuged for 2 min at 10.000 rpm. The aqueous phase was taken and once more mixed with two volumes of chloroform isoamyl alcohol solution. They were mixed well and centrifuged for 2 minutes at 10.000 rpm. The aqueous phase (100 µl) was transferred into new eppendorf tubes and 10 µl 3 M sodium acetate were added. The mixture was mixed well. Two and a half volumes of 99%ethanol was then added and mixed throughly. The samples were then centrifuged for 15 minutes at 10.000 rpm. The liquid phase was removed and the pellet was washed in 500 µl 70% ethanol. After the centrifugation for 5 minutes at 10.000 rpm, ethanol was removed without disturbing the pellets. The pellets were dried at room temperature and dissolved in 25 µl 1xTE. 5µl of the purified PCR product was electrophoresed to estimate the concentration of DNA.

2.2.6.1.4. Restriction Fragment Length Polymorphism (RFLP)

Two restriction endonucleases were selected and used. These were;

*Hae*III (GG ‘CC)

*Taq*I (T’CGA)

Approximately 700 ng of the amplification products, were digested with 5 units of each of the enzymes in a final volume of 50µl .Ten µl of each PCR product was transferred into eppendorf tubes and 40 µl restriction enzyme mixtures (Appendix C) were added. *Taq*I digestion samples were overlaid with mineral oil and the restriction reactions were carried out in a water bath at 65 ^{o}C. *Hae*III digestions were performed at 37 ^{o}C. Both reactions were incubated overnight. After the digestion, DNA was extracted as described in section 2.2.6.1.3. and dissolved in 15 µl 1xTE.

2.2.6.1.5. Electrophoresis of Restriction Fragments

Restriction fragments were separated in 2.5 % agarose gels. First, 2.5 gr agarose was dissolved in 100ml of 1× TAE buffer by boiling. 10µl of ethidium bromide solution (10mg/ml) was added after cooling the agarose solution to 40 ^{o}C. Then it was poured into gel casting stand and combs were placed. After the gel was cooled, the combs were removed. The casting tray carrying gel was placed into the tank. 1× TAE buffer was carefully added until the buffer covers the gel. 10µl of restriction fragments were taken and mixed with 2µl of 6x gel loading buffer. Then the samples and 4µl of DNA molecular weight marker were loaded into the wells of agarose gel.

Electrophoresis was performed for 30 minutes at 60 mA and for approximately 2.5 hours at 80 mA. Images of the gels were recorded and analysed in a gel documentation system (Vilber Lourmat, France).

2.2.6.2. Identification of Isolates by Plasmid Profiling

2.2.6.2.1. Plasmid Isolation

The method of Sullivan and Klaenhammer (1993) with some minor modifications, was used for isolation of plasmid DNA.

Bacterial cultures were streaked from frozen glycerol stocks onto nutrient agar plates and were then incubated overnight at 55 ^{o}C. The cells were scraped and suspended in 1.5 ml sterile water. They were centrifuged for 15 minutes at 5000 rpm. The supernatant was discarded and the pellet was suspended in 200µl lysozyme solution (25% sucrose containing 30mg/ml lysozyme). The solution was incubated for 15 minutes at 37 ^{o}C. After the incubation, 400 µl of alkaline SDS solution (3% SDS 0,2 N NaOH) were added and incubated at room temperature for 7 min. After this step 300 µl of ice cold 3 M sodium acetate (pH 4.8) were added. The solution was mixed immediately and centrifuged for 15 minutes at 12.000 rpm at 4 ^{o}C. The supernatant was then transferred into a new eppendorf and 650 µl isopropanol solution was added and mixed well. After centrifugation for 15 minutes at 12.000 rpm at 4 ^{o}C, the supernatant was removed and the pellet was resuspended in 300 µl TE. Two hundred µl of ammonium acetate (7.5 M) containing ethidium bromide (0.5mg/ml) were added onto

the solution and mixed. After this step 350 µl phenol/chloroform solution were added, mixed well and centrifuged for 5 min at 10000 rpm at 4 ^{o}C. The aqueous phase was transferred into a new eppendorf tube and 1 ml ethanol (99% at -20 ^{o}C) was added and mixed well. After centrifugation for 15 minutes at 12.000 rpm at 4, the pellet was washed in 500 µl 70% ethanol (at room temperature). DNA was pelleted and resuspended in 40 µl TER (TE + 0.1 mg/ml RNase).

2.2.6.2.2. Restriction Enzyme Digestion of Plasmid DNA

Two restriction endonucleases were used for the digestion of plasmid DNA. These were;

*Hae*III (5'-GG 'CC-3')

*Taq*I (5'-T'CGA-3')

Ten µl plasmid DNA of each plasmid were electrophoresed to visualise the concentration of the material. The template volumes were adjusted according to the concentrations. The plasmid DNA was digested with 10 units of each of the enzymes in a final volume of 50µl. Adjusted volume of each plasmid DNA was transferred into eppendorf tubes and restriction enzyme mixtures were added. *Taq*I digestion reactions were overlaid with mineral oil and carried out in a water bath at 65 ^{o}C. *Hae*III digestion was performed at 37 ^{o}C. Both reactions were incubated overnight. After the digestion, DNA was extracted as described in section 2.2.6.1.3. and dissolved in 8 µl 1xTE.

2.2.6.2.3. Electrophoresis of Plasmid DNA

Restriction fragments were separated in 1.25 % agarose gels. The gels were prepared as described in section 2.2.6.1.2. Eight µl (300-500ng) of digested plasmid DNA were taken and mixed with 2 µlt of 6x gel loading buffer.

Gel electrophoresis was performed for 30 minutes at 60 mA and for approximately 2.5 hours at 80 mA. Images of the gels were recorded and analysed in a gel documentation system (Vilber Lourmat, France).

2.2.6.3. Pulsed Field Gel Electrophoresis RFLP

2.2.6.3.1. Preparation of Agarose Embedded Bacterial DNA

Bacterial cultures were streaked from frozen glycerol stocks, onto nutrient agar plates and were then incubated overnight at 55 ^{o}C. The reusable 15 welled plug mold holder (BIO-RAD) was unscrewed and agarose plugs were prepared using only one side of the mold holder. The cells were embedded into the agarose as follows. Onto the open molds, 100 µl of cell suspension buffer (10 mM Trizma base, pH 7.2; 20 mM NaCl; 50 mM EDTA, pH 8) were pipetted. The colonies were then scraped with a platinum loop into this cell suspension buffer. Using the edge of a steel spatula, the cells were then suspended by gentle mixing. After the cells were suspended homogenously, 100 µl of 1.5% low melting temperature agarose solution (in sterile water, at 50 ^{o}C) were added dropwise onto the cell suspension. Agarose and cells were mixed by using the edge of the spatula and gentle stirring. The plugs were then allowed to solidify.

The cells were lysed by transferring the plugs into 1.5 ml eppendorf tubes containing 500µl lysozyme solution (10 mM Trizma base, pH 7.2; 50 mM NaCl; 0.2 % sodium deoxycholate; 0.5% sodium lauryl sarcosine; and 1 mg/ml lysozyme), and were then incubated for 1h at 37 ^{o}C without agitation. The plugs were transferred into 50 ml falcon tubes containing 2.5 ml of 1x wash buffer (20 mM Trizma base, pH 8; 50 mM EDTA, pH 8) and rinsed by gentle agitation for 45 min at room temperature on an orbital shaker. The plugs were then transferred into 1.5 ml eppendorf tubes containing 500 µl proteinase K buffer (100mM EDTA, pH 8; 0.2% sodium deoxycholate; 1% sodium lauryl sarcosine; 1 mg/ml proteinase K), and were incubated for 18 h at 50 ^{o}C without agitation.

Prior to restriction enzyme digestion, the plugs were washed four times in 2.5 ml wash buffer by gentle agitation for 45 min at room temperature on an orbital shaker. First and second washes were in 1x wash buffer (20 mM Trizma base, pH 8; 50 mM EDTA, pH 8) plus 1 mM NaCl. Third wash was in 1x wash buffer plus 1 mM PMSF. Fourth wash was in 1x wash buffer.

2.2.6.3.2. Restriction Enzyme Digestion of Agarose Plugs

Sma I restriction endonuclease (5'-CCC'GGG-3') was used for the restriction enzyme digestion of genomic DNA.

After the washing steps the plugs were equilibrated in 1 ml of 1x *Sma*I restriction enzyme buffer for 1h at room temperature with gentle agitation.The buffer was then removed. Genomic DNA was then digested with 30 units of *Sma*I in 300 µl reaction volume overnight at 30 ^{o}C. Before electrophoresis the plugs were equilibrated in 1 ml of 1xTBE for 30 minutes at room temperature on an orbital shaker.

2.2.6.3.3. Pulsed Field Gel Electrophoresis

The electrophoresis was performed in 1% PFGE grade agarose (Biorad) gel. One gram of agarose was dissolved in 100 ml 1x TBE buffer by boiling. After the gel was cooled, it was poured onto the platform in the casting stand provided by CHEF DRII equipment. The 15 well comb was attached to the comb holder. After the gel was solidified, the comb was removed. The plugs were sliced to a comb size and loaded into the wells by using a spatula. The plugs were placed onto the front walls of the wells and care was taken to ensure that the height of the plugs should not be more than 90 % height of the wells.

% 1 low melting agarose was dissolved in 1x TBE by boiling. After it was cooled to approximately 40 ^{o}C. The agarose wells were then covered with this agarose solution. The gel was allowed to solidify for 15 minutes. Into the electrophoresis chamber approximately 2 L of 1xTBE at 4 ^{o}C was poured and the chiller and the pump were switched on 30 min before the electrophoresis to adjust the buffer temperature to 14 ^{o}C.

After the chiller and the pump were switched off, the gel was removed from the casting stand with a platform and placed into the electrophoresis cell.

Electrophoresis was performed in a CHEF DRII system (BIO-RAD) with 1-8 pulse times, for 26 h at 4 V/cm at 14 ^{o}C.

2.2.6.3.4. Staining the PFGE Gels

When the electrophoresis was complete, the gel was removed. Staining was performed in 200 ml dH_2O containing 100µl (10mg/ml) ethidium bromide for 30 min with gentle agitation. The gel was then destained with deionized water for 1-2 h with gentle agitation. The image of the gel was analyzed in a gel documentation system (Vilber Lourmat, France).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Isolation of Thermophilic Bacteria

Eight water and mud samples were collected from Balçova Geothermal Region for isolation of thermophilic bacteria. Both dilution plate and enrichment method were used as described in section 2.2.1. A number of different media were tried for isolation. For preparation of isolation media sterilised reenjection water was used. The isolates were selected according to differences in their colony morphology. The isolated strains showing extracellular enzyme activities were selected for further analysis. In total, 112 isolates were obtained after purification studies (Table 3.1). The 45% of the isolates were from uncontrolled thermal leak, 31% of the isolates were from reenjection water samples and 24% of the isolates were obtained from the mud samples used in Balçova Physical Therapy and Rehabilitation Centre.

Table 3.1 Samples, names and numbers of isolates.

Sample	Isolate Number	Isolate Name
Uncontrolled thermal leak	50	1,2,3,4,5,6,8,9,10,11,28a,13,14,16,17,18,19,20,21a,23,24,64b,26,651a,28,36,37,42,43,44,45,46,47,651b,50,53,58,59,60,61,63,64,65,66,67,70,86,87,90,92
Reenjection Water	35	75A,38y,21,62B,74B,30,75B,32,33,34,35,38,39,40,41,49,52,54,55,56,57,62,71,72,73,74,75,76,77,78,79,80,84,85,94
Mud	27	Ç1A, Ç1,Ç-2, Ç-3, Ç-4, Ç-5, Ç-6, Ç-7, Ç-9, Ç-10, Ç-11, Ç-13, Ç-14, Ç-16, Ç-17, Ç-19, Ç-20, Ç-21, Ç-22, Ç-23, Ç-24,Ç-31,Ç-33, Ç-34,Ç-32,Ç-35,Ç-30

3.2. Determination of Phenotypic Characteristics

3.2.1. Gram staining

Isolates and the reference strains were all Gram stained. As, they were observed as violet cells under the light microscope, they were all accepted to be Gram positive cells. All the isolates were rod shaped. Appearance of some gram (+) cells were shown in Figure 3.2.

3.2.2. Examination of Endospores

Isolates and the reference strains were examined for the presence of endospores. All the isolates were found to produce endospores. The position of the spores in the cells were subterminal or terminal. Appearance of cells and spores of some isolates and reference strains under phase contrast microscope were shown in Figure 3.1.

3.2.3. Catalase and oxidase tests

All the isolates and the reference strains were found to be catalase and oxidase positive. Weak reactions were also accepted as positive result. Although the reference strain *Bacillus kaustophilus* has been described as oxidase negative (Sunna *et al.,* 1997), the type strain used in the study was oxidase positive.

3.2.4. Growth at Different Temperatures

The isolates and reference strains were grown for 1-3 days at 37°C, 45 °C, 65°C, 70 °C. All the isolates and reference strains were able to grow at 45 °C and 65°C except one reference strain. *Bacillus thermoglucosidasius* CECT 4038 was not able to grow at 65°C. Weak observations of growth were also taken as positive result.

None of the reference strains could grow at 37 °C. Growth at 37 °C however for *Bacillus stearothermophilus* and *Bacillus thermoglucosidasius* have also been observed (Nazina *et al*., 2001). *Bacillus kaustophilus* CECT 4264, *Bacillus thermoglucosidasius* CECT 4038, *Bacillus thermoglucosidasius* NRRL 1407 were able to grow at 70 °C.

