Isolation and Molecular Characterization of Extracellular Lipase and Pectinase Producing Bacteria from Olive Oil Mills

By

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ABSTRACT

Lipases and pectinases are industrially important enzymes. These enzymes are produced by a variety of microorganisms. However there are few studies on the production of these enzymes by thermoacidophilic *Bacillus* species.

The aim of this research was the isolation of extracellular lipase and pectinase producing thermoacidophilic *Bacillus* from olive oil mills and their identification by phenotypic tests, 16S-ITS rDNA RFLP and DNA sequencing.

Eighty-six thermoacidophilic strains were isolated from olive, olive husk and soil contaminated with alpechin collected within different olive oil mills in Ayvalık. The strains were screened for the presence of 5 extracellular enzyme activities. These were lipase, pectinase, amylase, xylanase and cellulase. In total, 69 lipase (Tween 20 as subtrate), 32 pectinase and 68 amylase activities were detected. None of the isolates were able to produce xylanase or cellulase enzyme. All of the isolates were Gram(+) endospore forming rods, thus they were identified as *Bacillus* sp.

Taq I was used for 16S-ITS rDNA based RFLP. The isolated strains were clustered into four groups by *Taq* I restriction profiles of 16S-ITS rDNA.

One representative isolate among the members of each of the 16S-RFLP homology groups was chosen and used for 16S rRNA gene partial sequence analysis. Sequencing results were submitted to GenBank. So far the indicated accession numbers were obtained: AY601903 (isolate H 22 of G-3, 679 nucleotides), AY606276 (isolate S1 of G1, 330 nucleotides)

ÖZ

Lipazlar ve pektinazlar entüstriyel öneme sahip enzimlerdir. Bu enzimler çeşitli mikroorganizmalar tarafından üretilmektedirler. Bununla birlikte bu enzimlerin termoasidofilik *Bacillus*'lar tarafından üretilmesiyle ilgili yapılan çalışmalar az sayıdadır.

Bu çalışmada ekstraselüler lipaz ve pektinaz üreten termoasidofilik *Bacillus*'ların zeytinyağı işleyen fabrikalardan izolasyonu ve fenotipik, 16S-ITS rDNA'ya dayalı RFLP yöntemi ve DNA dizi analiziyle tanımlanmaları amaçlanmıştır.

Ayvalık Bölgesi'ndeki farklı zeytinyağı fabrikalarından alınan zeytin, pirina ve karasu ile kontamine olmuş topraktan 86 adet termoasidofilik suş izole edilmiştir. İzolatlar 5 adet ekstraselüler enzim aktivitesi yönünden incelenmiştir. Bu enzimler; lipaz, pektinaz, amilaz, ksilanaz ve selülazlardır. Toplam 69 adet lipaz (Tween 20 substrat), 32 adet pektinaz 68 adet amilaz aktivitesi saptanmıştır. İzolatlardan hiçbiri ksilanaz ve selülaz aktivitesi göstermemiştir. Tüm izolatlar Gram (+) ve endospor oluşturan çubuk şeklinde bakteriler olduklarından, *Bacillus* türü olarak tanımlanmışlardır.

16S-ITS rDNA RFLP için *Taq* I enzimi kullanılmıştır. İzole edilen suşlar *Taq* I restiriksiyon enzimi profillerine göre 4 farklı gruba ayrılmışlardır.

Her bir 16S-ITS RFLP homoloji grubunun üyeleri arasından bir temsilci izolat seçilmiş ve bu izolatlar 16S rRNA geni kısmi dizi analizinde kulanılmışlardır. Dizi analizi sonuçları GenBank'a gönderilmiştir ve aşağıda belirtilen giriş numaraları alınmıştır: AY601903 (izolat H 22, Grup G-3, 679 nucleotit), AY606276 (izolat S1, Grup,G-1, 330 nukleotit).

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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
min	minute
Mb	Mega base
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymophism
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sp.	Species
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

1.1 Thermoacidophiles

Thermoacidophilic prokaryotes can be found in both Archeae and Bacteria. Thermoacidophilic Archeal species are included in six genera: *Acidianus, Stygiolobus, Sulfolobus, Desulfurolobus, Metallospheraera*, and *Picrophilus*. Thermoacidophilic eubacteria can be grouped in eight genera: *Acidimicrobium, Acidothermus, Alicyclobacillus, Sulfobacillus, Acetogenium, Clostridium, Moorella and Thermoaerobacterium* (Hirashi *et al*., 1997).

1.1.1 Thermoacidophilic *Bacillus* (Genus *Alicyclobacillus*)

The genus *Bacillus* includes aerobic or facultatively anaerobic, rod shaped, Gram + (to Gram variable), endospore forming bacteria that are widely distributed in the environment (Goto *et* al., 2000; Slepecky and Hemphill., 1991; Holt *et al*., 1994; Nazina *et al*., 2001). There are many kinds of species which have thermophilic, psychrophilic, acidophilic, alkalophilic and halophilic properties in the genus (Nazina *et al*., 2001). The reclassification of genus *Bacillus* began in 1991 and yielded eight genera: *Alicyclobacillus*, *Aneurinibacillus*, *Bacillus*, *Brevibacillus*, *Gracilibacillus*, *Paenibacillus*, *Salibacillus* and *Virgibacillus* (Goto *et al*., 2000). These eight genera include more than 100 species that have similar phenotypic characteristics. Thus identification of them is not easy. In the past *Bacillus* species have been identified mainly by morphological and physiological criteria. However the discrimination power of phenotypic methods is limited. Randomly amplified polymorphic DNA (RAPD) method and the hybridization method were effective for detection of a small number of *Bacillus* species. Over the years, a data base of 16S rRNA gene has been constructed and it was successfully used in the differentiation of bacteria (Goto *et al*., 2000).

The genus *Alicyclobacillus* consists of thermoacidophilic, aerobic, Gram (+), rod-shaped, low GC content bacteria (Matsubara *et al*, 2002; Goto *et al.,* 2002a; Nicolaus *et al*., 1998; Albuquerque *et al*., 2000).

Until 1992 the three thermoacidophilic species, *B. acidocaldarius, B. acidoterrestris,* and *B. cycloheptanicus* were placed in the genus *Bacillus* (Goto *et al*., 2002a, 2002b). They were then reclassified as a seperate genus named *Alicyclobacillus* because of their distinct 16S rDNA sequences and cellular fatty acid profiles. They had unique fatty acids (ω-cyclohexane or ω-cycloheptane fatty acids) as the major components of their cellular membrane (Goto *et al*., 2002b). These ω-alicyclic fatty acids have terminal cyclohexyl or cycloheptyl rings

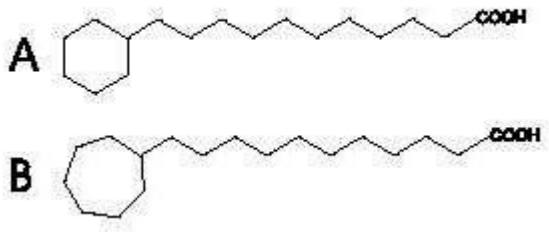


Figure 1.1. The structure of aliphatic, cyclic fatty acids found in the cytoplasmic membrane of *Alicyclobacillus* species. (A) ω-cyclohexylundecanoic acid (B) ω-cycloheptylundecanoic acid (Deinhard *et al*., 1987a).

A. acidocaldarius mutants are unable to synthesize cyclohexyl fatty acids. This indicates the importance of these lipids for growth at temperatures above 50ºC and below pH 4. These lipids adapt membranes to extreme pH and temperatures. They have been found in a variety of thermoacidophilic bacilli (Hippchen *et al*., 1981). *A. acidocaldarius, A. acidoterrestris* (Wisotzkey *et al*., 1992), *A. hesperidum* (Albuquerque *et al*., 2000), *A. acidiphilus* (Matsubara *et al*., 2002), and *A.* sendaiensis (Nishino *et al*., 2001) possess ω-cyclohexane fatty acids. *A. cycloheptanicus* (Deinhard *et al*., 1987) and *A. herbarius* (Goto *et al*., 2002) have ω-cycloheptane fatty acids. Electron migrographs of *A. acidocaldarius* have shown that the surface structure of the bacterium is composed of protein subunits arranged in a crystalline array termed as S-Layer (Messner, 1994) (Figure1.2). An S-Layer is present in a large number of species that comprises all the major groups of bacteria (Engerhardt and Peters, 1998). It can play a protective role, since S-Layer proteins are extremely resistant to harsh conditions. It may also participate in the adherence of bacteria to various surfaces (Neidhardt *et al*., 1990).

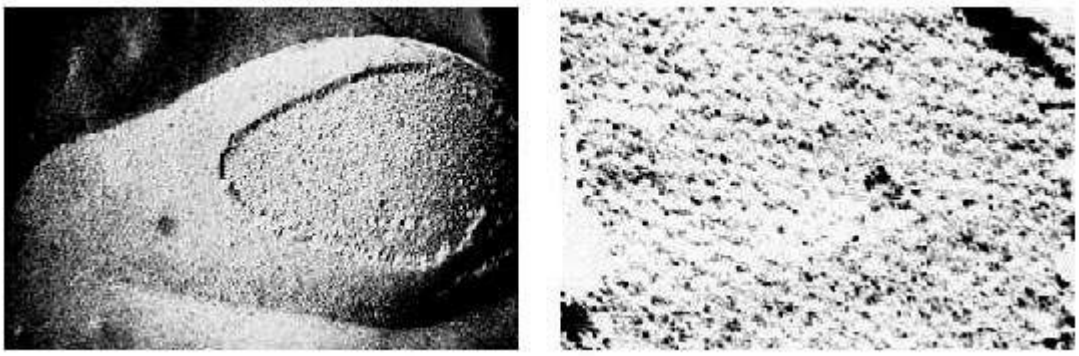


Figure 1.2. Electron micrographs of *A. acidocaldarius* (Source: Engelhardt, 1998)

1.1.2 Distribution of Thermoacidophilic *Bacillus*

The *Alicyclobacillus* species have been isolated from natural sources such as hot springs and soil (Uchino and Doi, 1967; Darland and Brock, 1971; Hippchen *et al*., 1981; Deinhard *et al*., (1987a, 1987b), Hiraishi *et al*.,1997; Nicolaus *et al*., 1998; Albuquerque *et al*., 2000; Goto et al., 2002b, Tsuruoka *et al*., 2003), as well as spoiled fruit based beverages (Yamazaki *et al*., 1996; Goto *et al*., 2002a; Matsubara *et al*., 2002).

1.2 Protein Functioning at low pH

Acidophiles do not adapt the entire cellular machinary to acidic conditions. Instead they have a near neutral intracellular pH (Bakker, 1990). Since it is not needed to adapt intracellular proteins to low pH, only the components of the cell which are exposed to the acidic environment (extracellular enzymes and exposed regions of membrane proteins) should be acidophilic.

Although some acidophilic proteins have been characterized (Inagaki *et al*., 1998; Kimura *et al*., 2000; Hlsman *et al*., 2000a), the mechanism of protein adaptation to low pH is unclear. For example a thermostable isomerase with an acidic pH optimum from *Thermoanaerobacterium* (Liu *et al*., 1996) was compared with two other thermostable isomerases. Except for the low pH optimum, it had similar biochemical characteristics to those of two other isomerases. The primary sequence differed only in 7 or 10 amino acids over a lenght of 439 residues. A similar situation has also been

described for an acidophilic xylanase (Ohta *et al*., 2001). All acidophilic enzymes described, showed overall sequence similarities to the mesophilic counterparts.

Despite the difficulties mentioned, some theories have been suggested to explain acidostability. For example one theory, proposed by Schwermann *et al* (1994), suggests a lower charge density in acidophilic as the reason for stability. In neutrophilic proteins, exposure to low pH leads to protonation of all basic and acidic residues. Basic residues remain positively charged, whereas the carboxyl group of the acidic amino acids become uncharged. This results in an excess positive charge, leading to denaturation of the protein. In acidophilic proteins, this is circumvented by reducing positive and negative charges. Indeed, extracellular proteins from acidophiles including *A. acidocaldarius* were found to have a low charge density, especially on the surface of the protein.