The results were in agreement with the other works (Nazina *et al*., 2001; Sunna *et al.,* 1997). *Bacillus stearothermophilus* CECT 43 was able to grow at 70 °C well, while other *Bacillus stearothermophilus* type strains growed weakly.

Most of the isolates (70 %) were able to grow at 37 °C (Table 3.2), they were therefore called as moderate thermophiles or facultative thermophiles. (Baker *et al*., 2001; Hughes and Williams 1977). Growth at 37 °C was detected for the rest of the isolates. Ten of the isolates were not able to grow at 70 °C (Table 3.2).

Table 3.2 Differentiation of isolates according to growth at different temperatures.

Isolates do not grow at 37 °C	33, 37, 61B,64, 64b,66, 70,75, 75A 76, 90,92,94
Isolates grow at 37 °C weakly	3, 4, 14, 16, 19, 21a, 23, 24, 651a, 74b, 75b, 36, 37, 38, 38y,45, 46, 53, 54, 55, 56, 60, 61, 65, 68, 71, 72, 73, 74, 84, ç-2, ç-3, ç-30, ç-34,
Isolates do not grow at 70 °C	26, 28, 32, 34, 35, 39, 53, 57, 64, Ç-9

3.2.5. The Ability of the Isolates and Reference Strains to Grow at Different NaCl Concentrations

The isolates and reference strains were tested for growth at 0,5 %, 1 %, 3 %, 6% NaCl concentrations. All the isolates and reference strains were able to grow at 0,5 %, 1 % NaCl concentrations. None of the isolates and reference strains except Isolate 2, and 9, 10 and 62 (weakly) could grow at 6% NaCl concentration.

None of the reference strains except *Bacillus thermoglucosidasius* CECT 4038 could grow at 3 % NaCl concentration. The isolates that were able to grow at 3 % NaCl were 1, 2, 4, 5, 8, 9, 10 ,11, 28a, 16, 17, 21a, 26, 28, 40, 41, 42, 43, 52, 58, 62, 65, 66, 67, 68, 70, 73, 75, 92, 94, ç-1a, ç-10, ç-13, ç-23, ç-24 that account for 35 % of the isolates. The isolates 58, 59, 72,74, 78, 80, 90, ç-6, ç-7, 64b, 651a could not be determined exactly for growth at 3 % NaCl concentration. It would be better if the NaCl range have been enlarged.

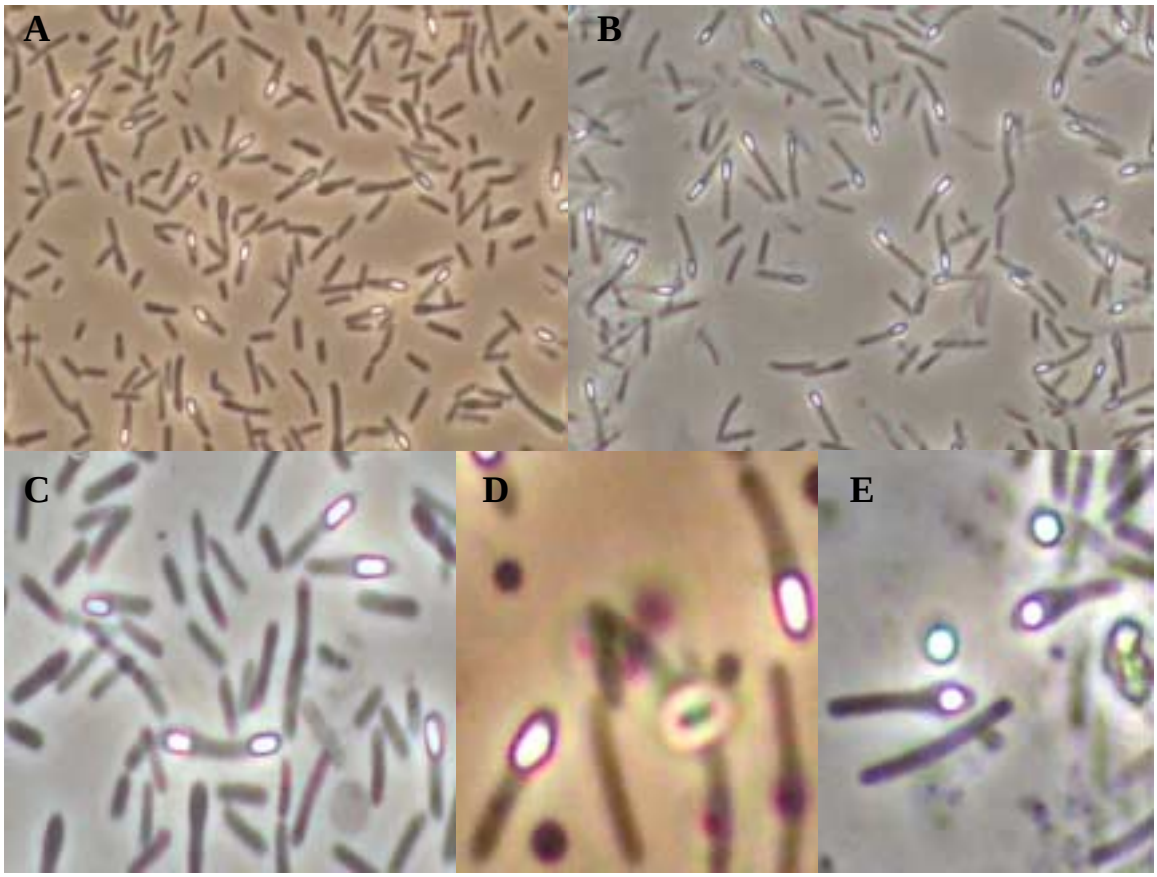

Figure 3.1 Appearance of cells and spores of some isolates and reference strains under phase contrast microscope. A-Isolate 8 (l/w, 7.8-2.5µm;e,1.4µm) B-Isolate Ç-24 (l/w, 6.4-1.6µm;e,1.0µm), C-Isolate 28 (l/w, 6.5-2.4µm;e,1.2µm), D-Bacillus *stearothermophilus* CECT 48 (l/w, 6.4-1.4µm;e,1.1µm), E- *Bacillus thermoglucosidasius* NRRL 1407 (l/w, 6.60-1.5µm;e,1.4µm). l/w; length/width, e;length of endospore.

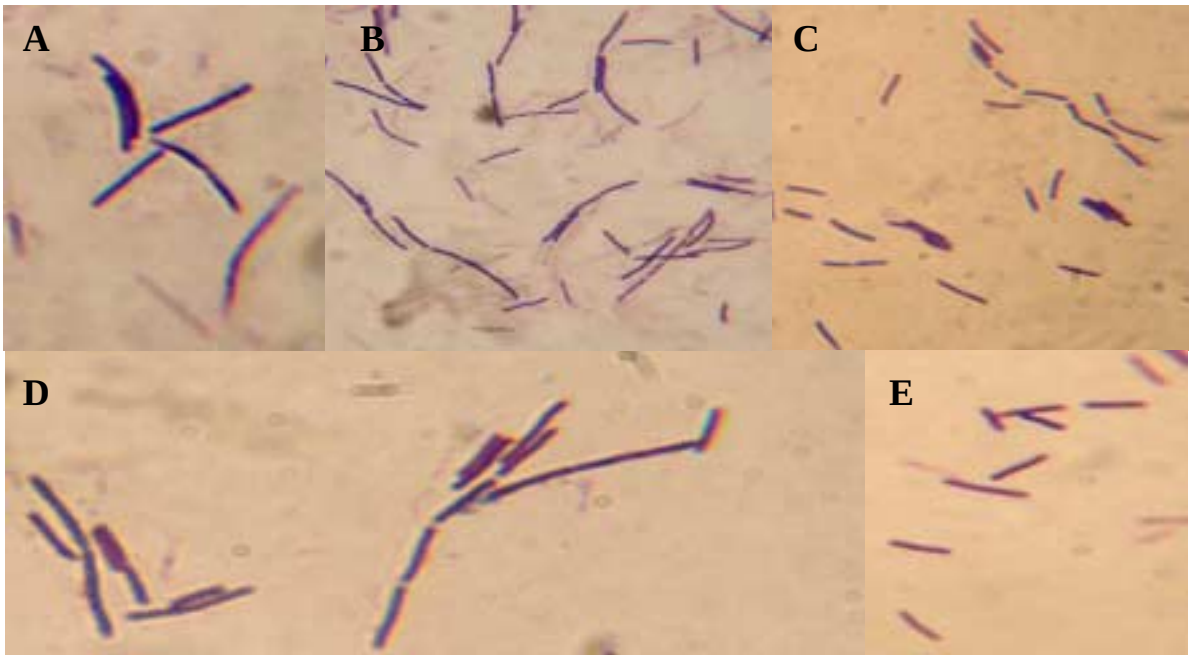

Figure 3.2 Appearance of gram (+) cells of some isolates. A-Isolate 24, B-Isolate Ç-22, C-Isolate 38, D-Isolate 42 , E- Isolate Ç-2.

3.2.6. Growth at Different pH Ranges

The isolates and reference strains were tested for growth at pH 8, 9, 10. All the isolates and reference strains were able to grow at pH 8. None of the reference strains were detected to grow at pH 10. It was also observed that the reference strains except *Bacillus thermoglucosidasius* CECT 4038 were not able to grow at pH 10. The isolates that were not able to grow at pH 8 and pH 10 were listed in the Table 3.3. The remaining gave positive results for these parameters. The isolates 24, 651a, 36, 21a, 85, 90, Ç-20 could not be determined exactly.

Table 3.3 Differentiation of Isolates according to growth at different pH ranges.

Isolates not grow at pH 9	5, 8, 9, 10, 11, 13, 17, 23, 28a, 32, 33, 38, 38y, 45, 47, 61b, 64b, 651a 75a , Ç-4, Ç-11, Ç-14, Ç-16, Ç-31, Ç-33, Ç-34, Ç-32, Ç-35.
Isolates not grow at pH 10	5, 8, 9, 10, 11, 13, 14, 16, 17, 18, 21, 23, 28a, 74b, 32, 33, 34, 35, 38, 38y , 42, 45, 46, 47, 61b, 62b, 64b, 67, 68, 75a, 76, 77, 79, 84, Ç-4, Ç-11, Ç-14, Ç-16, Ç-31, Ç-33, Ç-32, Ç-35.

3.3. Extracellular Enzyme Profiles of Isolated and Reference Strains

All the isolates and reference strains were screened for lipase, pectinase, amylase, protease, xylanase and cellulase activity. The presence of these enzymes were detected by the using the substrates, Tween 80 and Tween 20, pectin and polygalacturonic acid, starch, casein, xylan and carboxymethylcellulose, respectively. The enzyme screening studies were performed two times for each isolate.

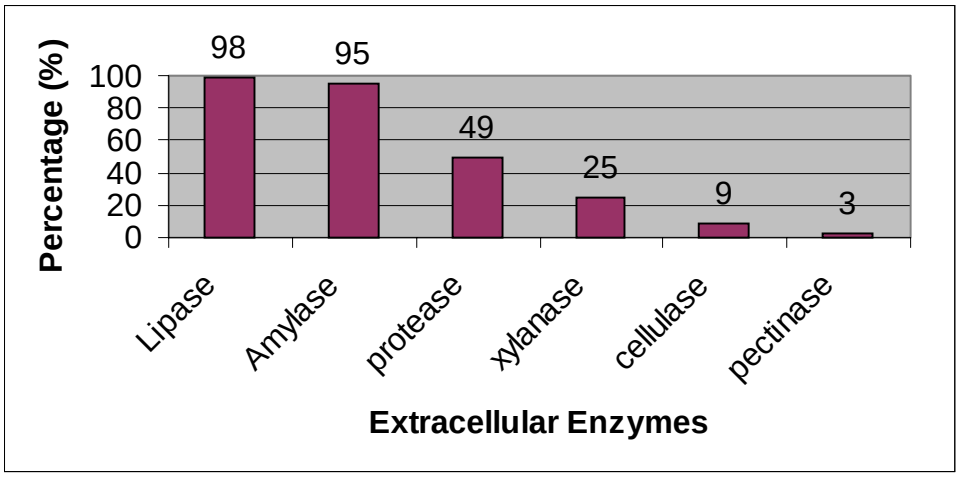

Figure 3.3 Percentage of extracellular enzymes detected.

In total 112 isolates, 110 lipase producing strains (98%) (Figure 3.3.) were detected. None of the isolates were found to hydrolyze Tween 80. The hydrolysis of tween 20 were taken as the presence of lipase activity. One hundred and six isolates that account for 95% (Figure 3.3.) of isolates were able to hydrolyze starch. Protease activity was detected in 55 isolates (49%) (Figure 3.3.). Twenty eigtht of the isolates (25%) (Figure 3.3.) were able to produce xylanase enzyme. Presence of pectinolytic activity was detected for 3 isolates. All those isolates were able to hydrolyze both polygalacturonic acid and pectin.