1.3 Industrially Important Extracellular Enzymes

1.3.1 Pectinases

Pectin or other pectic substances are heterogeneous group of high molecular weight, complex acidic structural polysaccharides with a backbone of galacturonic acid residues linked by α- (1-4) linkages. (Kapoor *et al*., 2000; Kashyap *et al*., 2001). They constitute major components of the middle lamella, a thin layer of adhesive extracellular material found between the primary cell walls of adjacent young plant cells (Singh *et al*., 1999; Kapoor *et al*., 2000; Naidu and Panda, 1998; Hoondal *et al*., 2002).

Figure 1.3. Structure of pectin molecule (Source: Alkorta *et al*., 1998)

Pectic substances are classified into four main types based on the type of modifications of the backbone chain which are; protopectin, pectic acid, pectinic acid and pectin (Kashyap *et al*., 2001).

Protopectin is the water insoluble parent pectin substance found in the middle lamella of plant tissues. It yields soluble pectic substances such as pectin or pectinic acid upon restricted hydrolysis.

Pectic acid is a group designation applied to pectic substances mostly composed of galacturonans containing negligible amounts of methoxyl groups. The salts of pectic acid are called pectates.

Pectinic acids are the galacturonans containing various amounts of methoxyl groups. The salts of pectinic acids are either normal or acid pectinates. Under suitable conditions, pectinic acids are capable of forming gels with sugars and acids or if suitably low in methoxyl content, with certain metallic ions.

Pectins are the soluble polymeric materials containing pectinic acids as the major component. They can form insoluble protopectins with other structural polysaccharides and proteins located in the cell wall (Kashyap et al., 2001).

There are basically three types of pectic enzymes; de–esterifying enzymes (pectinesterases), depolymerizing enzymes (hydrolases and lyases), and protopectinases. They can be further classified according to the following criteria; whether they cause random cleavage (endo-, liquefying or depolymerizing enzymes) or whether the cleavage is endwise (exo- or saccharifying enzymes) (Alkorta *et al*., 1998 ; Kashyap *et al*., 2001).

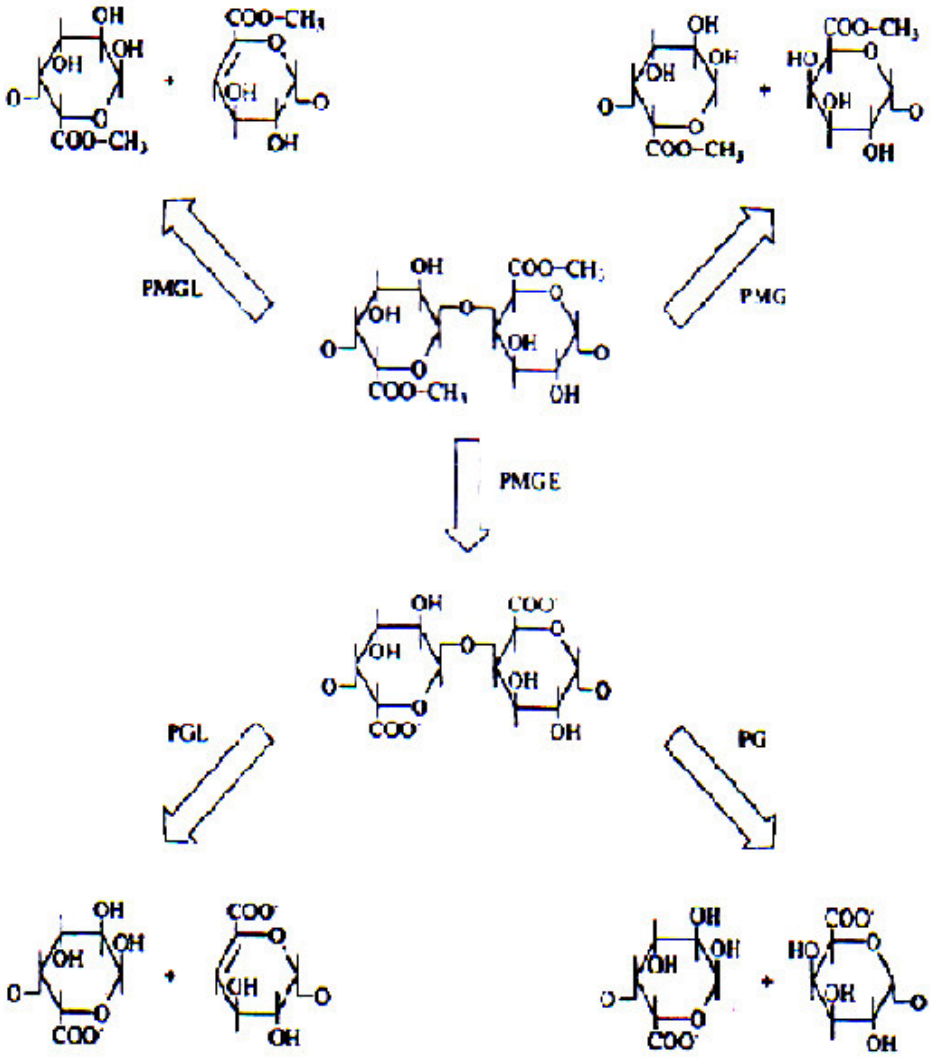


Figure 1.4. Enzymatic mode of action of PMGL, PMG, PGL and PG on the pectin molecule

PMGL : polymethylgalacturonate lyase (pectin lyase)
PMG : polymethylgalacturonase (pectin hydrolase)
PMGE : polymethylgalacturonate esterase (pectin esterase)
PG : polygalacturonase (pectate hydrolase)

(Source: Alkorta *et al*., 1997).

Table 1.1. Some examples of microbial, acidic and alkaline pectinases

	Microorganism	Type of pectinase	Optimum pH	Optimum temp. (^{o}C)
Acidic Pectinases	*Aspergillug niger* CH4	Endo-pectinase Exo-pectinase	4.5-6.0	Below 50
	Penicillium frequentans	Endo-PG	4.5-4.7	50
	Sclerotium rolfsii	Endo-PG	3-5	55
	Rhizoctonia solani	Endo-PG	4.8	50
	Mucor pusilus	PG	5	40
	Clostridium thermosaccharolyticum	Polygalacturonate hydrolase	5.5-7.0	30-40
Alkaline Pectinases	*Bacillus sp.* RKG	PGL	10	-
	Bacillus sp. NT-33	PG	10.5	75
	Bacillus polymxa	PG	8.4- 9-4	45
	Bacillus pumilis	PATE	8.0-8.5	60
	Amuloca sp.	Pectate lyase	10-12.5	70
	Bacillus sp. P-4-N	PG	10-10.5	65
	Penicillium italicum	PMGL	8	50
	Bacillus sp. DT7	PMGL	8	60
	Bacillus subtilis	PAL	9.5	60-65

(Source: Kashyap *et al*., 2001)

Pectinases are mainly produced by plants and microorganisms (Naidu and Panda, 1998) Several microbial acidic and alkaline pectinases are listed in Table 1.1. Acidic pectinases are widely used in the production and clarification of fruit juices. They are also very important in maceration and solubilization of fruit pulps (Naidu and Panda, 1998). Alkaline pectinases have been used in several areas, including retting and degumming of fiber crops, textile proccessing, coffee and tea fermentations, paper and pulp industry, and oil extraction (Hoondal *et al*., 2002).

1.3.2 Lipases

Lipases are the enzymes capable of catalysing the hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids (Sharma *et al*., 2001; Sunna *et al*., 2002; Svendsen *et al*., 2000).

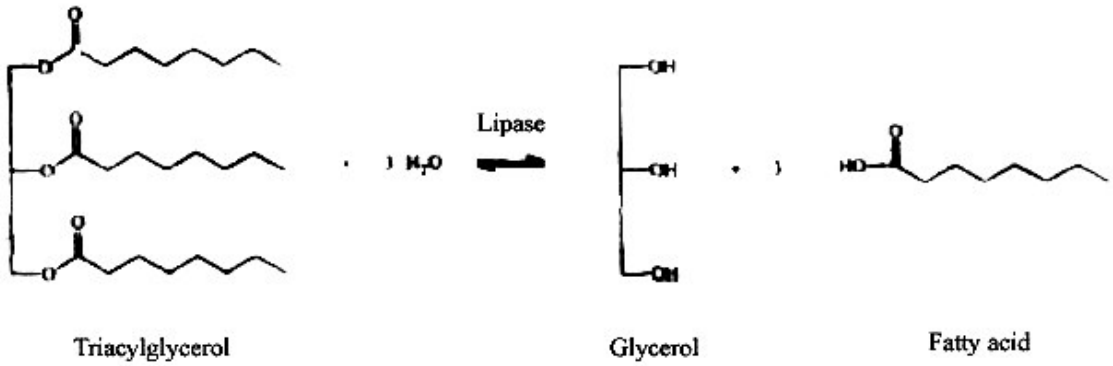


Figure 1.5. Hydrolysis or synthesis of a triacylglycerol substrate catalysed by a lipase enzyme (Source: Jaeger *et al*., 1994)

Lipases have a number of unique characteristics such as 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). They are a versatile group of enzmes. They often express other activities such as phospholipase, isophospholipase, cholesterol esterase, cutinase, amidase and other esterase type of activities (Svendsen, 2000). Recently several lipases from thermophilic bacteria, *Bacillus thermoleoverans* (Lee *et al*., 1999 ; Lee *et al* ., 2001; Markossian *et al*., 2000), *Bacillus stearothermophilus* (Sinchaikul *et al*., 2001), thermoacidophilic bacteria, *Bacillus acidocaldarius* (D'Auria *et al*., 2000), and alkaliphilic bacteria, *Bacillus* sp. strain A 30-1 (Wang *et al*., 1995), *Bacillus*, have been purified and characterized. However at present commercially used lipases are mostly of fungal origin (Jaeger *et al*., 1994).

Lipases are widely used in fat and oil processing, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, production of cosmetics (Sharma *et al*., 2001), and lipid rich waste water treatment (Markossian *et al*., 2000).

1.3.3 Starch Degrading Enzymes

Starch is a complex polysaccharide composed of α–glucose units which are linked by α–1,4- or α-1,6- glycosidic bonds. The main fractions of starch are amylose and amylopectin. Amylose is a linear polymer of glucopyranose units that are linked by α–1,4–glycosidic bonds. Amylopectin is a branched polymer containing α–1,4–

glycosidic linkages and α – 1,6 linked branched points occuring every 17-26 glucose units (Bertoldo and Antranikian, 2002).

Starch degrading enzymes can be classified into two groups, endo-acting and exo-acting enzymes. Endoamylases (α–amylases) cleave interior linkages of starch randomly and yield linear and branched oligosaccharides. Exoamylases hydrolyse the substrate from the non-reducing end. Debranching enzymes include pullunases and isoamylases and they are able to hydrolyse α –1,6- bonds in pullunan and amylopectin. (Bertoldo and Antranikian, 2002; Niehaus *et al*., 1999).

Recently several amylolytic enzymes from *A. acidocaldarius* (Schwermann *et al*., 1994), *Bacillus* sp. IMD 434 (Hamilton *et al*., 1999), and *Bacillus* sp WNII (Mamo *et al*., 1999), have been purified and characterized .

Amylases are widely used in food, textile, detergent and paper industries and in starch processing (Pandey *et al*., 2000). There are two main steps in starch processing: liquefaction and saccharification. Both of the steps require high temperature. In liquefaction, concentrated suspension of purified granular starch is converted into a solution of soluble, shorter chain-length dextrins. Thermostable amylases from *B. amyloliquefaciens*, *B. stearothermophilus* and *B. licheniformis* are used in the liquefaction step. Although these enzymes are able to show activity at high temperatures, the process cannot be performed below pH 5.9, since thermostability of the operating amylases decreases at low pH. To be compatible with the pH optima of these enzymes, the pH of the starch slurry must be adjusted. Amylases however able to show activity at lower pH. Amylases from thermoacidophiles have promising properties but none have been produced at commercially viable level (Crabb and Mitchinson, 1997; Vielle and Zeikus, 2001).

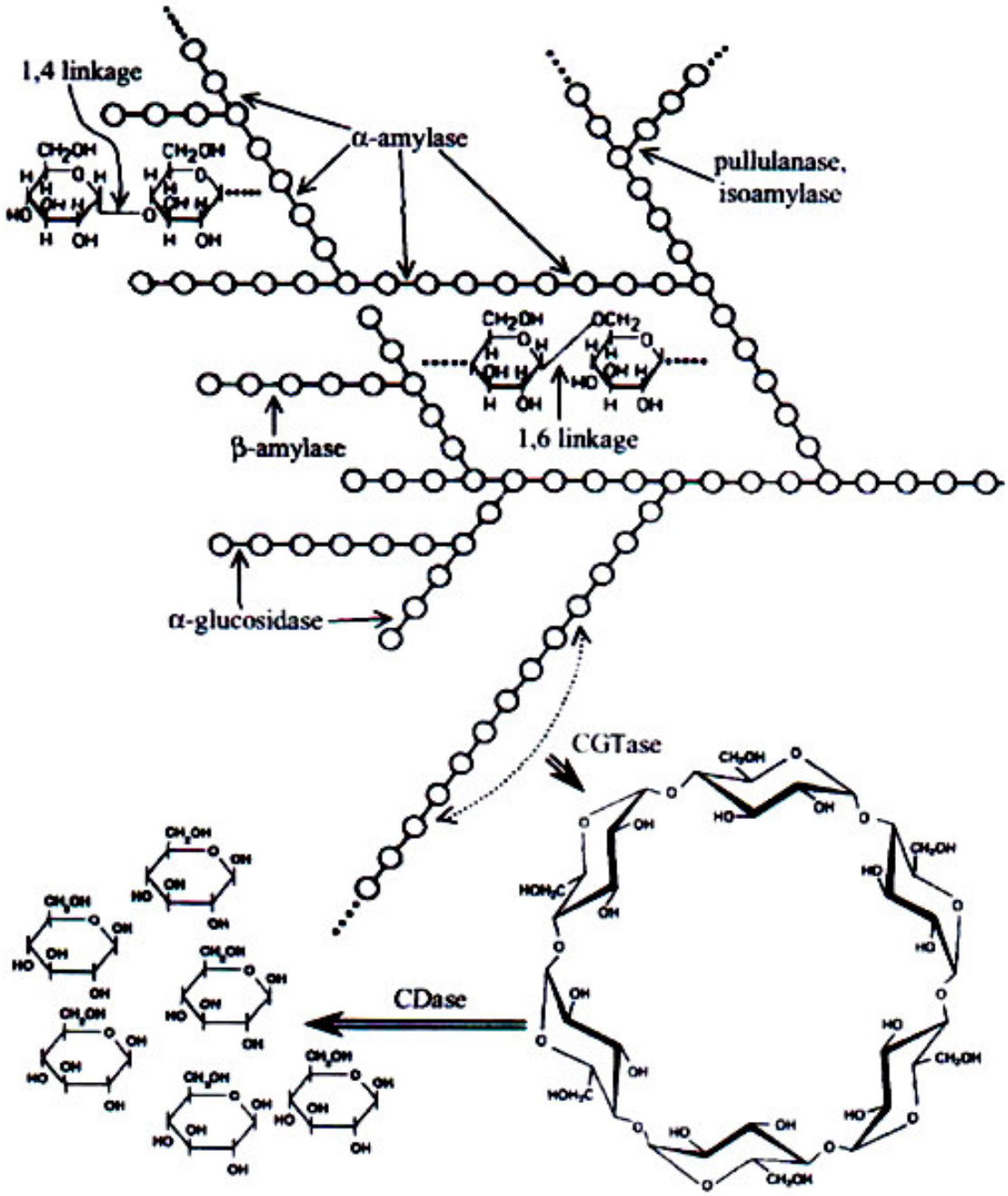


Figure 1.6. Hydrolysis sites of starch depolymerizing enzymes produced by members of the genera *Bacillus*, *Paenibacillu*s , *Alicyclobacillus* and *Brevibacillus*. (Source: Pirttizarvi *et al*., 2000)

1.3.4 Xylanases

Xylan is a complex polysaccharide having a backbone of xylose residues linked by β–1,4 – glycosidic bonds. It constitutes the major hemicellulose component of the

plant cell. The main chain of xylan consists of β–xylopyranose residues. Most xylans contain different substituent groups such as acetyl, arabinosyl, and glucuronysyl residues in their backbone and side chains (Beg *et al.*, 2001).

Many reports on thermostable xylan digesting *Bacillus* species, *Bacillus amyloliquefaciens* (Breccia *et al*., 1998), Bacillus sp. strain SPS-0 (Bataillon *et al*., 2000), are available.

Alicyclobacillus acidocaldarius (ATCC 27009) has been reported to produce extracellular thermoacidophilic xylanase (Eckert *et al*., 2002).

Xylanases are biotechnologically important enzymes since the hydrolysis products of xylans are used as thickeners or as fat substitutes in food industry. Xylose and xylooligosaccharides are also used to obtain liquid fuel, single cell proteins, and solvents (Eckert *et al*., 2002).

Xylanolytic enzymes are also used in extraction and clarrification of juices and wines, modification of cereal flours to enhance the volume, textural and staling properties of bread, prebleaching of paper pulps, retting of flax, hemp, and jute (Gilbert and Hazlewood, 1993).

1.3.5 Cellulases

Celluloses are complex and heterogeneous polymer formed of D-glucose residues linked by β-1,4-glycosidic bonds. Cellulases are classified into three groups: exoglucanases, endoglucanases and β–D–glucosidases. Exoglucanases cleave the cellobiosyl units from the nonreducing ends of the cellulose chains. Endoglucanases hydrolyse the internal cellulosic linkages and β–D–glucosidases specifically cleave glucosyl units from the nonreducing ends of cellooligosaccharides (Schülein *et al*., 2000).

Cellulases are widely used in textile industry for bio-polishing of fabrics, house hold laundry detergents, animal feeds, fruit juice processing, baking and in de-inking of paper (Mavadza *et al*., 2000).

1.4 Characterization Methods for Bacteria

1.4.1 Phenotypic Methods

For determining the major groups to which a new isolate is likely to belong, certain phenotypic characteristics have primary importance. Some of these are morphology (rod, coccus, vibrioid, helical or other), Gram status, motility, temperature and pH requirements, pigment formation, nutritional classification (phototrophic, chemoautotrophic, chemoheterotrophic), oxygen demand (aerobic, anaerobic, microaerophilic), cell wall structure, the presence of spores (endospores, exospores), antibiotic sensitivity, ability to use various carbon, nitrogen and sulfur sources, pathogenicity, immunological characteristics, and habitats (Smibert and Krieg, 1994).

Some other phenotypic methods for identification of bacteria are biotyping, antibiotic susceptibility, phage typing, serotyping, immunoblotting, and multilocus enzme electrophoresis (MLEE) (Bush and Nitschko, 1999).

Biotyping is used to detect the biochemical reactivity of a bacteria towards a variety of substrates. An organism can be classified at genus and/or species level using various biochemical reagents. The pattern of utilization of carbon sources plays an important role in differentiating species. The utilization of glucose, lactose or other sugars can be detected by the production of gas or change in color of an indicator dye which is caused by the production of acids (Bush and Nitschko, 1999). Antibiotic susceptibility test or antibiogram provides the analysis of growth of an isolate in the presence of a given antibiotic. It is the most standardized typing method. However it is not very discriminatory because antibiotic susceptibility of an organism can change rapidly by the horizontal transformation of plasmids containing resistance genes (Bush and Nitschko, 1999). In phage typing, bacteria are differentiated by examining their infection capability by specific phages. In serotyping microorganisms are differentiated according to their reaction to special antibodies. This method has a limited use. In immunoblotting, proteins from whole-cell lysate are separated by SDS PAGE. Whole cell protein patterns are used for typing and differentiation of strains (Busch and Nitschko, 1999). Multilocus enzyme electrophoresis (MLEE) is a technique which is based on the separation of water soluble cellular enzymes on starch gels or cellulose acetate sheets by electrophoresis. Differences in the amino acid sequence cause

differences in the mobilities of enzyme variants. Each unique protein variant (Electrophoretic type; ET) is compared with those of reference strains.

The use of phenotypic techniques is limited by the appearance of phenotypic differences of isolates of the same strain (Busch and Nitschko, 1999). Also some phenotypic characteristics may not be expressed under certain conditions. Therefore classical phenotypic methods are often not suitable or enough for differentiation of microorganisms (Farber, 1996). Therefore genotypic and phenotypic methods must be used together in order to classify a bacterium.

1.4.2 Molecular Characterization Methods

Molecular characterization methods often rely on the analysis of chromosomal or extrachromosomal DNA (Farber, 1996). These methods have many advantages over phenotypic methods in terms of better discriminatory power, reproducibility and typeability (Farber 1996). The main advantages can be summarized below:

- Higher discriminatory power, since they can distinguish between two closely related strains (Farber, 1996).
- Since DNA can always be extracted from an organism, all strains can be typeable (Farber 1996).
- Analytical strategies in genotypic methods are similar and can be applied to DNA of any source (Farber, 1996).
- Genomic DNA is highly stable and its composition does not change according to the cultural conditions and methods of preparation (Olive and Bean, 1999; Farber, 1996).
- The results can be analyzed statistically and convenient for automation (Farber, 1996; Bush and Nitschko, 1999).

The most common genotypic characterization methods are: plasmid profiling, nucleotide sequencing, pulsed-field gel electrophoresis, polymerase chain reaction based methods, REP PCR, PCR ribotyping, RAPD, 16S-ITS (Internally Transcribed Spacer) rDNA region –RFLP (Restriction Fragment Length Polymorphism) (Farber, 1996).

1.4.2.1 Plasmid Typing

Plasmids are small, self replicating, usually supercoiled, and double stranded, extrachromosomal DNA (Farber, 1996; Busch and Nitschko, 1999). They usually encode product(s) and or function(s) which modify the phenotype of the cell. Their size ranges from 1.5 kb to 300 kb. Plasmids encode many different genetic determinants which are not essential for the survival of bacteria. For example F plasmids have information for their own transfer from one cell to another. R plasmids encode resistance to antibiotics. Cryptic plasmids often have no functional coding genes (Crosa *et al*., 1994).

In plasmid typing, plasmids of the isolates are extracted and then separated electrophoretically in an agarose gel. The isolates are differentiated according to the differences in their plasmid number and size. However different plasmids can be of the same size. To circumvent this problem, plasmids are digested with a specific restriction enzyme. A specific restriction enzyme digests plasmids at different sites revealing different fragment patterns on the gel (Farber, 1996). Plasmid typing is the oldest and the simplest method among the other molecular typing methods. It is relatively fast and easy.

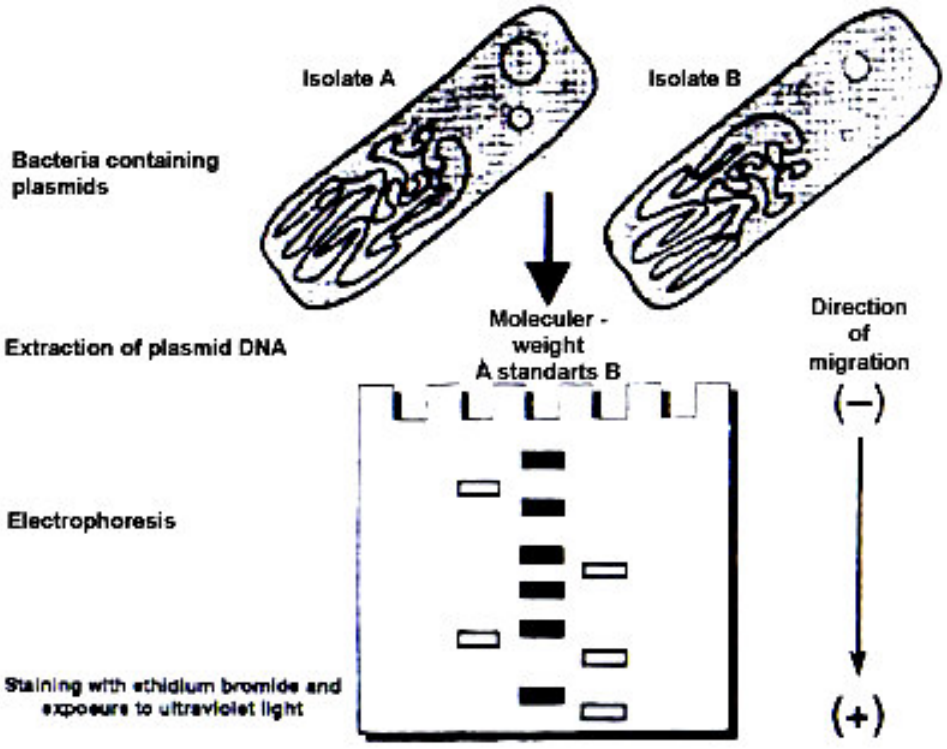


Figure 1.7. Schematic illustration of plasmid- profile analysis (Source: Farber, 1996)

The results can be standardized by using DNA size markers. However plasmid profile analysis has some drawbacks. Plasmids are usually unstable, some organisms contain few or no plasmids. The main disadvantage of plasmid typing is the loss of plasmids or transfer of plasmids between the strains and between the species (horizontal gene transfer) (Farber, 1996).