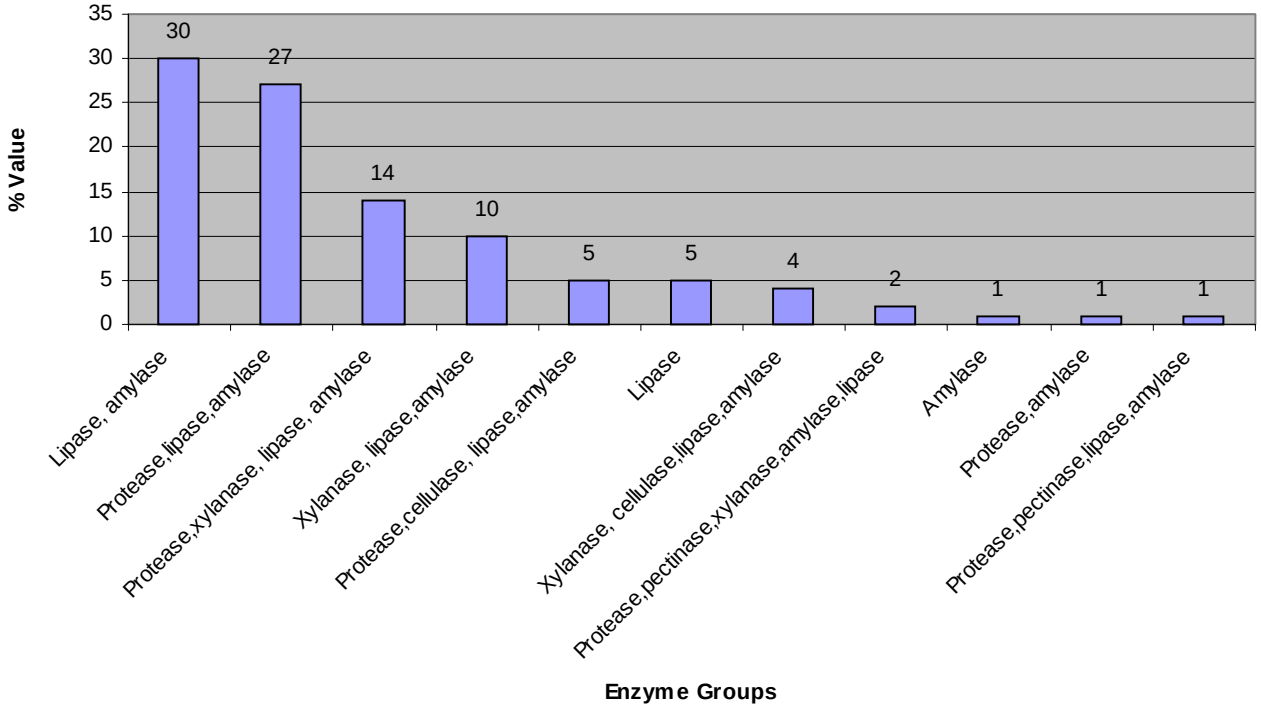

Figure 3.4 Percentage of the enzyme groups obtained.

Most of the isolates were found to produce more than one type of enzyme. Therefore, the isolates were further grouped according to all enzymes that they were able to produce (Table 3.4). Eleven different enzyme groups were obtained. The % distribution of the enzyme groups were shown in Figure 3.4.

Lipase and amylase producing 34 isolates were grouped together. This group represented 30% of the isolates (Figure 3.4).

The members of the second group were able to hydrolyze casein, tween 20 and starch. The protease zones were observed more clearly at the end of 2 – 3 days incubation at 55 ºC.

The third group included the isolates showing protease, xylanase, lipase and amylase activity. This group accounted for % 16 of the isolates (Figure 3.4).

Another group members showed xylanase, lipase, amylase activity which represented 11 % (Figure 3.4) of the isolates.

The fifth and the sixth group had 6 members which were able to hydrolyze casein, carboxymethylcellulose, tween 20, starch and only tween 20.

The seventh group members were able to produce xylanase, cellulase, lipase and amylase. These were 4% of the isolates.

The other group members were capable of hydrolyzing casein, carboxymethylcellulose, tween 20, starch, xylan, pectin and polygalacturonic acid.

The other groups were represented by only one isolate. These were isolate 77 which showed only amylase activity, isolate 53 that was able to hydrolyze casein and starch, isolate 20 showing protease, pectinase, lipase and amylase activity.

The reference strains were also screened for extracellular enzymes. *Bacillus stearothermophilus* CECT 43 showed lipase, amylase, xylanase, protease activity. Hydrolysis of pectin, polygalacturonic acid, tween 80, could not be detected for the reference strains. *Bacillus stearothermophilus* CECT 47,48,49 were able to degrade starch, casein and tween 20.The activity of xylanase, pectinase, cellulase were absent. *Bacillus thermoglucosidasius* NRRL 1407 showed protease, amylase (weak) and lipase activity. *Bacillus kaustophilus* CECT 4264 was also capable of degrading casein, starch and tween 20. Only *Bacillus stearothermophilus* CECT 48 was found to produce cellulase enzyme.

Table 3.4 Enzyme groups, numbers and names of the isolates.

Enzyme Groups	Number of Isolates	Name of Isolates
Lipase, amylase	34	1, 5, 6, 16, 18, 24, 38Y, 50, 75, 75A, 84, Ç-1a, Ç-1, Ç-2, Ç-3, Ç-5, Ç-6, Ç-7, Ç-9, Ç-10, Ç-14, Ç-16, Ç-17, Ç-19, Ç-20, Ç-21, Ç-22, Ç-23, Ç-24, Ç-30, Ç-31, Ç-32, Ç-34, Ç-35,
Protease,lipase,amylase	30	8, 9, 10, 11, 23, 26, 28, 28A, 36, 38, 41, 52, 54, 55, 56, 57, 58, 60, 61, 61B, 62, 62B, 63, 64, 65, 651A, 651B, 78, 79, 80
Protease,xylanase, lipase, amylase	16	21, 30, 32, 34, 35, 37, 39, 42, 43, 49, 70, 71, 72, 74, 75B, 94
Xylanase, lipase,amylase	11	2, 3, 4, 13, 59, 66, 68, 85, 87, 92, Ç-11
Protease,cellulase, lipase,amylase	6	14, 19, 33, 44, 67, 73
Lipase	6	17, 64B, 74B, Ç-14, Ç-13, Ç-33
Xylanase, cellulase,lipase,amylase	4	45, 46, 47, 76
Protease,pectinase,xylanase,amylase,lipase	2	90, 21a
Protease,amylase	1	53
Amylase	1	77
Protease,pectinase,lipase, amylase	1	40
	112	

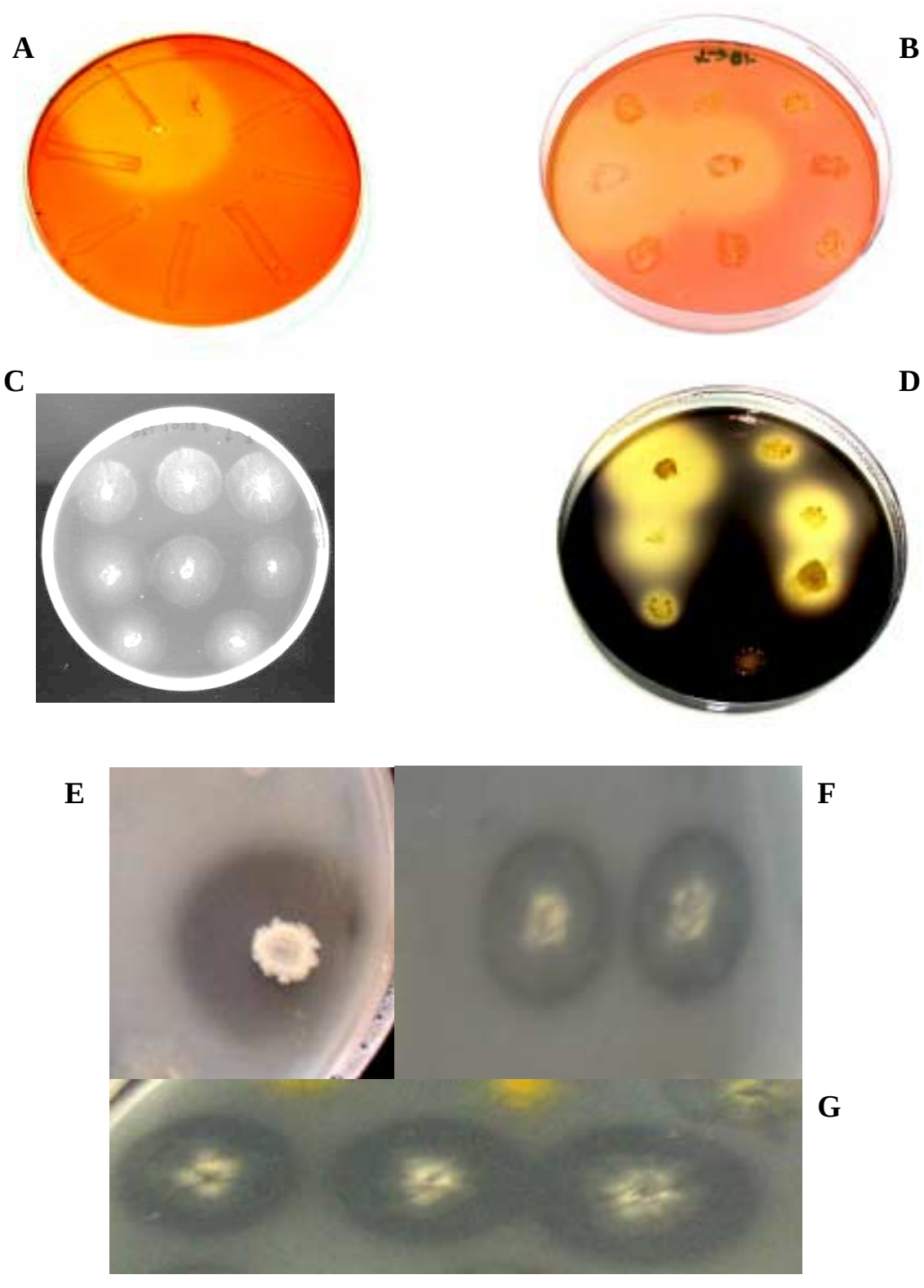

Figure 3.5 Detection of various extracellular enzyme activities. **A**. Xylanase activity of isolate 90, **B.** The cellulase activities of isolates 46 and 47, **C.** The hydrolysis zones of Tween 20 of the isolates 1, 70,6,71,30,34,38,41 **D.** The white halos indicating the amylase activity. The isolates 18,Ç-5,36,38,Ç-9,41 **E.** Clear zones occured by the activity of pectinase produced by the isolate 21a. **F.G.** Halos indicating proteolytic activity. The isolates 30, 53,41,49,52.

3.4. Preliminary Studies for Preparation of Genomic DNA

Two methods were performed in order to prepare genomic DNA to be used as a template for amplification of desired rRNA gene fragments. We observed that the method of *Bravo et al.,* (1998) which has been used for preparation of *Bacillus thuringiensis* genomic DNA did not give amplification products for thermophilic *Bacillus* species. Figure 3.6 shows the amplification patterns of 16S rDNA region of reference strains of thermophilic *Bacillus* sp**.** In the lanes 1, 3, 5, 7, 9, 11 where no amplification products were seen, the method of *Bravo et al.,* (1998) was used for preparation of template DNA. When genomic DNA prepared using CTAB/NaCl method was used as the template, 16S rDNA fragments could be amplified successfully (Lanes 2, 4, 6, 8, 10 and 12).

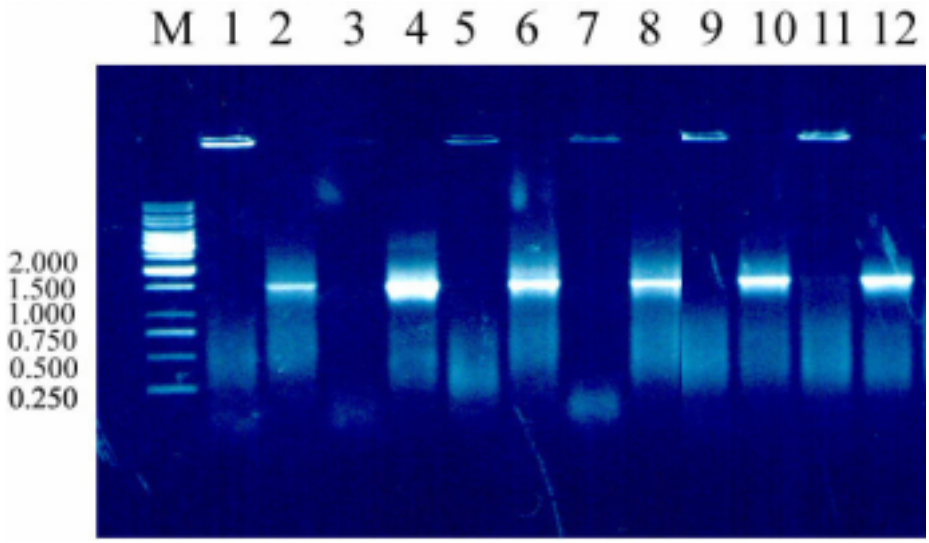

Figure 3.6 16S rDNA PCR fragments amplification results by using two different DNA preparation methods as template. Lanes with odd numbers represent PCR amplification results where method of Bravo *et. al.* was used for template DNA preparation and in even lanes template DNA was from CTAB/NaCl extraction method. M; 1 kb DNA ladder.