1.4.2.2 Ribotyping

Ribotyping is based on the use of nucleic acid probes to recognize ribosomal genes. The ribosomal RNA (rRNA) which is composed of three different molecules named 23S, 16S, 5S rRNA, constitutes nearly 82% of the total RNA in a typical bacterial cell. The genes encoding rRNA are highly conserved. While most bacterial genes are present in only one copy, multiple copies (from 2 to 11 copies) of rRNA operons (*rrn*) can also exist. Therefore as the number of copies of *rrn* operon increases ribotyping becomes more discriminative for that particular bacterium.

In ribotyping firstly bacterial chromosomal DNA is isolated and then restriction digestion is applied. After the DNA is electrophoresed onto an agorose gel, it is transferred onto a nylon or nitrocellulose membrane either electrophoretically or by capillary action (Farber, 1996). Hybridization is done by using labelled probes containing *E.coli* 23S, 16S, and 5S rRNA genes, and the membrane is exposed to an X-ray film. Resulting hybridization bands can be compared easily. The advantages of ribotyping are that the method is highly reproducible and all eubacteria can be subtyped by using a single probe (Farber, 1996). The disadvantages of the method are that it may not be applied to bacteria which have only one or two copies of ribosomal RNA genes, and its discriminatory power is not as high as the newer molecular methods (Olive and Bean, 1999 ; Farber 1996).

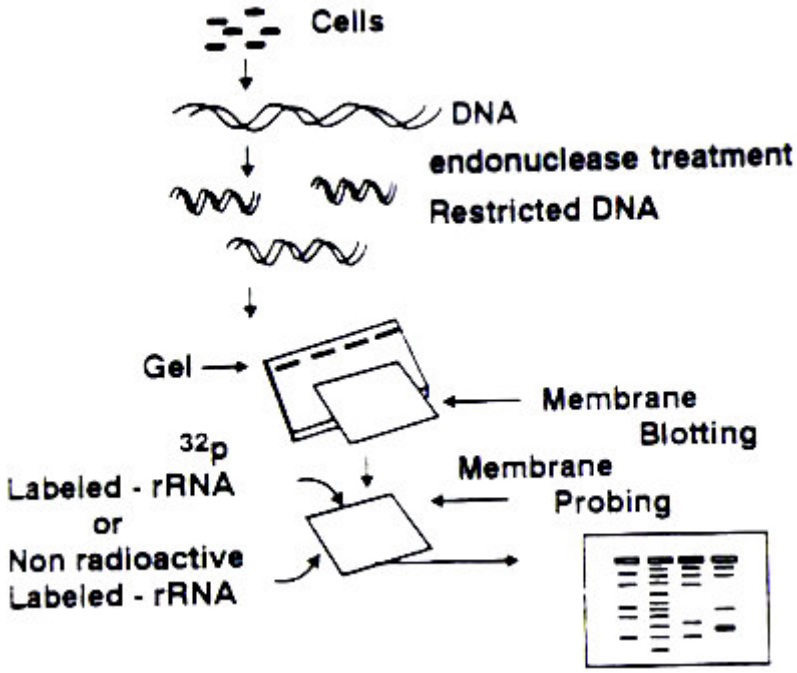


Figure1.8. Schematic representation of ribotyping (Source: Farber, 1996)

1.4.2.3 Polymerase Chain Reaction (PCR) Based Methods

PCR is based on the *in vitro* amplification of DNA by a thermostable DNA polymerase enzyme (usually *Taq* Polymerase from *Thermus aquaticus)*. Special primers are used in order to amplify the region of interest. PCR includes repeated cycles of high temperature for denaturing the DNA, primer annealing and an extention step in which the complementary DNA is synthesized by the action of a heat stable polymerase. At the end of each cycle the number of copies of the chosen sequence is doubled. Thus the amount of the target sequence is increased exponentially (Busch and Nitschko, 1999). The amplified target sequence of a given bacterium can be used for RFLP or sequenced.

1.4.2.3.1 Randomly Amplified Polymorphic DNA (RAPD)

The randomly amplified polymorphic DNA assay is also referred to as arbitrary primed (AP)–PCR. In a RAPD assay synthetic primers of 10 bases in length are used in the polymerase chain reaction. These short primers hybridize at 3-10 random genomic sites simultaneously and an amplicon is generated if the sites are nearby. The amplification products are separated by gel-electrophoresis and polymorphisms are

detected according to the presence or absence of band patterns of a particular size. Some advantages of RAPD are:

- Easy to perform
- Primer knowledge on the template DNA is not necessary because of random priming.
- Does not require isotopic labelling and also there is no need to use restriction endonucleases.
- It is suitable for automation
- Low quantities of template DNA (5-50 ng per reaction) are enough for the assay.

However RAPD assay has also some disadvantages; Some problems may arise in reproducibility if the method is not well standardized. Since large number of amplicons is obtained, the comparison of patterns can also be difficult.

1.4.2.3.2 16S-ITS rDNA RFLP

The bacterial ribosomal operon has been used as a genetic marker to study the evolution and phylogeny of microorganisms (Abd- El- Haleem *et al*., 2000; Luz *et al*., 1998) In most prokaryotes, the ribosomal genes constitute an operon with the order 16S- 23S – 5S and are transcribed in a single polycistronic RNA (Luz *et al*., 1998). The 16S rRNA gene is a good tool to evaluate bacterial phylogenies at genus level (Abd- El-Haleem *et al.,* 2000; Shaver et al., 2002). Since there is high 16S rDNA sequence similarity within microbial species, these sequences are insufficient for species identification. The use of 23S rDNA as a phylogenetic marker is limited because its size is large and there is inadequate data in the database (Abd-El-Haleem *et al*., 2002). The region between 16S and 23S is referred to as ISR (Intergenic spacer region) or ITS (Intergenic/ Internal transcribed spacer) (Abd-El-Haleem *et al*., 2002; Fischer and Triplett, 1999; Toth *et al*., 2001; Shaver *et al*., 2002; Daffonchio *et al.,* 2000). The ITS region is an important tool for the discrimination of bacterial species and the constitution of specific bacterial probes and primers (Daffonchio et al., 1998). It contains both conserved and highly variable sequences (Abd-El-Haleem *et al*., 2002) such as tRNA genes and *box*A (Garcia- Martinez et al., 1999).

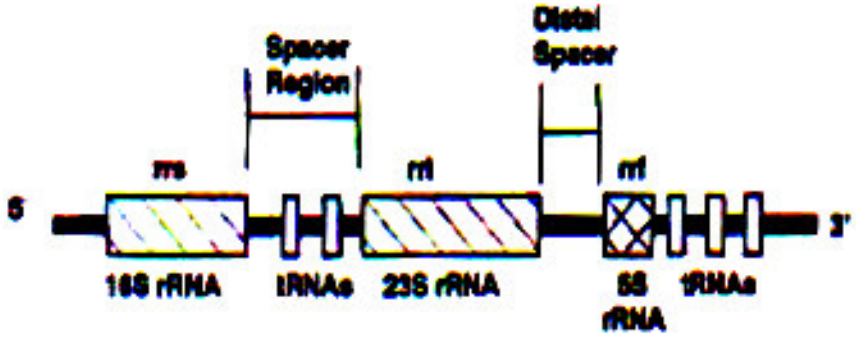


Figure 1.9. Schematic illustration of internal spacer region (Source: Garcia-Martinez at al., 1999)

16S-ITS rDNA region is amplified as a single amplicon by the use of specific primers. After the amplification restriction endonuclease digestion is performed. The restriction endonuclease is selected according to the nucleotide composition of 16S and ITS rDNA region. Frequent cutting restriction enzymes are used for restriction digestion. Finally restriction fragments are separated in an agarose gel by electrophoresis. Restriction patterns are then compared. This method distinguishes bacteria at species level (Garcia- Martinez at al., 1999).

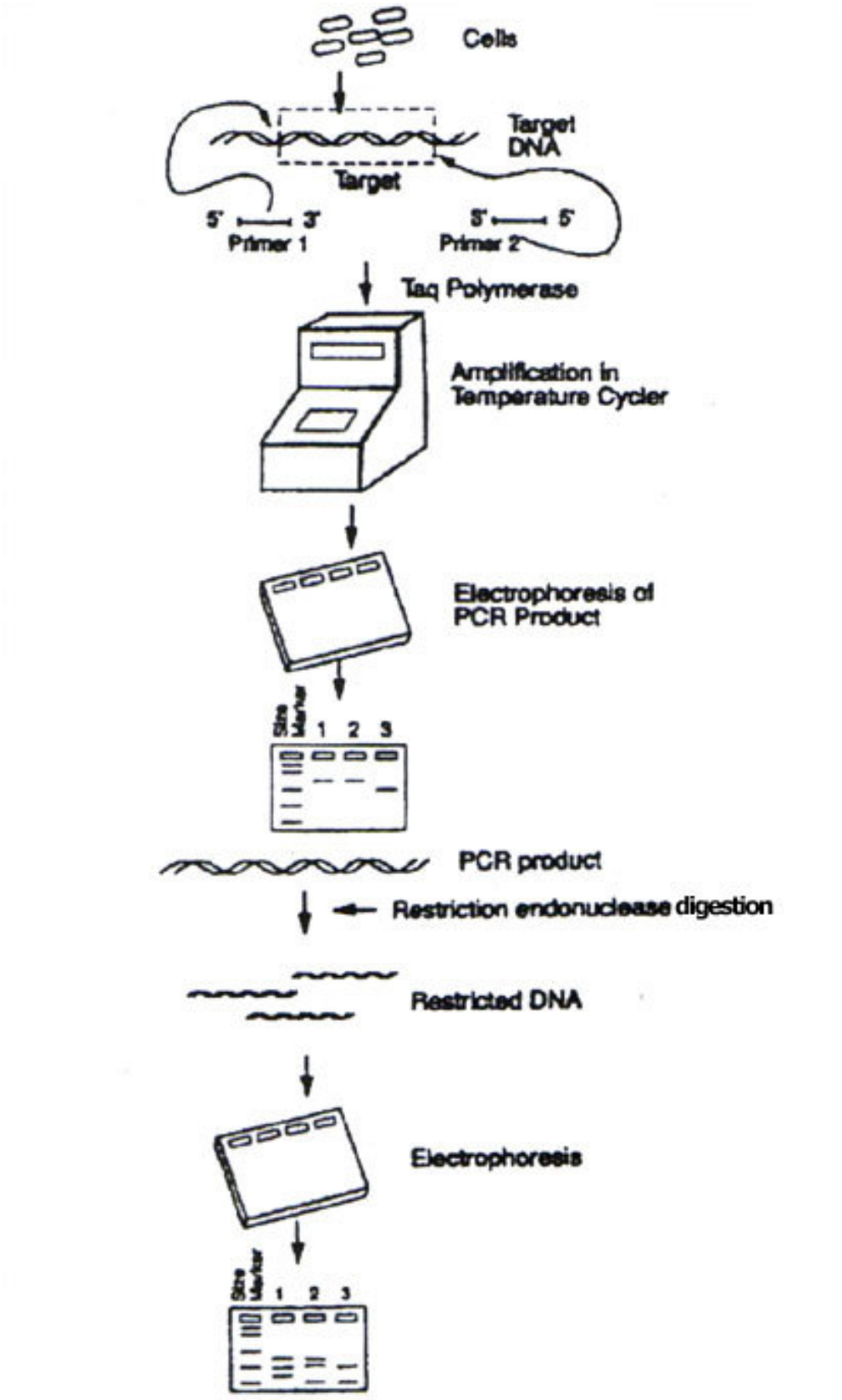


Figure 1.10. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (Source: Farber, 1996)

1.4.2.3.3 Repetetive-Polymerase Chain Reaction (Rep-PCR)

Bacterial genomes can also be typed by examining strain-specific chromosomal DNA patterns obtained by PCR. There are for example two main sets of repetitive elements within bacterial genomes commonly used for DNA typing. They are known as repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences. REP elements are 38-bp sequences consisting of 6 degenerate positions and 5 bp variable loops. ERIC sequences are 126-bp elements containing highly conserved central inverted repeat (Olive and Bean, 1999). REP-PCR is an easy technique to perform. It has comparatively better discriminatory power than plasmid profiling, 16S rDNA –RFLP and ITS-RFLP.

1.4.2.4 PFGE

PFGE is a molecular typing method which allows extremely large DNA molecules to be resolved. It has better discriminatory power than PCR-based methods. It can discriminate bacteria at subspecies level (Bush and Nitschko, 1999). In PFGE, the organisms are first embedded in agarose. Embedding the DNA in agarose avoids the random shearing of DNA into non-specific fragments. The embedded cells are first lysed by a suitable lytic agent and then the cells are deproteinized with proteinase K. Several washing steps are applied to avoid the inhibitory affects of the chemicals. The embedded DNA is then digested with an infrequent cutting restriction enzyme. The infrequent cutting restriction endonucleases recognize few sites in genomic DNA and create only a small number of large DNA fragments ranging from 10 to 800 kb in size (Busch and Nitschko, 1999; Olive and Bean, 1999). After inserting the digested bacterial plugs into the agarose gel, electrophoresis is performed. The restricted genomic DNA fragments are visualized under the UV light. Finally the restriction band patterns of the isolates can be compared. A schematic illustration of PFGE is shown in Figure 1.11.

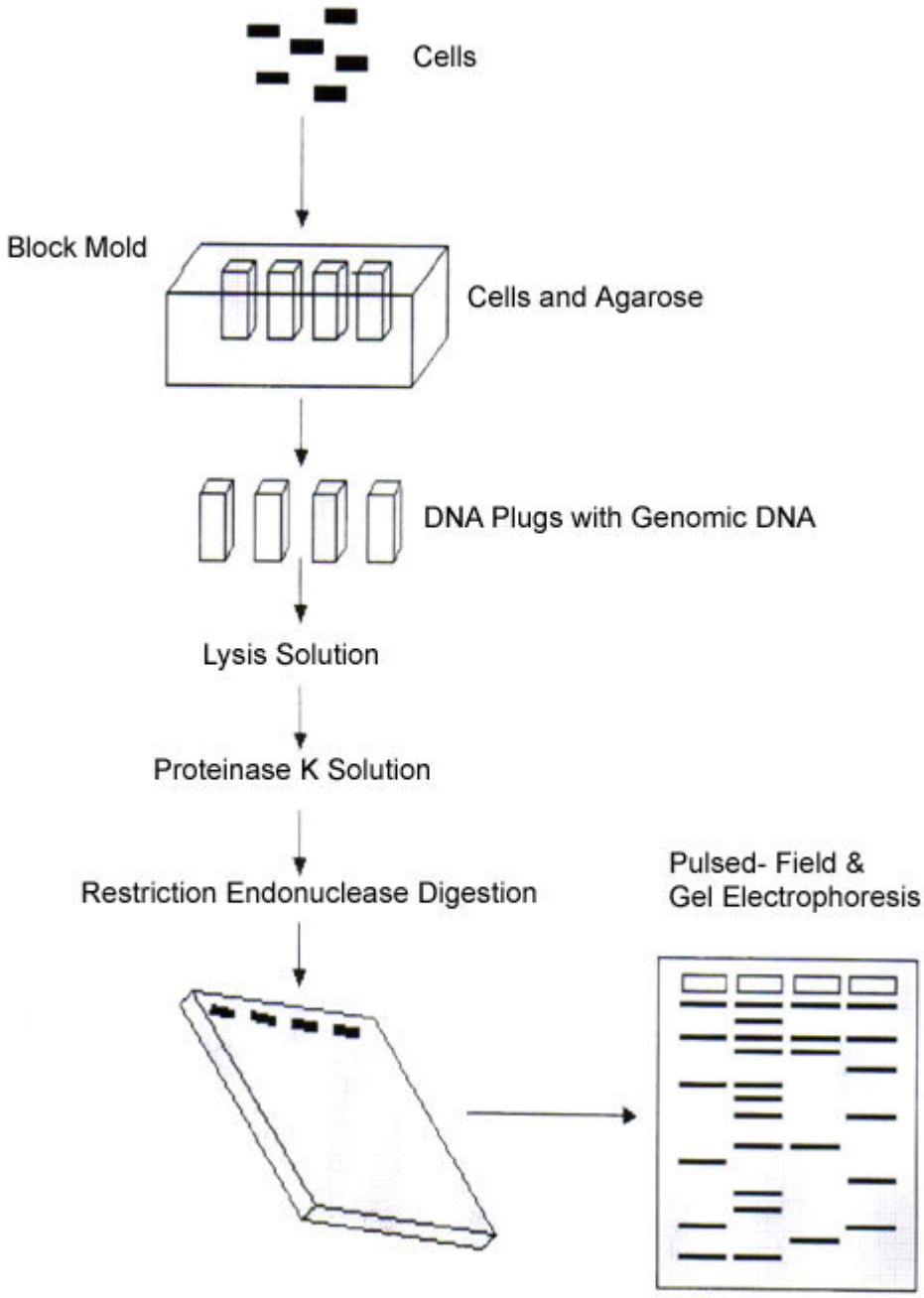


Figure 1.11. Schematic illustration of PFGE (Source: Farber, 1996)

PFGE system allows the separation of higher molecular weight DNA by alternating electrical field with predetermined intervals. These intervals are called switch times or pulse times. When the first electrical field (E_1) is turned on, DNA fragments begin to migrate in the porous gel. They elongate in the direction of the field. After a pulse time, another electrical field (E_2) with different direction is applied. DNA molecules therefore have to change their direction and reorient themselves. DNA migration follows in a straight way in the gel (Birren and Lai, 1993).

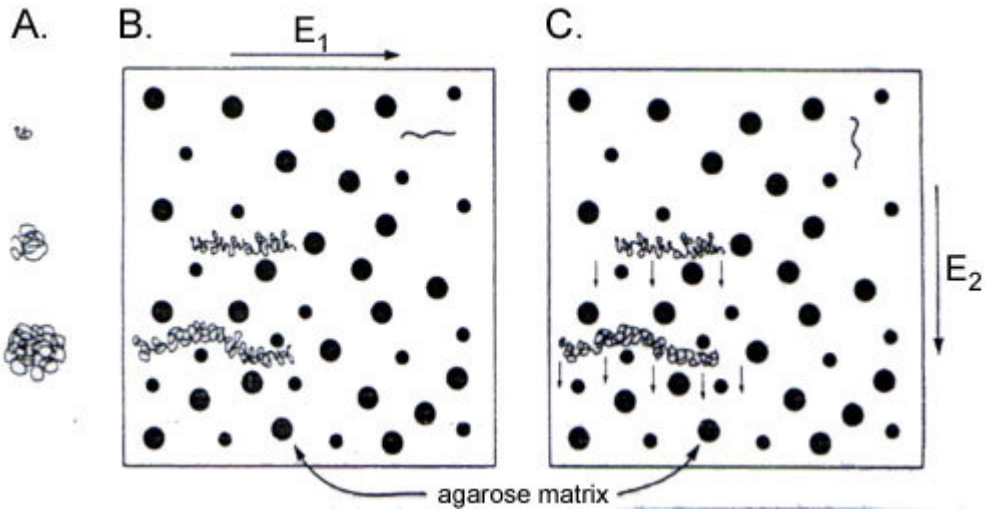


Figure 1.12. Schematic illustration of DNA separation in pulsed field gel electrophoresis (Source: Birren and Lai, 1993)

Clamped homogeneous electrical field system (CHEF) is the latest type and one of the most commonly used PFGE system. CHEF DR II system consists of twenty-four electrodes arranged in a hexagonal array and homogeneous electric fields are generated through these electrodes. One hundred and twenty degree reorientation angle is generated for separating DNA molecules ranging from 100 kb to 6 Mb in size (Birren and Lai, 1993; Bio-Rad Manual, 2001).

Resolution of high molecular weight DNA fragments are affected by several parameters (Bio-Rad Manual, 2001). These are:

- switch interval
- voltage gradient
- electrophoresis time
- buffer type and temperature
- agarose concentration
- running temperature

The size of the DNA which will be resolved on pulsed field gel is directly proportional to the pulse time used. Larger DNA molecules require higher switch times to be resolved since they need longer time to reorient themselves (Birren and Lai, 1993).

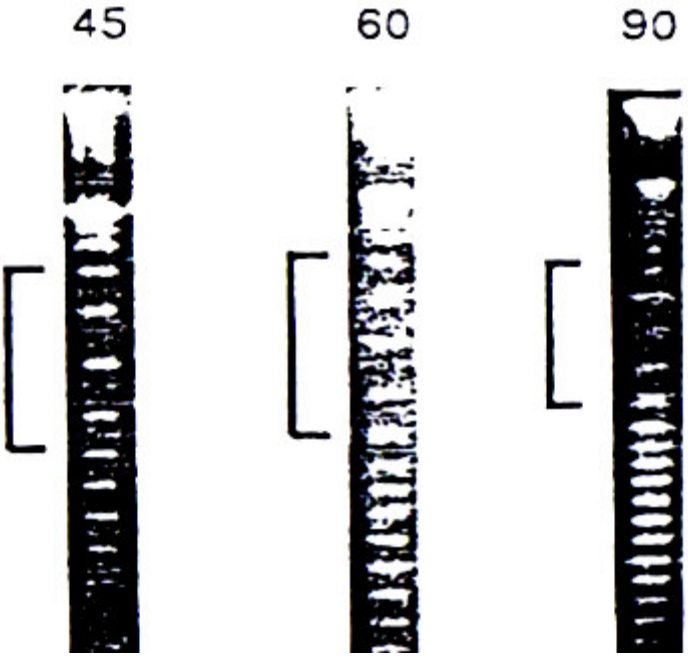


Figure 1.13. Effect of switch time on resolution of DNA fragments (Source: Birren and Lai, 1993).

Effect of switch intervals on resolution of the lambda ladder can be seen in Figure 1.13 (Birren and Lai, 1993). The size of the DNA separated in the indicated area is directly proportional with the pulse time applied. As the pulse time increases (45 sec; 60 sec; 90 sec) also the fragment sizes (350–550 kb; 550–750 kb; 650–900 kb respectively) increase (Birren and Lai, 1993).

The difference between the electrical potential of the electrodes in a gel box is known as voltage gradient. Increasing the voltage gradient also increases the DNA migration however band sharpness decreases. High voltage can reduce the resolution capacity of the system (Bio–Rad Manual, 2001). High voltage will cause the DNA to reorient rapidly; thus shorter pulse times are used to obtain comparable resolution. Similarly applying lower voltages requires longer switch intervals (Birren and Lai, 1993).

Buffer concentration, buffer type and buffer temperature also affect the mobility and resolution of DNA molecules. DNA migrates much more quickly when buffers with low ionic strength are used. When buffer temperature increases, the mobility of the DNA also increases, but resolution decreases. The recommended buffer temperature is 14^0C or an acceptable correlation between speed and resolution (Bio–Rad Manual, 200; Birren and Lai, 1993).

The speed of the separation and the size range of fragments resolved are also affected by the concentration of the agarose. Thus the change in agarose concentration must be balanced with the change in the switch interval to maintain resolution. When the agarose concentration is decreased, DNA migration rate increases (Birren and Lai, 1993). The optimum agarose concentration used to separate DNA fragments up to 3 Mb is 1%. In order to obtain more sharp DNA bands, the agarose concentration can be increased to 1.2–1.5 % but in this case electrophoresis time must be increased (Bio – Rad Manual, 2001).

1.4.2.5 DNA Sequencing

DNA sequencing is the determination of the exact order of nucleotides in a region of DNA molecule. Generally the 16S rRNA gene is sequenced since it contains variable and conserved regions among different bacterial species. Automated DNA sequencing generally includes PCR amplification of 16S rRNA gene, computer aided sequence analysis and interpretation. Databases of 16S rRNA sequences are constructed and bacterial isolates are identified by comparison with these sequences.