3.5. Genotypic Characterization

3.5.1. Identification of Isolates by 16S rDNA –ITS (Internally Transcribed Spacer) - RFLP (Restriction Fragment Length Polymorphism)

3. 5.1.1. Preliminary Studies for Amplification of Isolates by 16S rDNA –ITS

At the beginning of the study, 16S rDNA–RFLP was performed using approximately sixty isolates. The primers used were, forward 5'- AGAGTTTGATCCTGGCTCAG-3' and reverse 5'-CTACGGCTACCTTGTTACGA-3' (Mora *et al.,* 1998). The forward primer was complementary to the 5'- end of 16S rDNA and the reverse primer was complementary to the 3'- of the 16S rDNA. The PCR products were approximately 1400 basepairs in length. PCR products were digested with both *Hae* III and *Taq* restriction enzymes. However, restriction enzyme analyses were not able to differentiate the isolates (data not shown).

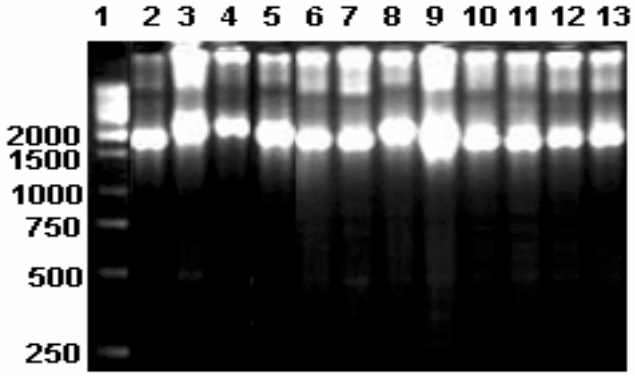

Figure 3.7 16S rDNA-ITS amplification products of some isolates. Lanes 1. 1 kb DNA ladder, 2. Isolate 90, 3. Isolate 15, 4. Isolate 59 5. Isolate 62, 6. Isolate Ç-4, 7. Isolate Ç-24, 8. Isolate 32, 9. Isolate Ç-43, 10. Isolate 17, 11. Isolate 75A, 12. Isolate 5 and lane 13. Isolate Ç-17.

We then extended 16S rDNA sequences at the 3'-end to include internally transcribed spacer region (ITS). This time another reverse primer 5'-CAAGGCATCCACCGT-3' (Jensen *et al*., 1993) which is complementary to the 5'-end of 23S rDNA, was used. Genomic DNA of 9 isolates were randomly chosen and their 16S rDNA-ITS regions were amplified. The amplification product varied, in

length, from 1.500 to 2.000 bp. (Figure 3.7). RFLP profiles of the amplification products with the above mentioned restriction enzymes provided much better discriminative data. It was then decided that the 16S rDNA-ITS RFLP profiles of the remaining isolates should also be produced.

3. 5.1.2. 16S rDNA –ITS –RFLP Profiles of Isolated and Reference Strains

In order to identify the 112 isolated strains according to their 16S-ITS rDNA restriction profiles, two restriction enzymes, *Taq* I and *Hae* III were used. First, all the digestion products were resolved by agarose gel electrophoresis. Isolates producing similar restriction profiles were grouped together. One restriction product representing each of these groups was then electrophoresed on another gel along with those of 7 reference strains and with a DNA size marker (Figures 3.8, 3.9). The name of the groups represented were indicated on each lane in Figures 3.8 and 3.9. Further analyses were performed on these gels by using BioRD^{++} computer programme. The fragment sizes obtained by both restriction enzymes were shown in Tables 3.10 and 3.11.

Restriction profiles of reference strains of thermophilic *Bacillus* with both *Taq* I and *Hae III* were also analysed. The three subspecies of *Bacillus stearothermophilus* CECT 47,48,49, gave similar restriction digestion patterns with both *Taq* I (Figure 3.8 Lanes 3,4,5) and *Hae* III (Figure 3.9 Lanes 2,4,8). The results were in agreement with those in the dendograms (Figures 3.10 and 3.11). Among the four subspecies of *Bacillus stearothermophilus*, both enzymes were able to differentiate *Bacillus stearothermophilus* CECT 43 (Figure 3.8 Lane 2, Figure 3.9 Lane 3) from *Bacillus stearothermophilus* CECT 47,48,49. According to *Taq* I dendrogram (Figure 3.10), *Bacillus stearothermophilus* CECT 43 showed approximately 75 % homology with the other subspecies of *Bacillus stearothermophilus* (Table 3.8). 90 % homology was observed according to *Hae* III dendrogram (Table 3.11).

Bacillus kaustophilus CECT 4264, produced specific restriction patterns by both *Taq* I (Figure 3.8 Lane 8) and *Hae* III (Figure 3.9 Lane 7) enzymes. This strain showed approximately 89 % homology with *Bacillus stearothermophilus* CECT 47, 48 and 49 and 67 % homology with *Bacillus stearothermophilus* CECT 43 according to *Taq* I dendrogram(Figure 3.10). This result was almost in accordance with the search of Goto *et al.,* (2000) since they have found 99% homology between *Bacillus kaustophilus* and *Bacillus stearothermophilus* strains according to the homology between 16Sr DNA

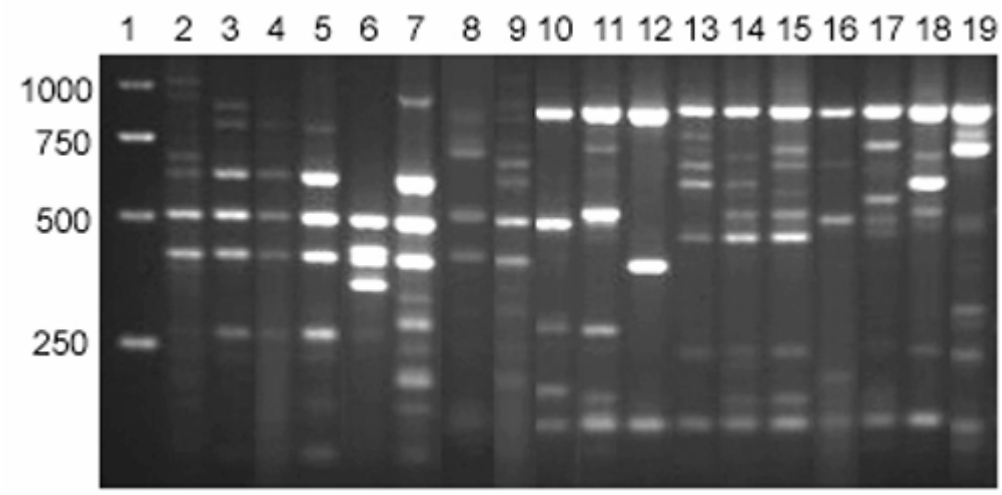

Figure 3.8 16S-ITS rDNA RFLP profiles of *Taq I* groups and reference strains. Lanes 1. 1 kb DNA ladder, 2. *Bacillus stearothermophilus* CECT 43, 3. *Bacillus stearothermophilus* CECT 47 , 4. *Bacillus stearothermophilus* CECT 48, 5. *Bacillus stearothermophilus* CECT 49, 6. *Bacillus thermoglucosidasius* CECT 4038, 7. *Bacillus kaustophilus* CECT 4264, 8. *Bacillus thermoglucosidasius* NRRL 1407, 9. Isolate 90(T-1), 10. Isolate 62 (T-2), 11. Isolate 94 (T-3), 12. Isolate 651a (T-4) , 13. Isolate Ç-4 (T-5), 14. Isolate Ç-19 (T-6), 15. Isolate 5 (T-7), 16. Isolate Ç-34 (T-8), 17. Isolate 3 (T-9), 18. Isolate 17 (T-10), 19. Isolate 76 (T-11).

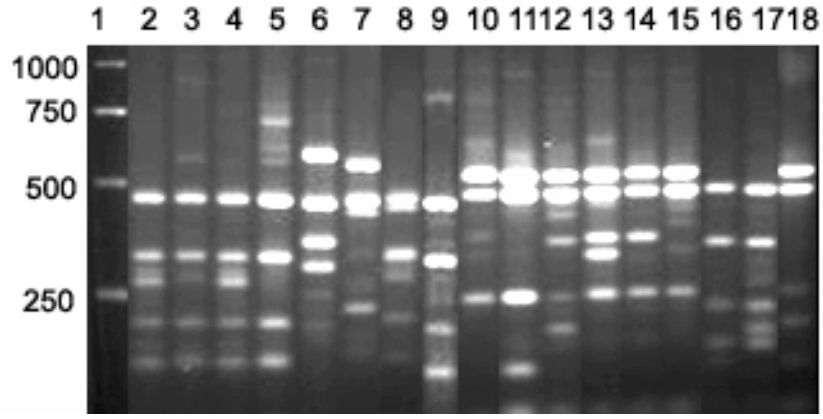

Figure 3.9 16S-ITS rDNA RFLP profiles of *Hae III* groups and reference strains. Lanes 1. 1 kb DNA ladder, 2. *Bacillus stearothermophilus* CECT 47, 3. *Bacillus stearothermophilus* CECT 43 , 4. *Bacillus stearothermophilus* CECT 49, 5. *Bacillus kaustophilus* CECT4264, 6. *Bacillus thermoglucosidasius* CECT 4038, 7 *Bacillus thermoglucosidasius* NRRL 1407, 8. *Bacillus stearothermophilus* CECT48, 9. Isolate 90 (H-1), 10. Isolate 53 (H-2), 11. Isolate 651a (H-3), 12. Isolate Ç-24 (H-4), 13. Isolate Ç-43 (H-5), 14. Isolate 17, 15. Isolate Ç-17 (H-6), 16. Isolate 8 (H-7), 17. Isolate 76 (H-8), 18. Isolate 59 (H-9).

sequences. The result was also confirmed by a number of other works (Sunna *et al.,* 1997; Nicolaus *et al.,* 1996; Rainey *et al.,* 1993).

Two *Bacillus thermoglucosidasius* reference strains obtained from CECT and NRRL showed quite different reassociation values (Tables 3.8, 3.9). This result strongly implied that one of these strains were not *Bacillus thermoglucosidasius.* This finding was also supported by data produced from biochemical experiments. *Bacillus thermoglucosidasius* NRRL 1407 was thought to be the right strain.

According to *Taq* I restriction profiles, the isolated strains were classified into eleven groups (T1- T11) (Tables 3.5). *Hae* III profiles on the other hand clustered the 112 isolates into 9 groups (H1-H9) (Table 3.6). The dendrograms of the isolates and of the reference strains with both *Taq* I and *Hae* III enzymes were shown in Figure 3.10 and 3.11

Two of the isolated strains which were included in both T1 and H1 groups (Tables 3.5 and 3.6), produced similar patterns with the type strain *Bacillus stearothermophilus* (CECT 43) (Figure 3.8, Lanes 2,9; Figure 3.9 Lanes 3,9). This similarity was also revealed in both dendrograms (Figures 3.10 and 3.11). These isolates were therefore identified as *Bacillus stearothermophilus* CECT 43.

Hae III profiles clustered 67 isolated strains into one large group (H2) (Table 3.6). *Taq* I restriction profiles however included these strains into five different groups with some exceptions. Three of the isolates for example were included in Group T-3 (Table 3.5). Similary, eleven isolated strains of H2 were included in T6 (Tables 3.5,3.6). All the isolates comprising group T7 (Table 3.5), were also distinguished. The 37 isolated strains of H2, were included in the group T1 (Tables 3.5 and 3.6).

The 6 isolates (24, 85, Ç-4, Ç-14, Ç-24, Ç-32) were clustered as a separate group by both of the enzymes. They were named as T-5 (Table 3.5) and H-4 (Table 3.6).

Group T-11 contained only one isolate (Isolate 76) (Table 3.5). *Hae* III also confirmed the result and clustered the isolate in H-8 as a separate group (Table 3.5).

The members of the T-10 were exactly the same with H-6. They were both clustered as a different group by both *Hae* III and *Taq* I restriction analyses.(Tables 3.5 and 3.6)

The thirteen isolates of T-4 (Table 3.5), except the isolates 10, Ç-10, Ç-31 were also clustered as distinct group (H-3) (Table 3.6) by *Hae* III profiles.

Three members of the H3 group (68, 78, 84) and one member of H2 (Ç-34) formed another distinct *Taq* I group (T-8).

Table 3.5 Groups obtained by *Taq* I restriction analysis of 16S-ITS ribosomal DNA region.

Groups of Isolates According to *Taq* I Digestion	Names of Isolates
T1	21a,90
T2	8, 9, 13, 14, 19, 21, 23, 26, 28A, 28, 32, 33, 36, 37, 38,38Y, 39, 40, 41,42, 43, 44, 45, 46, 47, 49, 50, 52, 53, 54, 55, 56, 57, 58, 61, 61B 64, 64B, 65, 651B, 67, 71, 72, 73, 80, 87
T3	30, 70, 94,59
T4	10, 60+, 62, 62B, 63, 651A, 74, 74B, 77, 79, Ç-10, Ç-16, Ç-21, Ç-23, Ç-30, Ç-31
T5	24, 85, Ç-4, Ç-14, Ç-24, Ç-32
T6	18, Ç-1-a, Ç-2, Ç-5, Ç-6, Ç-9, Ç-11, Ç-19, Ç-22, Ç-33, Ç-35
T7	5, Ç-7, Ç-13, Ç-17, Ç-20
T8	68, 78, 84, Ç-34
T9	1, Ç-1, 2, 3, 6, Ç-3
T10	16,17, 34, 35, 66, 75A, 75B, 75, 92.
T11	76

Table 3.6 Groups obtained by *Hae* III restriction analysis of 16S-ITS ribosomal DNA region.