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

Samples were taken from four olive oil mills in Ayvalık

Table 2.1. Sample type, location, sample number

Sample type	Location	Sample number
Soil contaminated with alpechin	Ahmet Ertem Olive Oil Factory Gömeç / Ayvalık	1
Soil contaminated with alpechin	Anfora Olive and Olive Oil Factory Gömeç/Ayvalık	1
Soil contaminated with alpechin; olive husk, olive	Doğuş Olive Husk / Olive Oil Factory Karaağaç/Ayvalık	3
Soil contaminated with alpechin	Karayaz Olive Oil Factory Karaağaç/Ayvalık	1

2.1.3 Reference Strains

Alicyclobacillus acidocaldarius (DSM 446)

Alicyclobacillus acidoterrestris (DSM 2498)

Alicyclobacillus Genomic Species 2 MIH 332

Alicyclobacillus pomorum 3A

Alicyclobacillus herbarius CP1

MB 1359

A. acidocaldarius DSM 446 and *A.acidoterrestris* DSM 2498 were kindly provided by Prof. Dr. Elmar Kannenberg from Mikrobiologisches Institut Mikrobiologie/ Biotechnologie, Eberhard Karls Universitat, Tübingen /Germany.

A. pomorum 3A, AGS2 MIH 332 and *A. herbarius* CP1 were kindly provided by Dr. Keiichi Goto from Food Research Laboratories, Mitsui Norin Co., Ltd., Fujieda/ Japan.

MB 1359 was kindly prodived by Prof. Dr. Marc Heyndricxks from Department Kwaliteit van Dierlijke Producten en Transformatietechnologie, Belgium.

2.2 Methods

2.2.1 Isolation of Thermoacidophilic *Bacillus*

Both dilution plate and enrichment method were used for isolation. For the enrichment method, 10 gr of samples were subjected to heat treatment for 10 min at 80ºC in a waterbath in order to kill most of the vegetative cells and thus to eliminate non-spore forming bacteria (Mora *et al.,* 1998). After heat treatment, the samples were transferred into 100 ml of *Bacillus acidocaldarius* Broth (Appendix B). Incubation was performed in a rotary shaker at 50^0C until a turbidity obtained. Then 500 µl of the broth was plated on *Bacillus acidocaldarius* medium (BAM) (Appendix B).

For the dilution plate method 10 gr of samples were transferred in 90 ml of 0.85% saline water. After pasteurization at 80^0C for 10 min, 1 ml aliquot from each of the samples was transferred in 9 ml of 0.85% saline water and 6 fold dilutions were prepared. One ml of dilutions was plated on BAM and YSG agar (Appendix B) plates and incubated for 48-72 h at 50^0C. The plates were covered with aluminium foil and

cooking bags in order to prevent drying of agar plates at 50^0C. Single colonies with different morphologies were picked and purified using streak plate method.

2.2.2 Preservation of Isolates

Glycerol stocks were prepared and stored at -80^0C for long term preservation. Pure cultures and reference strains were incubated at 50^0C for 48 h in isolation broth. Then 0.5 ml of each of the cultures were transferred into cryotubes and 0.5 ml broth containing 40% glycerol was added. The samples were mixed gently and stored at -80°C.

2.2.3 Phenotypic Characterization

2.2.3.1 Gram Staining

Gram method was used for staining of bacteria. A 5 µl drop of 1x TE was pipetted onto a microscope slide. A loopfull of overnight culture was then suspended in 1xTE on the slide. Smear was prepared by spreading the drop with a toothpick. After drying, the thin film on the slide was fixed by passing the slide three times through the flame of a Bunsen burner. The heat fixed smear was first stained with crystal violet for 1 min. After rinsing the slide, under the tap water for some seconds gently and indirectly, it was transferred into iodine solution and was kept for 1 min. Then the slide was again washed under the tap water and incubated in 95% alcohol for 6 s. After washing the slide under the tap water, it was stained with safranin for 30 s. It was again rinsed under tap water and dried on paper towels. The cells were then examined under the light miroscope. Gram (+) cells seemed purple while Gram (-) cells seemed pink or red. Cell morphology was also examined.

2.2.3.2 Examination of Endospores

Isolates grown on *B. acidocaldarius* medium (Appendix B) for 3-4 days were suspended in 3-5 µl of sterile 0.09% NaCl on a microscopic slide and covered with a coverslip. Endospores were observed as shiny bodies in the cells under the phase-contrast microscope.

2.2.3.3 Catalase Test

Isolates were grown in BAM (Appendix B) for 48-72 h at 50^0C. 3 % hydrogen peroxide was poured onto the colonies. Formation of air bubbles indicate the presense of catalase enzyme (Smibert and Krieg, 1994).

2.2.3.4 Oxidase Test

Isolates were grown in BAM for 48 h at 50^0C. 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 tapped lightly onto the wet fitler paper. Formation of a blue-purple colour was taken as the evidence for oxidase activity (Tarrand *et al.,* 1982).

2.2.3.5 Growth at Different Temperatures

Isolates were plated on BAM agar (Appendix B) and were then incubated at 37^0C, 45^0C, 65^0C, and 70^0C. Colony formation was checked periodically up to 4 days.

2.2.3.6 Growth at Different pH Ranges

The ability of the isolates to grow at pH 2, pH 3, pH 5, pH 6 was tested in TYG agar (Appendix C) plates. pH was adjusted with 10 N H_2SO_4. The growth was checked periodically upto 4 days.

2.2.3.7 Growth at Different NaCl Concentrations

The ability of the isolates to grow at %1, %2, %3, %4, %5, %7, and %9 NaCl was tested in BAM agar plates. The growth of bacteria was checked periodically at 50^0C up to 4 days.

2.2.4 Screening for Extracellular Enzymes

2.2.4.1 Screening for Lipase Activity

The media described in Appendix B were used in lipase screening. After inoculation of the isotales, the plates were incubated for 3-4 days at 50^0C. Opaque halos around the colonies were taken as the indication of lipase activity (Haba *et al.,* 2000).

2.2.4.2 Screening for Pectinase Activity

The isolates were inoculated in medium described in Appendix B. After 3-4 days incubation at 50^0C, 1% (w/v) cetyltrimethylammoniumbromide (CTAB) solution was poured onto the colonies. After 10 min incubation at room temparature, colonies with clear zones were taken as pectinase producers (Kobayashi *et al.,*1999).

2.2.4.3 Screening for Amylase Activity

Media used for amylase screening were listed in Appendix B. After inoculation of isolates, the plates were incubated for 3-4 days at 50^0C. Iodine solution (I_2= 1g, KI=2g /300ml) was poured on the plates. Clear zones around the colonies indicated the presence of amylase activity (Bragger *et al*., 1989).

2.2.4.4 Screening for Xylanase Activity

The medium described in Appendix B was used for xylanase screening. After inoculation and incubation for 3-4 days at 50^0C, 1% Congo red solution was poured onto the plates. The plates were incubated for 30 min at room temparature then they 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).

2.2.4.5 Screening for Cellulase Activity

Cellulase screening was similar to that of xylanase, except carboxymethylcellulose was used as substrate (Appendix B).

2.2.5 Preparation of Genomic DNA

The method described by Ausubel *et al*. (1994) was slightly modified and used for genomic DNA isolation. Fourty-eight hour cultures grown on BAM agar were scraped and suspended in 1.5 ml 1xTE buffer. Cells were pelleted by centrifugation for 5 min at 8000 rpm. Supernatant was discarded and pellet was resuspended in 567 µl of 1xTE buffer. Afterwards 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added. The eppendorf tubes were mixed throughly and the samples were incubated for 1h at 37^0C. Then 100 µl of CTAB/NaCl solution (10 cetyltrimethylammoniumbromide, 0.7 M NaCl) were added, mixed throughly and the samples were then incubated for 10 min at 65^0C. Chloroform extraction was performed twice using one equal volume of chloroform/isoamyl alcohol (24:1). First 1 equal volume of chloroform/isoamyl alcohol was added and the samples were centrifuged for 5 min at 10,000 rpm. The aqueous phase was transferred into a new eppendorf tube and chloroform extraction was repeated. The aqueous phase was transferred into a clean eppendorf tube. DNA wool was obtained by the addition of 0.6 volume isopropanol. The DNA wool was transferred into a new eppendorf tube containing 500 µl ethanol (70%) and washed. When DNA was not visible after the isopropanol addition, these samples were centrifuged for 10 min at 10,000 rpm to pellet genomic DNA. After discarding the isopropanol, genomic DNA was washed with 500 µl 70% ethanol. DNA was pelleted, dried (10 min at 37^0C) and dissolved in 200 µl 1xTE using alternating heat/cold shocks (10 min at 80^0C, 20 min at -20^0C twice). Afterwards phenol/chloroform extraction was performed in order to purify DNA. One and a half volume of phenol was added and mixed slowly. After that 1.5 volumes of chloroform/isoamyl alcohol were added, mixed and centrifuged for 2 min at 8,000 rpm. The aqueous phase was transferred into a new eppendorf tube and 300 µl chloroform/isoamyl alcohol were added and mixed. It was centrifuged for 2 min at 8,000 rpm and upper phase was transferred into a new tube. DNA was precipitated by adding 1/10 sample volume of 5 M NaCI. The sample was mixed well. Two volumes of 99% ethanol was then added and mixed throughly. The samples were then centrifuged for 15 min at 8,000 rpm. The liquid phase was removed and the pellet was washed with 300 µl 70% ethanol. After centrifugation for 5 min at 8,000 rpm, ethanol was removed without disturbing the pellets. The samples were centrifuged for 20 s at 8,000 rpm. Excess ethanol was removed and the pellets were dried for 10 min at 37^0C. Finally according to the pellet size, appropriate amount of 1xTE (50 µl, 100 µl, 150 µl, 200 µl)

was added and DNA was dissolved by alternating cold-heat shock (for 10 min at 80^0C and 20 min at -20^0C). Dissolved genomic DNA samples were stored at -20^0C.

2.2.6 Genotypic Characterization

2.2.6.1 Identification of Isolates by 16S–ITS rDNA RFLP

2.2.6.1.1 Amplification of 16S–ITS rDNA Region

PCR was performed for the amplification of 16S–ITS rDNA region of the isolates and reference strains. The reaction was performed in a final volume of 50 µl, containing 2 µl of genomic DNA as template and 48 µl of PCR mix. PCR mix was prepared as described in Appendix C. First, 2 µl of genomic DNA was transferred into 0.5 ml PCR tubes PCR sample was mixed gently and centrifuged for 2-3 s. After being prepared as described in Appendix C, 48 µl of the PCR mix was then distributed into each tubes. Then the tubes were overlaid with 60 µl mineral oil. They were centrifuged for 5 s at 6,000 rpm. All the steps were performed on ice. Finally the tubes were placed into the wells of the Mini Cycler PCR System. Amplification was performed using the following amplification conditions:

Step 1: 94^0C for 5 min (initial denaturation)
Step 2: 94^0C for 1 min (denaturation)
Step 3: 42^0C for 1 min (annealing)] 40 cycles
Step 4: 72^0C for 1 min (elongation)
Step 5: 72^0C for 10 min (final extention)

DNA oligoprimers (Appendix C) used in this experiment were:

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

Reverse Ege 1 : 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 upstream sequences of 23S rRNA gene sequences.

2.2.6.1.2 Electrophoresis of Amplified 16S-ITS rDNA Fragments

At the end of the PCR reaction, amplification products were checked by resolving the PCR products in 0.8% agarose gels. For this purpose 0.80 gr agarose were

dissolved in 100 ml of 1x TAE buffer by boiling. The agarose solution was cooled to 40 ^{0}C and 15 µl of ethidium bromide solution (10 mg/ml) was added. Then it was poured into the gel casting stand and combs were placed. After the gel was solidified the combs were removed. The casting tray carrying the gel was placed into the tank. One strenght TAE buffer was carrefully added until the buffer was 2-3 mm above the surface of the gel. Five µl of PCR products were taken and mixed with 1 µl of 6x gel 1oading buffer (Appendix C). The samples were loaded into the wells of agarose gel starting from the second well. Finally 3 µl of DNA molecular weight marker were loaded into the first well. Electrophoresis was performed for approximately 2.5 h at 60 mA. The PCR products were visualized on an UV illuminator (Vilber Lourmat, France). The images of gels were further analysed by a Bio ID ++ computer programme.