Groups of Isolates According to *Hae* III Digestion	Names of Isolates
H1	21a, 90
H2	1, 2, 3, 4, 5, 6, 14, 18, 19, 23, 26, 28, 28A 30, 36, 37, 38, 38Y 39, 41, 42, 44, 45, 46, 47, 49, 50, 52, 53, 54, 55, 56, 57, 58, 61,61B, 64, 64B, 65, 651B, 67, 70, 71, 72, 73, 80, 87, 94, Ç-1-A, Ç-1Ç-2, , Ç-3, Ç-5, Ç-6, Ç-7, Ç-9, Ç-10, Ç-11, Ç-13, Ç-17, Ç-19, Ç-20, Ç-22, Ç-31, Ç-33, Ç-34, Ç-35
H3	10, 60+, 62, 62B, 63, 651A, 68, 74, 74B, 77, 78, 79, 84, Ç-16, Ç-21, Ç-23, Ç-30
H4	24, 85, Ç-4, Ç-24, Ç-14,Ç-32
H5	9, 11, 13, 32, 43
H6	16, 17, 34, 35, 66, 75, 75A, 75B, 92
H7	8, 21, 33
H8	76
H9	40, 59

On the other hand, the isolates 9, 11, 13, 32, 43 were clustered as group H-5 (Table 3.6) , where as they were grouped in T-2 (Table 3.5) according to *Taq I* profiles.

The situation was also similar for the members of H-7 (Table 3.6). The isolates 8, 21 and 33 were differentiated although they were grouped in T-2 according to *Taq* I profiles (Table 3.5).

When a comparative analysis among the groups generated by two restriction enzymes were performed, 17 different groups (TH1-TH17) (Table 3.7) were obtained. In this point of view, the isolated strains could be clustered under 3 main topics.

1- Groups of isolated strains that can be differentiated by both of the restriction enzymes;

The members of TH1, TH2, TH3, TH4, TH5, TH6 and TH7 (Table 3.7) were described in this group. Both *Taq I* and restriction enzymes were discriminative for those 71 isolated strains. The isolated strains in groups TH1, TH2, TH3 and TH4 were exactly same with each other in terms of *Taq I* and *Hae* III digestions separately.

2- Groups of isolated strains that can be differentiated by *Taq I* restriction enzyme;

Seven TH groups (TH8-TH14) (Table 3.7), were included in this category. It was concluded that *Taq I* restriction enzyme was more discriminative for those 31 isolated strains.

3- Groups of isolated strains that can be differentiated by *Hae* III restriction enzyme;

Members of groups TH15, TH16 and TH17 (Table 3.7), were differentiated as separate groups by Hae *III* restriction enzyme.

It was therefore concluded that *Taq I* 16S-ITS rDNA – RFLP profiles were seemed to be more discriminative. However Hae *III* restriction enzyme digestion was also required for differentiation of some of the isolated strains. Combination of the results of two enzymes increased the differentiation power among the isolated strains.

Similar to this study, in a recent work on the identification of thermophilic bacilli from marine thermal vents of Eolian Islands (Caccamo *et al*.,2001), 73 thermophilic isolates have been compared according to their restriction patterns of amplified 16S rDNA with 8 type strains of *Bacillus*. They have obtained 13 different *Alu* I restriction patterns and %78 of isolates were recognized as representatives of different *Bacillus* species. In another work (Blanc *et a*l., 1997), The *Hae* III restriction profiles of 16S

Table 3.7 Groups obtained by comparison of *Taq* I and *Hae* III digestion of 16S-ITS rDNA region.

Groups of isolates when compared with both *Taq* I and *Hae* III enzymes	Groups they belong according to the restriction enzymes separately	The restriction enzyme enables differentiation	Names of Isolates
TH1	T1, H1	Both *Taq* I and *Hae* III	21A, 90
TH2	T5, H4	Both *Taq* I and *Hae* III	24, 85, Ç-4, Ç-24, Ç-14, Ç-32
TH3	T10, H6	Both *Taq* I and *Hae* III	16, 17, 34, 35, 66, 75, 75A, 75B, 92
TH4	T11, H8	Both *Taq* I and *Hae* III	76
TH5	T4, H3	Both *Taq* I and *Hae* III	10, 60+, 62, 62B, 63, 651A, 74, 74B, 77, 79, Ç-16, Ç-21, Ç-23, Ç-30
TH6	T2, H2	Both *Taq* I and *Hae* III	14, 19, 23, 26, 28, 28A, 36, 37, 38, 38Y, 39, 41, 42, 44, 45, 46, 47, 49, 50, 52, 53, 54, 55, 56, 57, 58, 61, 61B, 64, 64B, 65, 651B, 67, 71, 72, 73, 80, 87.
TH7	T3, H9	Both *Taq* I and *Hae* III	59
TH8	T6, H2	*Taq* I	18, Ç-1-A, Ç-2, Ç-5, Ç-6, Ç-9, Ç-11, Ç-19, Ç-22, Ç-33, Ç-35.
TH9	T7, H2	*Taq* I	5, Ç-7, Ç-13, Ç-17, Ç-20
TH10	T9, H2	*Taq* I	1, 2, 3, 6, Ç-1, Ç-3
TH11	T8, H3	*Taq* I	68, 78, 84
TH12	T4, H2	*Taq* I	Ç-10, Ç-31
TH13	T3, H2	*Taq* I	30, 70, 94
TH14	T8, H2	*Taq* I	Ç-34
TH15	T2, H5	*Hae* III	9, 13, 32, 43
TH16	T2, H7	*Hae* III	8, 21, 33
TH17	T2, H9	*Hae* III	40

rDNAs have been compared with four different reference strains. Five different groups have been found, four of the them including the reference strains.

Kuisine *et al.,* (2002) has evaluated the thermophilic proteolytic isolates from a geothermal site in Lithuania based on 16SrDNA-RFLP and ITS- PCR analyses separately while we analysed the 16S rDNA and ISR regions together as a single amplicon. On the basis of RFLP of 16s rDNA by *Alu* I and *Taq* I restriction enzymes, 42 strains have been divided into six distinct groups. ITS sequences have been found more variable and the isolates have been divided into 9 groups according to both analysis.

It has been concluded that high phenotypic intraspesific diversity existed in thermophilic bacilli. Phenotypically heterogeneous and genetically homogeneous groups have been described (Maugeri *et al.,* 2001; Ash *et al.,* 1991). It has also been reported that although thermophilic bacilli can be clearly grouped using molecular techniques, the species are difficult to differentiate using biochemical tests (Flint *et al.,* 2001). Confirming with these works, when the genotypic results were compared with the results of enzyme screening and phsyical tests, a correlation between a few groups were observed. An exact relation could not be obtained.

The isolates 21a and 90 for example were different from *Bacillus stearothermophilus* CECT 43, in terms of results of some physical and enzyme screening tests although they produced similar restriction patterns by both *Taq* I and *Hae* III enzyme. The isolates were able to produce protease, xylanase, amylase, pectinase and lipase enzymes while *Bacillus stearothermophilus* CECT 43 was not able to hydrolyze pectin or polygalacturonic acid. Similary, *Bacillus stearothermophilus* CECT 43 was not able to grow at 37 ºC, while isolate 90 was able to grow at 37 ºC but 21A showed a weak growth at this temperature (Section 3.2.4.)

It was observed that all members (except one) of enzyme group including protease, cellulase, lipase, and amylase activity (Table 3.4), were clustered as a separate group (TH6) (Table 3.7). All members of TH9 (Table 3.7), most of the isolates (except 2) in TH8 were also able to hydrolyze lipase and amylase (Table 3.4). Similarly all the isolates of TH13 (Table 3.7) were detected to produce protease, lipase and amylase (Table 3.4).

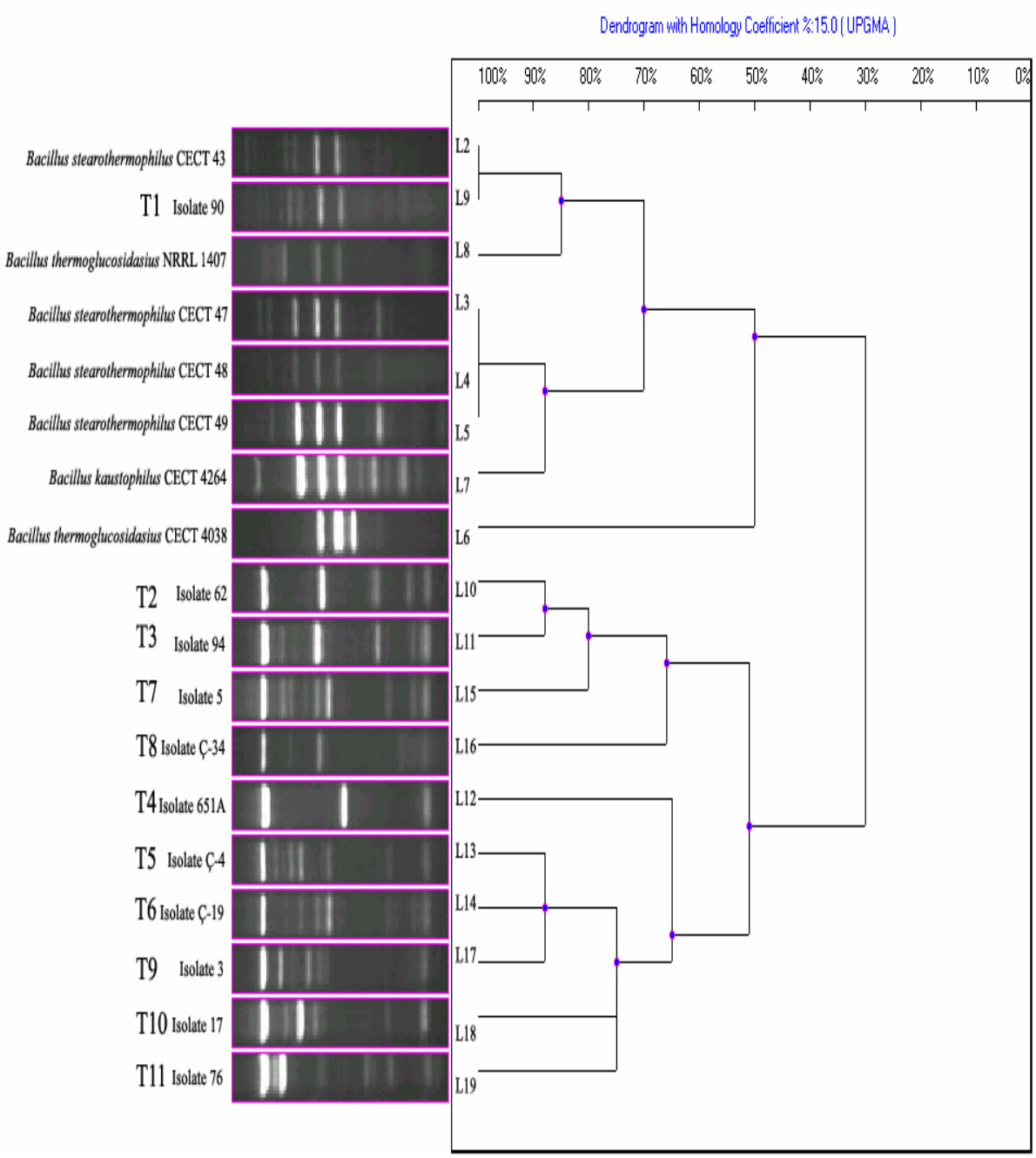

Figure 3.10 *Taq* I – RFLP Dendrogram of isolates and reference strains

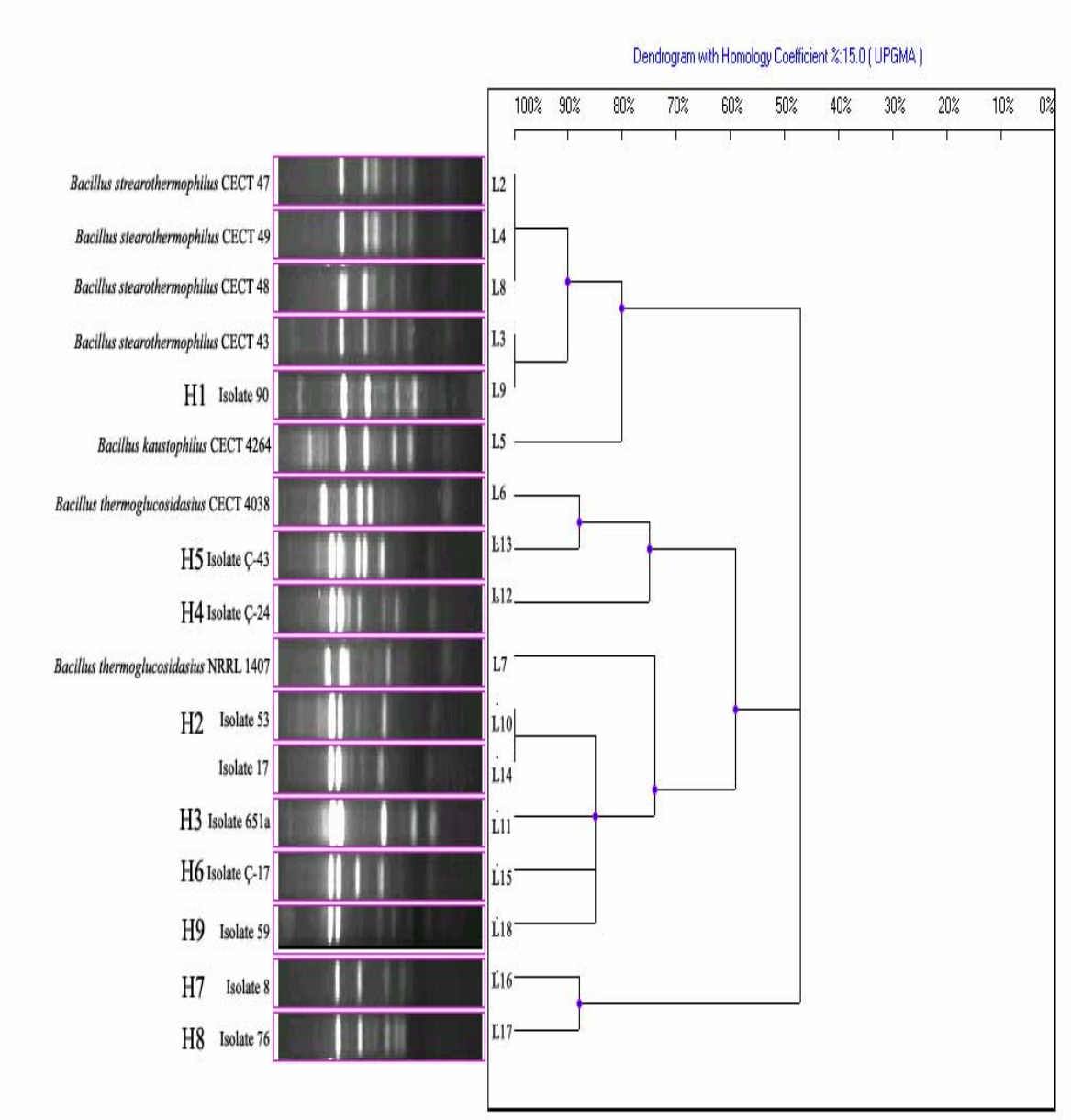

Figure 3.11 *Hae* III – RFLP Dendrogram of isolates and reference strains

Table 3.8 16S-ITS region similarity values for isolates and reference strains according to *Taq* I profiles.

	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19
L2	1.00																	
L3	0.75	1.00																
L4	0.75	1.00	1.00															
L5	0.75	1.00	1.00	1.00														
L6	0.50	0.50	0.50	0.50	1.00													
L7	0.67	0.89	0.89	0.89	0.44	1.00												
L8	0.86	0.86	0.86	0.86	0.57	0.50	1.00											
L9	1.00	0.75	0.75	0.75	0.50	0.67	0.86	1.00										
L10	0.22	0.44	0.44	0.44	0.22	0.40	0.25	0.22	1.00									
L11	0.25	0.50	0.50	0.50	0.25	0.44	0.29	0.25	0.89	1.00								
L12	0.29	0.29	0.29	0.29	0.29	0.25	0.33	0.29	0.50	0.57	1.00							
L13	0.67	0.67	0.67	0.44	0.22	0.40	0.75	0.67	0.60	0.67	0.50	1.00						
L14	0.50	0.50	0.50	0.50	0.50	0.44	0.57	0.50	0.67	0.75	0.86	0.89	1.00					
L15	0.44	0.44	0.44	0.44	0.44	0.40	0.50	0.44	0.80	0.67	0.50	0.80	0.89	1.00				
L16	0.25	0.25	0.25	0.25	0.25	0.44	0.29	0.25	0.67	0.75	0.57	0.67	0.75	0.67	1.00			
L17	0.50	0.50	0.50	0.50	0.25	0.44	0.29	0.50	0.67	0.75	0.57	0.89	0.75	0.67	0.75	1.00		
L18	0.29	0.29	0.29	0.29	0.00	0.25	0.00	0.29	0.50	0.57	0.67	0.75	0.57	0.50	0.57	0.86	1.00	
L19	0.29	0.29	0.00	0.00	0.00	0.00	0.33	0.29	0.50	0.57	0.67	0.75	0.57	0.50	0.57	0.57	0.67	1.00

Table 3.9 16S-ITS region similarity values of isolates and reference strains according to *Hae*III profiles

	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18
L2	1.00																
L3	0.91	1.00															
L4	1.00	0.91	1.00														
L5	0.80	0.89	0.80	1.00													
L6	0.60	0.44	0.60	0.50	1.00												
L7	0.20	0.22	0.40	0.25	0.50	1.00											
L8	1.00	0.91	1.00	0.80	0.60	0.20	1.00										
L9	0.91	1.00	0.91	0.89	0.44	0.22	0.91	1.00									
L10	0.44	0.50	0.44	0.29	0.57	0.86	0.44	0.50	1.00								
L11	0.40	0.44	0.40	0.25	0.50	0.75	0.40	0.44	0.86	1.00							
L12	0.60	0.67	0.60	0.75	0.75	0.50	0.60	0.67	0.57	0.50	1.00						
L13	0.55	0.60	0.55	0.44	0.89	0.89	0.55	0.60	0.75	0.67	0.67	1.00					
L14	0.60	0.67	0.60	0.50	0.75	0.75	0.60	0.67	0.86	0.75	0.75	0.89	1.00				
L15	0.44	0.50	0.44	0.29	0.57	0.57	0.44	0.50	1.00	0.86	0.57	0.75	0.86	1.00			
L16	0.40	0.44	0.40	0.50	0.50	0.50	0.40	0.44	0.57	0.50	0.50	0.67	0.75	0.57	1.00		
L17	0.55	0.60	0.55	0.67	0.44	0.44	0.55	0.60	0.50	0.44	0.67	0.60	0.67	0.50	0.89	1.00	
L18	0.60	0.67	0.60	0.50	0.75	0.75	0.60	0.67	0.86	0.75	0.75	0.67	0.75	0.86	0.50	0.44	1.00

Table 3.10 Fragment sizes obtained by *Taq* I restriction analysis of 16S-ITS rDNA genes of reference strains and isolates representing each group.

Strains	**Fragment Sizes (bp)**
Bacillus stearothermophilus CECT 43	412, 500, 617, 674
Bacillus stearothermophilus CECT 47	264 412, 500, 617
Bacillus stearothermophilus CECT 48	264 412, 500, 608
Bacillus stearothermophilus CECT 49	264, 400, 500, 600
Bacillus thermoglucosidasius CECT 4038	347, 400, 418, 480
Bacillus kaustophilus CECT 4264	179, 283, 400, 473, 575
Bacillus thermoglucosidasius NRRL 1407	406, 500, 684
Isolate 90 (T1)	400, 486, 591, 636
Isolate 62 (T-2)	87, 152, 278, 473, 850
Isolate 94 (T-3)	92, 269, 507, 863
Isolate 651a (T-4)	87, 389, 837
Isolate Ç-4 (T-5)	92, 448, 575, 636, 876
Isolate Ç-19 (T-6)	92, 435, 500, 863
Isolate 5 (T-7)	92, 130, 448, 500, 863
Isolate Ç-34 (T-8)	98, 179, 480, 850
Isolate 3 (T-9)	98, 486, 543, 863
Isolate 17 (T-10)	98, 591, 850
Isolate 76 (T-11)	92, 705, 850

Table 3.11 Fragment sizes obtained by *Hae* III restriction analysis of 16S-ITS rDNA genes of reference strains and isolates representing each group.

Strain	**Fragment Sizes (basepairs)**
Bacillus stearothermophilus CECT 47	102, 188, 276, 296, 328, 460
Bacillus stearothermophilus CECT 43	108, 188, 286, 328, 460
Bacillus stearothermophilus CECT 49	102, 193, 276, 296, 328, 460
Bacillus kaustophilus CECT 4264	102, 188, 333, 460
Bacillus thermoglucosidasius CECT 4038	312, 366, 448, 573
Bacillus thermoglucosidasius NRRL 1407	222, 429, 460, 543
Bacillus stearothermophilus CECT 48	333, 442, 467
Isolate 90 (H1)	108, 188, 286, 328, 448
Isolate 53 (H-2)	244, 473, 514
Isolate 651a (H-3)	85, 250, 473, 507
Isolate Ç-24 (H-4)	176, 360, 473, 514
Isolate Ç-43 (H-5	250, 333, 366, 473, 514
Isolate 17	255, 366, 473, 521
Isolate Ç-17 (H-6)	255, 473, 528
Isolate 8 (H-7)	142, 233, 360, 487
Isolate 76 (H-8)	148, 182, 227, 360, 473
Isolate 59 (H-9)	193, 265, 480, 543

3.5.2. Identification of Isolates by Plasmid Profiling

In order to identify thermophilic bacteria, plasmid profiling studies were also performed. After the plasmids were isolated as described in section 2.2.6.2.1.(Figure 3.12), two restriction enzymes *Taq* I and *Hae* III were used to identify the isolates according to their restriction patterns.

Similar to present study, Khalil *et al.,* (2003) investigated plasmid profiles of two thermophilic bacterial strains isolated from thermal spring in Jordan. They found approximately 3 kb and 7 kb plasmids of *Streptococcus thermophilus* and *Bacillus* according to *EcoRI* digests.

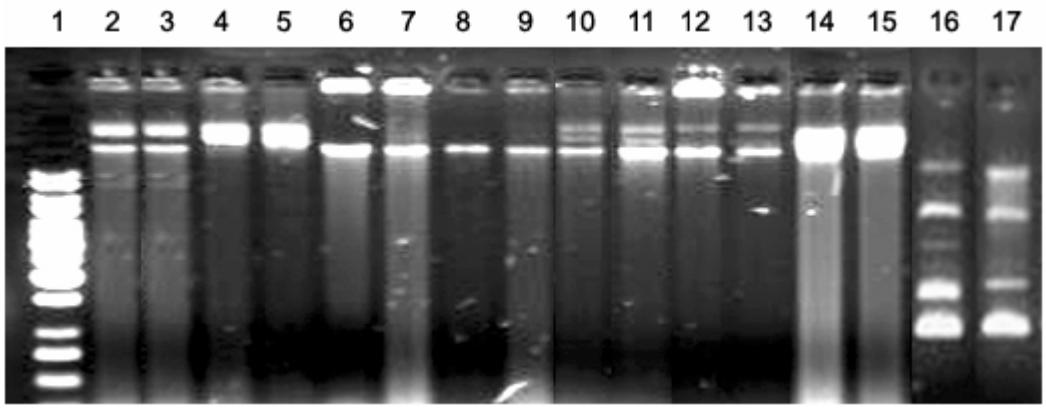

Figure 3.12 Undigested plasmids of isolates
Lanes 1. 1 kb DNA ladder, 2. Isolate 10, 3. Isolate Ç-13, 4. Isolate 18, 5. Isolate Ç-20, 6. Isolate 21a, 7. Isolate Ç-34, 8. Isolate 34, 9. Isolate 35, 10. Isolate 39, 11. Isolate 41, 12. 64, 13. 73, 14.Ç-1-a, 15.Ç-9, 16. Isolate 66 and 17. Isolate 92.

All the plasmids were digested with *Hae III*. Restriction digestion of plasmids with *Hae III* resulted three different restriction patterns (Table 3.12). Sixteen of the isolates produced similar profiles , 7 of these were shown in Figure 3.13 A. Isolates Ç-9, Ç-2, Ç-23, Ç-33, Ç-3, Ç-5, Ç-6, Ç-7 and Ç-24, also produced the same digestion profiles. The size of the plasmids were estimated to be around 12 kb.

Second group produced by *Hae III* digestion of plasmids were from the isolates 75, 17, 92, 66, 77 and 75a (Figure 3.13B). The size of the plasmids were approximately 8 kb.

Table 3.12 Groups obtained by *Hae* III restriction analysis of plasmid DNA

Groups of Plasmids According to *Hae* III Digestion	Names of Isolates
PH1	18, Ç-1-A, Ç-1, Ç-2, Ç-3, Ç-5, Ç-6, Ç-7, Ç-9, Ç-13, Ç-20, Ç-23, Ç-24, Ç-31, Ç-32, Ç-33.
PH2	17, 66, 75, 75A, 77, 92
PH3	10

Table 3.13 Groups obtained by *Taq* I restriction analysis of plasmid DNA of 35 isolates.

Groups of Plasmids According to *Taq I* Digestion	Names of Isolates
PT1	18, Ç-1-A, Ç-1, Ç-2, Ç-3, Ç-13, Ç-20, Ç-23, Ç-31, Ç-32, Ç-33.
PT2	17, 66, 75, 75A, 77, 92

The third group was represented by only one isolate (Figure 3.13 C). This plasmid seemed to be larger than other ones. The plasmid profiles of other isolates did not give interpretable results. This may be due to the lack of plasmids.