2.2.6.1.3 Chloroform Extraction of Amplified 16S-ITS rDNA Fragments

The phase below the mineral oil (PCR product) was removed gently using a micropipette and transferred into 1.5 ml eppendorf tubes. The volume of the PCR product was adjusted to 100 µl with 1xTE buffer. Two volumes of chloroform/ isoamyl alcohol solution (chloroform /isoamy alcohol: 24 /1) were added and mixed throughly. They were centrifuged for 2 min at 8,000 rpm. The lower phase was discarded with a micropipette and the rest was mixed again with two volumes of chloroform/isoamyl alcohol solution. They were centrifuged for 5 min at 8,000 rpm. The aqueous phase (100 µl) was transferred into new eppendorf tubes containing 10 µl of 3M sodium acetate. The mixture was mixed well. Two and a half volume of ethanol was then added and mixed throughly. The samples were centrifuged for 25 min at 10,000 rpm. The liquid phase was removed and the pellet was washed with 200 µl 70% ethanol. After centrifugation for 25 min at 10,000 rpm, ethanol was removed without disturbing the pellets. The pellts were dried at room temparature and dissolved in 25 µl 1xTE.

2.2.6.1.4 Restriction Fragment Lenght Polymorphism (RFLP)

Five µl of the purified PCR products were electrophoresed in 1% agarose gel to estimate the concentration of DNA. *Taq* I was used as restriction endonuclease. Ten µl of the PCR products were digested with 5 units of each of the enzymes in a final volume of 50 µl. Ten µl of each PCR product was transferred into eppendorf tubes then 40 µl

restriction enzyme mixture (Appendix C) were added. Samples restricted with *Taq* I were overlaid with mineral oil to prevent evaporation at 65^0C. The reaction was incubated overnight. After the digestion, the restriction products were purified as described in section 2.2.6.1.3 and dissolved in 15 µl 1xTE.

2.2.6.1.5 Electrophoresis of Restriction Fragments

For the seperation of restriction fragments, 2.5% agarose gels were prepared. Ten µl of restriction fragment were mixed with 2 µl of 6x gel loading buffer. First the samples and then 3 µl of DNA molecular weight marker were loaded. Electrophoresis was carried out for 30 min at 60 mA then the current was adjusted to 80 mA and the electrophoresis was continiued further for 2.5 h. At the end of the electrophoresis, the gel was removed and DNA was visualised on a UV illuminator. The image of the gel was analysed in a gel documentation system (Vilber Lourmat, France).

2.2.6.2 Identification of Isolates by DNA Sequencing

2.2.6.2.1 Amplification of 16S rDNA Region

16S rRNA gene was amplified using the following DNA oligoprimers:

EGE 1 5'-AGAGTTGATCCTGGCTCAG-3' (Jensen *et al*.,1993)

EGE 2 5'-CTACGGCTACCTGTTACGA-3' (Jensen *et al*.,1993)

Two µl of genomic DNA (approx. 500 µg) were transferred into 0.5 ml eppendorf tubes, as the template. PCR mix was prepared and after the addition of *Taq* DNA polymerase (1.25 U), it was mixed gently and centrifuged for 3 s. Forty-eight µl of the mix was distributed into the tubes. All the manipulations were performed on ice. The tubes were then overlaid with 60 µl mineral oil, centrifuged for 3 s at 6,000 rpm. 16S rRNA gene was amplified using the following programme:

Step 1: $94C^0$ for 5 min (initial denaturation)

Step 2: 94^0C for 1 min (denaturation) ⎫

Step 3: 42^0C for 1 min (annealing) ⎬ 40 cycles

Step 4: 72^0C for 1 min (elongation) ⎭

Step 5: 72^0C for 10 min (final extention)

2.2.6.2.2 Separation of Amplification Products by Agarose Gel Electrophoresis

Amplified fragments were separated in 0.8% agarose gel. First, 0.80 gr of agarose were dissolved in 100 ml of 1×TAE buffer by boiling and then 15µl of ethidium bromide solution (10 mg/ml) was added after cooling the solution to 40^0C. 1×TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. All the PCR products were mixed with 6× gel loading buffer. The samples were then loaded into the wells of agarose gel starting from the second well. Finally, 5 µl of DNA molecular weight marker was loaded into the first well and they were electrophored at 40 mA constant voltage until the blue dye migrated 2/3 of the gel lenght.

2.2.6.2.3 Extraction of DNA Fragments from Agarose Gel

DNA isolation kit (Applichem A3421) was used for extraction of amplification products from agarose gel slices. DNA bands (approximately 1,600 bp) were excised from the agarose gel by a rasor blade. Three gel slice volume of 6M NaI (for TAE gels) was added. The final concentration of NaI should be at least 4M. The samples were incubated for 5 min at 55^0C, mixed and further incubated for 2 min. Ten µl of well mixed glass powder suspension was then added, mixed well and incubated for 5 min at 55^0C by occasional mixing. The samples were then centrifuged for 10 s at 10,000 rpm and the supernatant was discharged. The glass pellet was rinsed with 500 µl wash buffer. The glass pellet was then resusupended by pipetting or gently flicking the tube. The samples were then centrifuged for 10 s at 10,000 rpm. The washing step was repeated twice. After the last wash, the supernatant was removed completely. The pellet was suspended in 25 µl of 1×TE buffer. The samples were eluted for 5 min at 55^0C with occasional mixing. Centrifugation was performed for 45 s at 10,000 rpm. The supernatant was finally transferred carefully into a new eppendorf tube. The eluted DNA samples were stored at -20^0C until use.

2.2.6.2.4 Sequencing Reactions

The sequencing reactions were performed by using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit. Four eppendorf tubes were first labeled as “A”,

"C", "G", and "T" for respective termination reactions. Two µl of corresponding Cy5.5 ddNTP termination mix was then dispensed into each of the labelled tubes. The tubes were then capped to prevent evaporation. A master mix was prepared in an eppendorf tube for each template to be sequenced. The contents of master mix were mixed thoroughly and 7 µl of the mix were dispensed into each labelled tube. The samples were mixed throughly and overlaid with 10 µl mineral oil. The tubes were capped and placed into the mini cycler system. The amplification conditions were:

Step1: 95^0C for 1 min (initial denaturation)

Step2: 94^0C for 30 s (denaturation)

Step3: 56^0C for 30 s (annealing)

Step4: 72^0C for 3 min (elongation)

40 amplification cycles for steps 2, 3 and 4 were performed.

The primer used was EGE 1 5'-AGAGTTTGATCCTGGCTCAG-3' (Jensen *et al.,* 1993).

2.2.6.2.5 Purification of Sequencing Reactions

The volume of each of the sequencing reaction sample was adjusted to 50 µl by adding 1×TE and were then transferred into fresh eppendorf tubes. Twenty µl of 7.5 M ammonium acetate and 200 µl (3 times the reaction volume) of chilled 100% ethanol were added into each tube. The tubes were then mixed thoroughly by vortexing and placed on ice for 20 min to precipitate the DNA. Centrifugation was then performed at 12,000 rpm for 20-30 min at 4^0C. Each supernatant was removed carefully and the pellets were washed with 200 µl of chilled 70% ethanol. After the centrifugation for 5 min, the supernatants were removed and the pellets were dried. Each pellet was then resuspended in 6 µl of formamide loading dye and vortexed vigorously (2 min) to ensure complete resuspension. Prior to electrophoresis each sample was heated at 70^0C for 3 min for denaturation and then placed on ice. Two µl of each sample were then loaded into lanes of the sequencing gel.

2.2.6.2.6 Sequencing

The sequencing reactions were performed in a SEQ4×4 Personal Sequencing System (Amersham Pharmacia Biotech). The system consists of SEQ4×4 personal

sequencer, SEQ4×4 Rapidset gel polymerizer, RapidGel QuickFill cassettes, RapidGel-XL-6% cartridge, filling gun, and filling fixture.

Preparing a gel involves attaching the RapidGel-XL cartridge to the casting gun, filling the RapidGel cassettes, polymerizing the acrylamide in the gel polymerizer. It is important that the RapidGel-XL cartridge should be taken from 4^0C at least 30 min before it was used. After the polymerization the comb was removed from the cassette. After cleaning the cassette, it was placed properly into the buffer chamber. At this step pre-run was performed to measure the background reading. Once the gel cassette was aligned and the pre–run was complete, 1×TE buffer was loaded into the upper and lower buffer chambers until the fill lines. The denatured samples (2 µl) were then loaded to the wells with a micropipette. Then the analysis was started. After the run finished, data were analysed by SEQ4×4 software.

The sequences obtained were then evaulated in GenBank using BLAST programme and accession numbers were obtained.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation of the environmental strains of thermoacidophilic *Bacillus*

Isolation was carried out using samples of soil contaminated with alpechin, olive husk and olive, on BAM (*B. acidocaldarius* medium) and YSG (yeast extract, soluble starch, glucose) agar plates. Both dilution plate and enrichment method were used as described in section 2.2.1. Single colonies were purified by repeated streaking. In total 86 isolates were obtained.

Table 3.1 Samples, number of isolates, isolate names.

Sample	Number of Isolates	Isolate name
Soil contaminated with alpechin	34	A-1, A-2, A-3, A-4, A-5, A-6, A-7, A-8, A-9, A-10, A-21, A-24, A-30, A-36a, A-36b, A-42, A-44, A-51, A-53, A-56, A-71a, A-71b A-76 , A-80, A-89, A-100, A-101, A-102, A-150, A-241
Olive husk	20	H-1, H-2, H-3, H-4, H-5, H-7, H-8, H-12, H-16a, H-16b, H-17, H-19, H-2O, H-21, H-22, H-28, H-29, H-33, H-50, H-120
Olive	32	S-1, S-4, S-6, S-7, S-8, S-10, S-17, S-19, S-22, S-23, S-24, S-25, S-26, S-27, S-28, S-32, S-35, S-36, S-37, S-40, S-42, S-43, S-45, S-50, S-51, S-52, S-55, S-56, S-57, S-62, S-63, S-64

3.2 Determination of Phenotypic Characteristics

3.2.1 Gram Straining

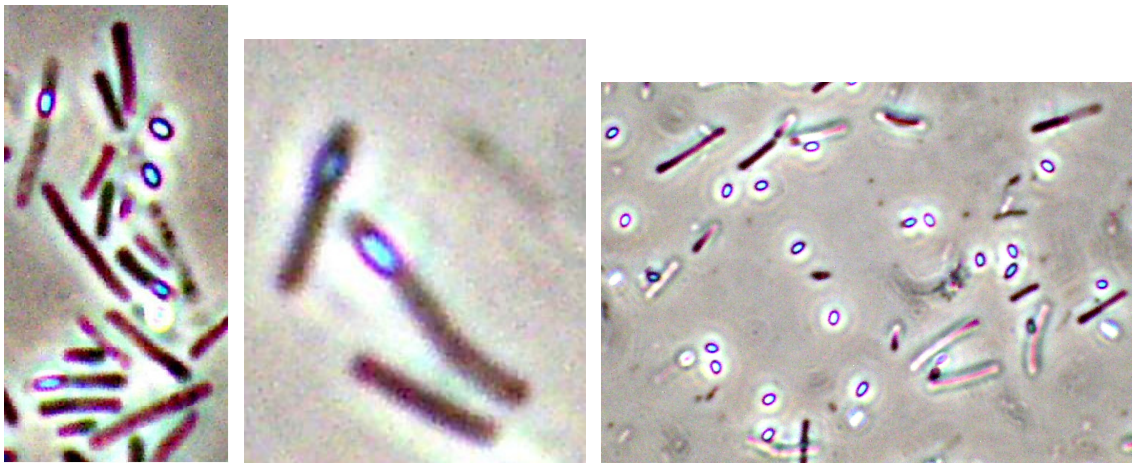
Isolates and reference strains were Gram stained. They were all observed as purple cells under the light microscope. Thus, they were accepted to be Gram positive. Some of the Gram(+) cells were shown in Figure 3.1.