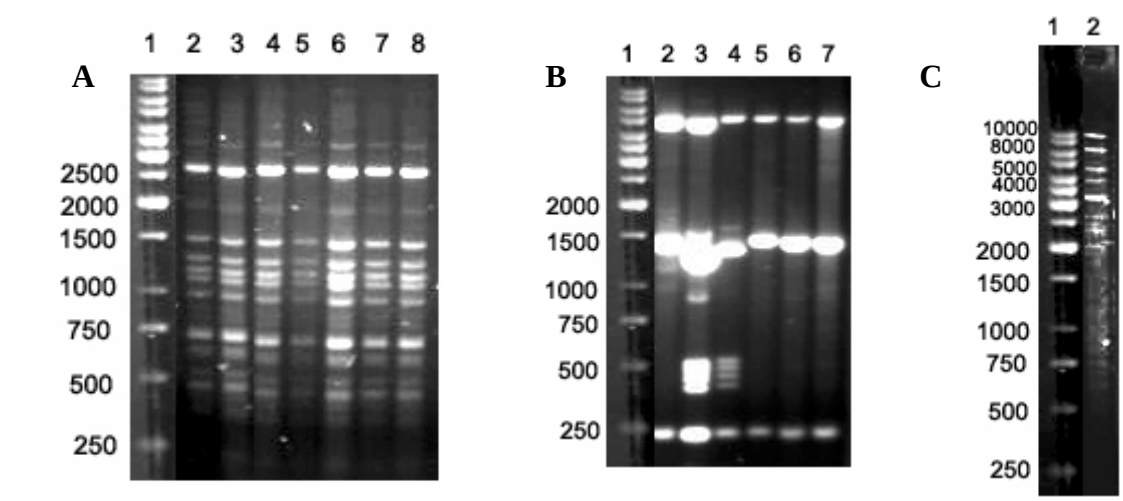

Figure 3.13 *Hae* III digests of plasmids of the isolates
A. Lanes 1. 1 kb DNA ladder, 2. Isolate Ç-13, 3. Isolate Ç-32, 4. Isolate ç-31, 5. Isolate Ç-1, 6. Isolate 18, 7. Isolate Ç-20, 8. Isolate Ç-1-a. **B.** Lanes 1. 1 kb DNA ladder, 2. Isolate 75, 3. Isolate 17, 4. Isolate 92, 5. Isolate 66, 6. Isolate 77, 7. Isolate 75a. **C.** Lanes 1. 1 kb DNA ladder, 2. Isolate 10.

Thirthy five of the plasmids were digested with *Taq* I restriction enzyme. *Taq* I digestion patterns produced two distinct profiles (Table 3.13). These profiles both matched with the *Hae III* digests of the same plasmids. The plasmid profiles of isolates Ç-13, Ç-32, Ç-31, Ç-1, 18, Ç-20, Ç-1 a, Ç-3, Ç-2, Ç-23 and Ç-33 were similar as in *Hae III* digests of these plasmids (Figure 3.14 A). The size of the plasmids were approximately 12 kb. *Taq* I profiles of the second group also differentiated the isolates as grouped with *Hae* III restriction digests (Figure 3.14 B). The *Taq* I plasmid profile of the isolate 10 which formed another group according to *Hae* III digestion patterns, did not give an interpretable result.

Members of groups PH2 and PT2 (Tables 3.12) were all included into T10 and H6 groups which were distinct groups according to 16S-ITS rDNA RFLP results (Tables 3.5 and 3.6). The members of PH1 were represented by 5 distinct (T4, T5, T6, T7, T9) groups according to *Taq* I 16S-ITS rDNA RFLP results and 3 distinct *Hae* III (H2, H3, H4) groups were obtained . Isolate 10 was included into group T4 and H3. The classification results for PT1 according to 16S-ITS rDNA RFLP patterns were also same as PH1.

The results showed that only 23 of the isolated strains contained plasmid DNA. It was concluded that plasmid profiling was not discriminative for the isolates. However, when we focused on the plasmid RFLP results, it was observed that the plasmid profiles of the groups, PH1 and PT1, were almost (except 18) from the strains isolated from mud (Table 3.1.). Since mud is used for therapeutic treatment, the plasmids of these isolates may contain genes coding for important metabolites related to balneotherapy. The members of the other groups (PH2, PH3, PT2) were all isolated from liquid samples (Table 3.1.).

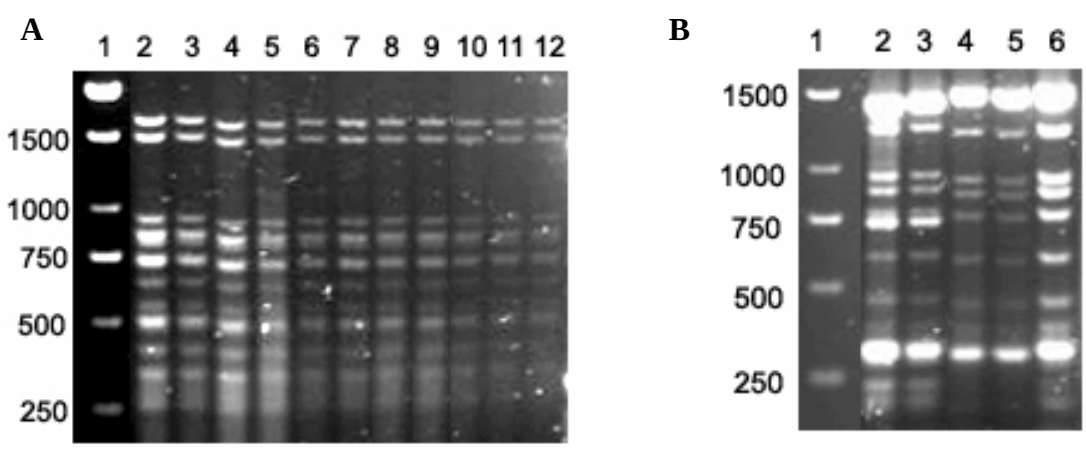

Figure 3.14 *Taq* I digests of plasmids of the isolates
A. Lanes 1. 1 kb DNA ladder, 2. Isolate ç-13, 3. Isolate Ç-32, 4. Isolate Ç-31, 5. Isolate Ç-1, 6. Isolate 18, 7. Isolate Ç-20, 8. Isolate Ç-1-a, 9. Isolate Ç-3, 10. Isolate Ç-2, 11. Isolate Ç-23, 12. Isolate Ç-33. **B.** Lanes 1. 1 kb DNA ladder, 2. Isolate 75, 3. Isolate 17, 4. Isolate 92, 5. Isolate 66, 6. Isolate 77.

3.5.3. Optimization of PFGE-RFLP Conditions For Thermophilic *Bacillus*

In the first PFGE experiments, reference strains of thermophilic *Bacillus* were used. Unfortunately we were not able to obtain interpretable DNA profiles by using a number of published methods (Birren and Lai, 1993; Manufacturer's manual, BIO-RAD, Bouton et al., 1998). The organisms seemed to be readily damaged upon mechanical manipulations. Thus we needed to develop a procedure for the preparation of agarose plugs that avoids the problem.

At the beginning of the study after observing uninterpretable DNA profiles, we made minor modifications on the method from last step to first step by changing one parameter at a time to find out where the DNA was degraded. These included, elimination of centrifugation step, decreasing the amount of lysozyme, decreasing the

EDTA concentration in the cell suspension buffer, decreasing the amount of enzyme and the incubation time, eliminating proteinase K, decreasing the number of washing steps (data not shown). It was finally observed that DNA was degraded at the very beginning of the procedure.

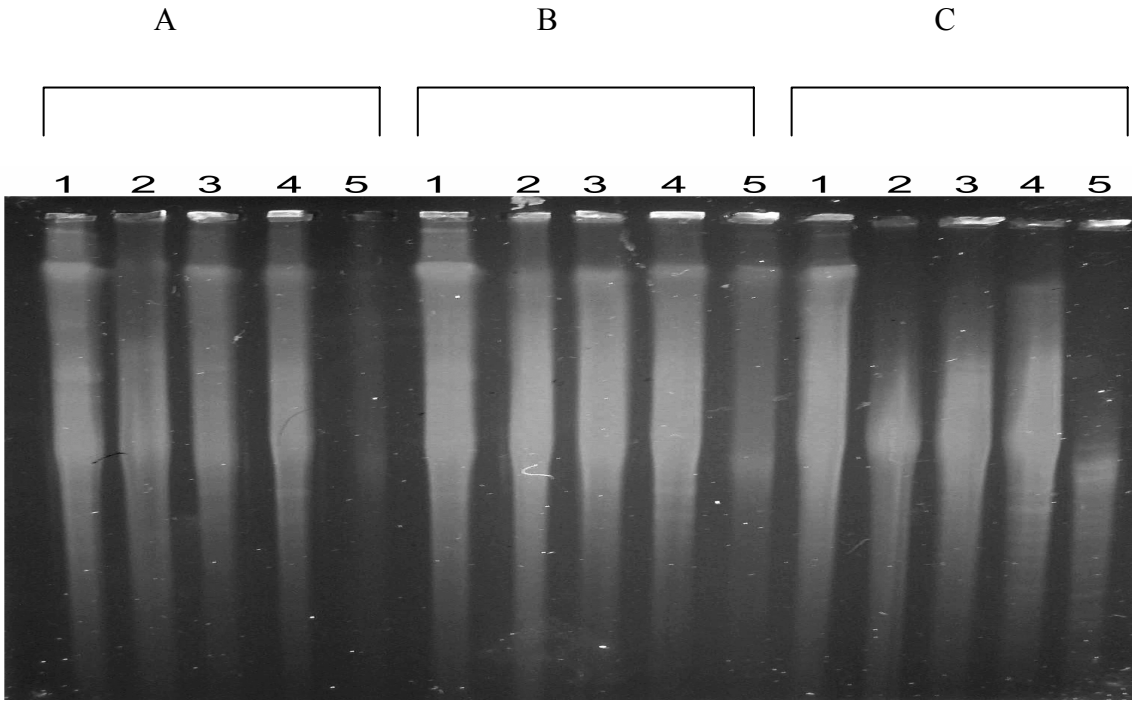

Figure 3.15 Non-specific degradation of the chromosomal DNA. A: Incubation of the agarose plugs overnight at 4°C. B: Incubation of the agarose plugs in *SmaI* buffer overnight at 30 °C. And C: *Sma*I digested plugs. All the groups include the following *Bacillus* cells in the same order: Lane 1, *Bacillus thermoglucosidasius* (CECT 4038T); lane 2, *Bacillus kaustophilus* (CECT 4264); lane 3, *Bacillus stearothermophilus* (CECT43); lane 3, isolate 4; and lane 5, isolate 9.

In the Figure 3.15 five different *Bacillus* members, *Bacillus thermoglucosidasius* (CECT 4038T), *Bacillus kaustophilus* (CECT 4264), *Bacillus stearothermophilus* (CECT 43), 4 and 9 (isolated strains) were first embedded in agarose by known procedures. In order to detect in which step DNA degradation occurred those plugs were loaded into agarose gel after different PFGE steps. As shown in Figure 3.15. A, the strains were first loaded into agarose gel just after the plug preparation step. The same strains were also treated with lysozyme and proteinase K as described in section 2.2.6.3. After the washing steps, they were kept overnight at 4 °C in cell suspension buffer instead of restriction enzyme digestion (Figure 3.15.B). The third group of

agarose plugs were digested with *Sma* I restriction endonuclease (Figure 3.15. C). Since non-specific degradation of DNA was observed in all lanes, we concluded that embedding bacteria into agarose using ordinary plug molds and the plug making procedures was damaging the cells and the chromosome.

We overcame this problem using the new approach described in section 2.2.6.3. As shown in Figure 3.16 the three thermophilic *Bacillus* species, *Bacillus thermoglucosidasius* (CECT 4038), *Bacillus kaustophilus* (CECT 4264), and *Bacillus stearothermophilus* (CECT 43), lanes 1, 7, and 8, respectively, could be readily differentiated.

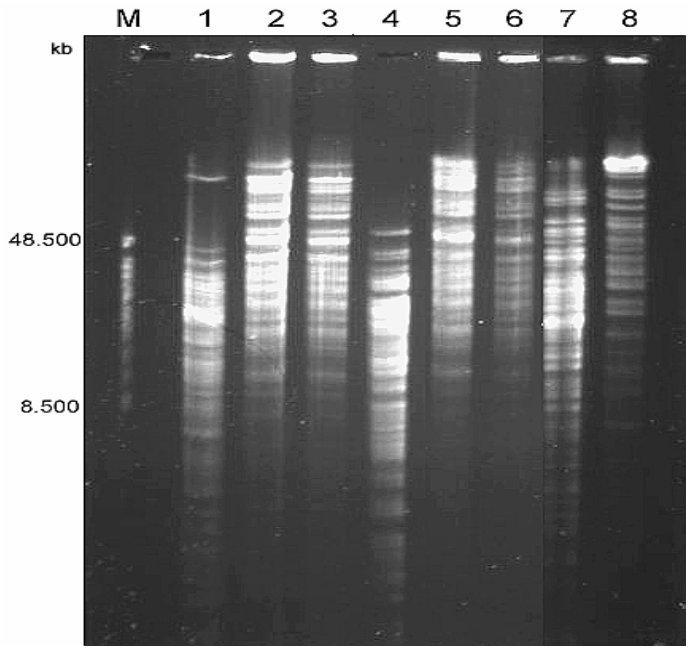

Figure 3.16 Pulsed field gel electrophoresis of *Sma*I digests of genomic DNA : Lane 1, *Bacillus thermoglucosidasius* (CECT 4038T); lane 2, isolate 4; lane 3, isolate 9; lane 4, isolate 20 ; lane 5, isolate 3; lane 6, isolate 92; lane 7, *Bacillus kaustophilus* (CECT 4264); and lane 8, *Bacillus stearothermophilus (CECT 43).*

We also tried different electrophoresis conditions (voltage gradient, pulse times, electrophoresis time) in order to obtain a better resolution of DNA fragments. The electrophoresis in Figure 3.16 was performed with 1-8 pulse times for 26 h at 4 V/cm at 14 º C.

CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, genotypic diversity among thermophilic bacilli producing industrially important extracellular enzymes isolated from Balçova Geothermal Region was investigated. Three genotypic methods were performed. These were 16S-ITS rDNA RFLP, plasmid profiling and pulsed field gel electrophoresis.

In total, 112 thermophilic strains were isolated from various environmental samples collected within Balçova Geothermal Region. The isolated strains were screened for the presence of 6 industrially important extracellular enzymes. For these studies, Tween 20-Tween 80, soluble starch, casein, xylan, carboxymethylcellulose, pectin-polygalacturonic acid were used as substrates for determination of lipase, amylase, protease, xylanase, cellulase, pectinase activities, respectively. In total, 110 lipase, 106 amylase, 55 protease, 28 xylanase, 10 cellulase and 3 pectinase producing strains were detected. Some other phenotypic tests were performed for these isolates. Since the genus *Bacillus* has been described as a diverse collection of aerobic and facultatively anaerobic, rod- shaped, gram positive, endospore forming bacteria (Nazina et. al.,2001; Ash *et al*., 1991; Goto *et al*., 2000), isolated strains were identified as *Bacillus* sp according to results of phenotypic tests.

16S-ITS rDNA RFLP and plasmid RFLP profiles were produced by using two restriction endonucleases *Taq* I and *Hae* III . The isolated strains were clustered into eleven groups by *Taq* I restriction profiles of 16S-ITS rDNA while nine groups were obtained by *Hae* III digestion profiles. In general, *Taq* I restriction enzyme, seemed to be more discriminative. *Hae* III however was also required for discrimination of some isolates. When a comparative analysis was performed among the groups generated by two restriction enzymes, ıt was concluded that 17 genotypically different strains were present in total 112 isolates. Two of the isolates yielded similar RFLP profiles to those of *Bacillus stearothermophilus* (CECT 43) reference strain. This similarity was also confirmed by both of the restriction enzymes.

Twenty three of the strains contained plasmid DNA. *Hae* III restriction profiles indicated the existence of three different types of plasmids while *Taq* I divided the plasmids into two groups.

PFGE optimization studies by *Sma* I restriction endonuclease for thermophilic Bacilli were also performed. A new method for preparation of agarose plugs was developed.

In the future, the PFGE profiles of the isolates and of the reference strains may be undertaken to identify the isolates at species and/or at subspecies level.

Sequencing of 16S rDNA of the isolated strain groups will also enable a further characterization of the isolates.

The analysis of microbial products to be extracted from mud, which has been used for therapeutic treatment in Balçova Physical Theraphy and Rehabilitation Center, could also be important. It has been reported that microorganisms could cause important changes in the physical and chemical composition of the mud used for theraphy. For example, a gluco-sulfo-lipid, which has been thought to be produced during maturation process, has been found to exert anti-imflammatory functions (Bellometti, 2000). In the lights of this finding, the isolates and their products may be studied to discover the role of the bacteria in mud pack theraply.

Extracellular enzymes produced by the isolated strains could be studied in respect of enzyme activity in aqueous/nonaqueous environments, enzyme purification and production. The biochemical and biophysical characterization of the selected enzymes could also be searched.

The genes coding for selected enzymes could also be cloned to initiate recombinant thermophilic enzyme design, development and production.

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APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Experiments

NO	CHEMICAL	CODE
1	Agar- 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	NaCl	AppliChem A2942
8	K_2HPO_4	AppliChem A2945
9	$MgSO_4.7H_2O$	Merck 1.05886
10	Xylan from birchwood	Sigma X-0502
11	Pectin from citrus peel	Fluka 76280
12	Polygalacturonic acid	Fluka 81325
13	Carboxymethylcellulose sodium salt	Fluka biochemika 21902
14	Soluble Starch	Merck 1.01252.0250
15	Disodium hydrogen phosphate	Applichem A2943
16	Ammonium sulfate	Applichem A3485
17	KH_2PO_4	Merck 1.04871
18	Immersion oil	Applichem A0699
19	Cetyl trimethylammonium bromide	Applichem A0805
20	Calcium chloride	Applichem A3652
21	Crystal violet	Sigma C3886
22	Safranin O	Merck 1.15948
23	Nutrient broth	Merck 1.05443
24	Congo Red	Sigma C6767
25	Potassium Iodide	Sigma P8256

26	Tween80	Applichem A1390
27	Tween 20	Applichem A1389
28	Sodium carbonate	Merck 1.06392
29	Tris Base	Sigma T6066
30	EDTA	AppliChem A2937
31	Isopropanol	AppliChem A3928
32	Proteinase K	AppliChem A3830
33	Ethidium bromide	AppliChem A1151
34	Ethanol	AppliChem A3678
35	Taq DNA polymerase	MBI, Fermentas EP0401
36	dNTP set	MBI, Fermentas, R0181
37	*Taq* I	Fermentas, ER0671
38	*Hae* III	Fermentas, ER0151
39	*Sma* I	Fermentas, ER0662
41	Low melting agarose	AppliChem A3762
42	Molecular Biology Certified Agarose	Bio-Rad 162-0134
43	Lysozyme	AppliChem A3711
44	Sodium lauryl sulfate	AppliChem A1163
45	Sodium deoxycholate	AppliChem A1531
46	Proteinase K	AppliChem A3830
47	Chloroform	AppliChem A3633
48	Isoamyl alcohol	AppliChem A2610
49	Bromophenol blue	Merck 1.08122
50	Boric acid	AppliChem A2940
51	Standard agarose (low electroendoosmosis)	AppliChem A2114

APPENDIX B

MEDIA

B.1. MEDIA USED FOR ISOLATION

Media 1

Solid Media For Dilution Plate Technique

	g/l
Yeast extract	1
Glucose	1
Agar agar	15

All ingredients were dissolved in sterilised balçova water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

Broth Media Used For Enrichment Technique

	g/l
Yeast extract	1
Glucose	1

All ingredients were dissolved in sterilised balçova water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

Media 2

Solid Media For Dilution Plate Technique

	g/l
Yeast extract	2.5
Tryptone	2.5
Agar agar	15

All ingredients were dissolved in sterilised balçova water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

Broth Media Used For Enrichment Technique

	g/l
Yeast extract	2.5
Tryptone	2.5

All ingredients were dissolved in sterilised balçova water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B.2. MEDIA FOR EXTRACELLULAR ENZYME SCREENING

B.2.1. Media Used For Protease Screening

	g/l
Nutrient broth	8
Skim milk	10
Agar agar	15

Ingredients except skim milk were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes. Skim-milk is autoclaved separately at 110 °C for 5 minutes (two times) and added to the medium.

B.2.2. Media Used For Amylase Screening

Media 1

	g/l
Yeast extract	1
$MgSO_4.7H_2O$	0.1
K_2HPO_4	7
KH_2PO_4	2
$(NH_4)_2SO_4$	1
NaCl	5
Starch	5
Agar agar	15

All ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

Media 2

	g/l
Yeast extract	1
Soluble starch	5
Agar agar	15

Ingredients were dissolved in sterilised balçova water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B.2.3. Media Used For Lipase Screening

Media 1

	g/l
Nutrient broth	8
$CaCl_2 2H_20$	0.1
Tween 80	10ml
Agar agar	15

Ingredients except Tween 80 were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes. Tween 80 was autoclaved separately and added to the medium.

Media 2

	g/l
Nutrient broth	8
$CaCl_2 2H_20$	0.1
Tween 20	10ml
Agar agar	15

Ingredients except Tween 20 were dissolved in deionized water. Medium was

sterilised by autoclaving at 121°C for 15 minutes. Tween 20 was autoclaved separately and added to the medium.

B.2.4. Media Used For Xylanase Screening

	g/l
Yeast extract	1
Birchwood xylan	5
Agar agar	15

Ingredients were dissolved in sterilised balçova water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B.2.5. Media Used for Cellulase Screening

	g/l
Yeast extract	1
Carboxymethylcellulose sodium salt	5
Agar agar	15

Ingredients were dissolved in sterilised balçova water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B.2.6. Media Used For Pectinase Screening

Media 1

	g/l
Yeast extract	1
Ammonium sulfate	2
Na_2HPO_4	6
KH_2PO_4	3
Polygalacturonic acid	5
Agar agar	15

All ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

Media 2

	g/l
Yeast extract	1
Ammonium sulfate	2
Na_2HPO_4	6
KH_2PO_4	3
Pectin from citrus peel	5
Agar agar	15

All ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

B.3. MEDIA USED FOR EXAMINATION OF SPORES

Nutrient broth	8g/l
Yeast extract	5g/l
Agar agar	15g/l
$CaCl_2$	$7x10^{-4}$ M
$MgCl_2$	$1x10^{-3}$ M
$MnCl_2$	$5x10^{-5}$

All ingredients were dissolved in deionized water. Medium was sterilised by autoclaving at 121°C for 15 minutes.

APPENDIX C

PCR-RFLP RECIPIES

C.1 PCR MIXTURE

Mg free Taq DNA polymerase buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Sterile deionized water	35 µl
Oligo forward 10 picomole/ µl	1 µl
Oligo reverse 10 picomole/ µl	1 µl
dNTP (2mM each) 10X	5 µl
Taq DNA polymerase	0.25 µl (1.25 U)

C.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.

C.3 dNTP (10X)

10 µl 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.

C.4. RESTRICTION ENZYME MIXTURE

Restriction enzyme buffer	5 µl
Sterile deionized water	35
DNA	10 µl
Restriction Enzyme	0.5 µl (5U)

C.5. OLIGONUCLEOTIDE PRIMERS

L1: 5'- CAAGGCATCCACCGT -3'

350 µg primer L1 was dissolved in 175 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Four microliter 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'

590 µg primer EGE 1 was dissolved in 295 µl of sterile deionized water to obtain 2 µg / µl stock solutions. Five microliter 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 D

STAINS AND INDICATORS

D.1 SOLUTIONS FOR GRAM STAINING

D.2.1 CRYSTAL VIOLET STAINING REAGENT

Solution A	
Crystal violet	2g
Ethanol (95%)	20ml
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. Water was then added slowly and the solution was stirred until the iodine was dissolved.

D.2.3. SAFRANIN SOLUTION

Safranin (2,5% in 95% alcohol)	10ml
Distilled water	100ml

APPENDIX E

BUFFERS AND STOCK SOLUTIONS

E.1 50 X TAE

242 g 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.

E.2 1XTAE

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

E.3 10 X TBE

108 g 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 1 L with deionized water.

E.4 1M Tris-HCl pH 7.2

121.1 g Tris base was dissolved in 800 ml of deionized water. pH was adjusted to 7.2 with concentrated HCl. Volume is brought to 1l with deionized water.

E.5 1M Tris-HCl pH 8

121.1 g Tris base was dissolved in 800 ml of deionized water. pH was adjusted to 8 with concentrated HCl. Volume is brought to 1l with deionized water.

E.6 1X TBE

100 ml 10X TBE was taken and the volume was brought to 1 liter with deionized water to obtain 1liter 1X TBE buffer.

E.7 0.5 M EDTA pH 8.0

186.12 g EDTA was dissolved in 800 ml of deionized water and pH was adjusted to 8.0 with 10 N NaOH. Volume was brought to 1000 ml with deionized water.

E.8 1X TE

10 mM Tris, pH 8, 1mM EDTA

E.9 SODIUM ACETATE (3M, pH 5.2)

408.1 g sodium acetate (3 H_2O) was dissolved in 800 ml deionized water and pH was adjusted to 5.2 by glacial acetic acid. Volume was brought to 1000ml.

E.10 ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

0.5 g ethidium bromide was dissolved in 50 ml of deionized water.

E.11 CHLOROFORM-ISOAMYL ALCOHOL SOLUTION

48 ml of chloroform was mixed with 2 ml of isoamyl alcohol.

E.12 PHENOL

Liquified phenol firstly, was removed from the freezer. After allowing to warm at room temperature, it was melted at 68 ˚C. Equal volume of buffer (usually 0.5 M Tris.Cl pH 8 at room temperature) was added to the melted phenol. The mixture was stirred for 15 minutes. When the two phases have separated, the aqueous (upper) phase was removed using a separation funnel. Equal volume of 0.1 M Tris.Cl pH 8 was then added to the phenol. The mixture was again stirred for 15 minutes. The aqueous phase was removed as described before. The extractions were repeated until the pH of the phenolic phase was > 7.8. The pH was measured by using pH paper. After the phenol was equilibrated, the mixture was divided into aliquots and they were stored at -20 ˚C. Before use, the phenol was melted at room temperature. Hydroxyquinoline and β-mercaptoethanol were added to a final concentration of 0.1% and 0.2 %, respectively. The phenol solution can be stored in this form at 4 ˚C.

E.13 PHENOL: CHLOROFORM: ISOAMYL ALCOHOL (25:24:1)

Equal volume of phenol and chloroform isoamyl alcohol (24:1) solution were mixed.

E.14 CTAB/NaCl SOLUTION

4.1 g NaCl was dissolved in 80 ml water. 10g CTAB was added slowly while heating and stirring. If necessary the solution was heated to 65 ˚C to dissolve. The final volume was then adjusted to 100 ml.

E.15 PHENYL METHYL SULFONYL FLORIDE (PMSF) STOCK SOLUTION (100Mm)

17.4 mg PMSF was dissolved in 1 ml isopropanol and stored at – 20 ˚C.