3.2.2 Examination of Endospores

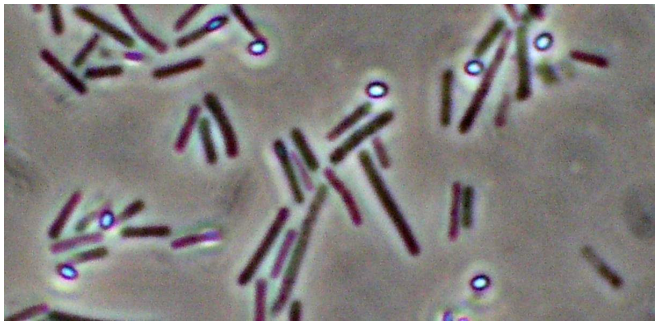
Isolates and reference strains were examined for the presence of endospores under the phase constract microscope. All the isolates were found to produce endospores. Appearence of spores of some isolates and reference strains under phase contrast microscope were shown in Figure 3.2.

3.2.3 Catalase and Oxidase Tests

All the isolates and reference strains (except *A. acidocaldarius* DSM 446) were found to be catalase positive. Oxidase presence was observed in isolates A-97, A-100, A-101, A-150, A-241, H-3, H-4, A-36a, A-56, A-80, A-89, and *A. pomorum* 3A.



A B C



D

Figure 3.1. Appearance of some isolates under phase contrast microscope. A: H 22, B: A-90, C: A-102, D: S-7

A B C

Figure 3.2. Appearance of Gram (+) cells under light microscope. A: A-6, B: H-12, C: H-120.

Table 3.2. Growth at Different Temperatures

Strain name	Growth at 37^0C	Growth at 45^0C	Growth at 65^0C	Growth at 70^0C
Alicyclobacillus acidocaldarius DSM 446	-	+	+	+
Alicyclobacillus acidoterrestris DSM 2498	+	+	+	+
AGS 2 MIH332	+	+	+	+
Alicyclobacillus pomorum 3A	+	+	-	-
Alicyclobacillus herbarius CP1	+	+	+	-
MB 1359	-	+	+	+
A-1	-	+	+	+
A-2	+	+	+	-
A-3	-	+	+	+
A-4	-	+	+	+
A-5	+	+	+	-
A-6	+	+	w.	w.
A-7	+	+	w.	w.
A-8	+	+	+	-
A-9	-	+	+	+
A-10	+	+	+	-
A-21	-	+	+	+
A-24	+	+	+	-
A-30	+	+	w.	w.
A-36a	+	+	+	-
A-36b	+	+	w.	w.
A-42	+	+	+	-
A-44	+	+	w.	w.
A-51	+	+	w.	w.
A-53	+	+	w.	w.
A-56	+	+	w.	w.
A-71a	+	+	+	-
A-71b	-	+	+	+
A-76	+	+	+	-
A-80	+	+	w.	w.
A-89	-	+	+	-
A-92	-	+	+	+
A-94	-	+	+	+
A-96	+	+	+	-
A-97	-	+	+	+
A-100	-	+	+	+
A-101	+	+	+	-
A-102	+	+	w.	w.

Table 3.2. (cont.)

Strain name	Growth at 37^0C	Growth at 45^0C	Growth at 65^0C	Growth at 70^0C
A-150	+	+	w.	w.
A-241	-	+	+	+
H-1	-	+	+	-
H-2	+	+	w.	w.
H-3	-	+	+	-
H-4	-	+	+	-
H-5	+	+	w.	w.
H-7	-	+	+	+
H-8	+	+	w.	w.
H-12	+	+	w.	w.
H-16a	+	+	w.	w.
H-16b	-	+	+	+
H-17	+	+	+	-
H-19	+	+	+	-
H-20	+	+	w.	w.
H-21	+	+	w.	w.
H-22	+	+	+	-
H-28	-	+	+	+
H-29	-	+	+	+
H-33	-	+	+	+
H-50	-	+	+	+
H-120	-	+	+	+
S-1	+	+	+	+
S-4	+	+	+	+
S-6	+	+	+	w.
S-7	+	+	+	w.
S-8	+	+	+	+
S-10	+	+	+	+
S-17	+	+	+	+
S-19	+	+	+	+
S-22	+	+	+	+
S-23	+	+	+	+
S-24	+	+	+	+
S-25	+	+	+	+
S-26	+	+	+	+
S-27	+	+	+	+
S-28	+	+	+	+
S-32	+	+	+	+
S-35	+	+	+	+
S-36	+	+	+	+
S-37	+	+	+	+
S-40	+	+	+	+
S-42	+	+	+	+

Table 3.2. (cont.)

Strain name	Growth at 37^0C	Growth at 45^0C	Growth at 65^0C	Growth at 70^0C
S-43	+	+	+	+
S-45	+	+	+	+
S-50	+	+	+	+
S-51	+	+	+	+
S-52	+	+	+	+
S-55	+	+	+	+
S-56	+	+	+	+
S-57	+	+	+	+
S-62	+	+	+	+
S-63	+	+	+	+
S-64	+	+	+	+

w: weak growth

Table 3.3. Differentiation of the isolates according to growth temperatures

Isolates did not grow at 37^0C	A-1, A-3,A-4,A-9, A-21, A-71b, A-89, A-92, A-94, A-97, A-100, A-241,H-1,H-3,H-4 H-7, H-16b, H-28, H-29, H-33, H-50, H-120
Isolates grew weakly at 65^0C	A-6, A-7, A-30, A-36b, A-44, A-51, A-53, A-56, A-80, A-102, A-150, H-2,H-5,H-8,H-12, H-16a, H-20, H-21
Isolates grew at 37^0C, 45^0C, 65^0C, 70^0C	S-1, S-4, S-8, S-10, S-17, S-19, S-22, S-23, S-24, S-25, S-26, S-27, S-28, S-32, S-35, S-36, S-37, S-40, S-42, S-43, S-45, S-50, S-51, S-52, S-55, S-56, S-57, S-62, S-63, S-64
Isolates did not grow at 70^0C	A-2,A-5, A-8, A-10, A-24, A-36a, A-42, A-71a, A-76, A-96, A-101, H-17, H-19, H-22
Isolates grew at 65^0C and grew weakly at 70ºC	S-6, S-7

Table 3.4. Growth at Different pH-Ranges

Strain name	Growth at pH 2	Growth at pH 3	Growth at pH 5	Growth at pH 6	Growth at pH 7
Alicyclobacillus acidocaldarius DSM 446	+	+	+	+	-
Alicyclobacillus acidoterrestris DSM 2498	w.	+	+	+	-
AGS 2 MIH 332	+	+	+	+	-
Alicyclobacillus pomorum 3A	-	+	+	+	-
Alicyclobacillus herbarius CP1	-	-	+	+	-
MB 1359	+	+	+	+	-
A-1	-	+	+	+	-
A-2	+	+	+	+	-
A-3	+	+	+	+	-
A-4	+	+	+	-	-
A-5	+	+	+	+	-
A-6	-	+	+	-	-
A-7	+	+	+	+	-
A-8	-	+	+	+	-
A-9	+	+	+	-	-
A-10	+	+	+	+	-
A-21	-	+	+	+	-
A-24	+	+	+	+	-
A-30	+	+	+	-	-
A-36a	-	+	+	+	-
A-36b	+	+	+	-	-
A-42	+	+	+	+	-
A-44	+	+	+	-	-
A-51	+	+	+	-	-
A-53	+	+	+	-	-
A-56	+	+	+	-	-
A-71a	-	+	+	+	-
A-71b	-	+	+	+	-
A-76	+	+	+	+	-
A-80	+	+	+	-	-
A-89	-	+	+	+	-
A-92	-	+	+	+	-
A-94	-	+	+	+	-
A-96	+	+	+	+	-

Table 3.4. (cont.)

Strain name	Growth at pH 2	Growth at pH 3	Growth at pH 5	Growth at pH 6	Growth at pH 7
A-97	-	+	+	+	-
A-100	-	+	+	+	-
A-101	+	+	+	+	-
A-102	-	+	+	-	-
A-150	+	+	+	-	-
A-241	-	+	+	+	-
H-1	+	+	+	-	-
H-2	+	+	+	+	-
H-3	+	+	+	-	-
H-4	+	+	+	-	-
H-5	+	+	+	+	-
H-7	-	+	+	+	-
H-8	+	+	+	+	-
H-12	+	+	+	-	-
H-16a	+	+	+	-	-
H-16b	-	+	+	+	-
H-17	+	+	+	+	-
H-19	+	+	+	+	-
H-20	+	+	+	-	-
H-21	+	+	+	-	-
H-22	+	+	+	+	-
H-28	-	+	+	+	-
H-29	-	+	+	+	-
H-33	-	+	+	+	-
H-50	-	+	+	+	-
H-120	-	+	+	+	-
S-1	-	w.	+	+	+
S-4	-	w.	+	+	+
S-6	-	-	+	+	+
S-7	-	-	+	+	+
S-8	-	w.	+	+	+
S-10	-	w.	+	+	+
S-17	-	w.	+	+	+
S-19	-	w.	+	+	+
S-22	-	w.	+	+	+
S-23	-	w.	+	+	+
S-24	-	w.	+	+	+
S-25	-	-	+	+	+
S-26	-	w.	+	+	+
S-27	-	w.	+	+	+
S-28	-	w.	+	+	+
S-32	-	w.	+	+	+

Table 3.4. (cont.)

Strain name	Growth at pH 2	Growth at pH 3	Growth at pH 5	Growth at pH 6	Growth at pH 7
S-35	-	w.	+	+	+
S-36	-	w.	+	+	+
S-37	-	w.	+	+	+
S-40	-	w.	+	+	+
S-42	-	w.	+	+	+
S-43	-	w.	+	+	+
S-45	-	w.	+	+	+
S-50	-	w.	+	+	+
S-51	-	w.	+	+	+
S-52	-	w.	+	+	+
S-55	-	w.	+	+	+
S-56	-	-	+	+	+
S-57	-	w.	+	+	+
S-62	-	-	+	+	+
S-63	-	-	+	+	+
S-64	-	-	+	+	+

Table 3.5. Differentiation of the isolates according to pH tolerance

Isolates grew at pH 2, pH 3, pH 5, and pH 6	A-2, A-3, A-5, A-10, A-24, A-36a, A-42, A-76, A-96, A-101, H-2, H-5, H-8, H-17, H-19, H-22
Isolates did not grow at pH 2	A-1,A-6, A-8, A-21, A-71b, A-92, A-94, A-97, A-100,A-102, A-241, A-36a, A-71a, A-89, H-7, H-16b, S-1, S-4, S-6, S-7, S-8, S-10, S-17, S-19, S-22, S-23, S-24, S-25, S-26, S-27, S-28, S-32, S-35, S-36, S-37, S-40, S-42, S-43, S-45, S-50, S-51, S-52, S-55, S-56, S-57, S-62, S-63, S-64
Isolates grew weakly at pH 3	S-1, S-4, S-8, S-10, S-17, S-19, S-22, S-23, S-24, S-26, S-27, S-28, S-32, S-35, S-36, S-37, S-40, S-42, S-43, S-45, S-50, S-51, S-52, S-55, S-57
Isolates did not grow at pH 6	A-4, A-7, A-9, A-30, A-36b, A-44, A-51, A-53, A-56, A-80, A-150, H-1, H-3, H-4, H-12, H-16a, H-20, H-21
Isolates grew weakly at pH 2	H-28, H-29, H-33, H-50, H-120
Isolates did not grow at pH 3	S-6, S-7, S-25, S-56, S-62, S-63, S-64

Table 3.6. Growth at Different NaCl Concentrations

Strain name	1% NaCl	2%NaCl	3% NaCl	4% NaCl	5% NaCl	6% NaCl
Alicyclobacillus acidocaldarius DSM 446	+	-	-	-	-	-
Alicyclobacillus acidoterrestris DSM 2498	+	+	+	+	+	-
AGS 2 MIH332	+	-	-	-	-	-
Alicyclobacillus pomorum 3A	+	+	-	-	-	-
Alicyclobacillus herbarius CP1	+	+	+	+	+	-
MB 1359	+	-	-	-	-	-
A-1	+	+	+	-	-	-
A-2	+	+	+	+	+	-
A-3	+	+	+	+	+	-
A-4	+	+	+	-	-	-
A-5	+	+	+	w.	-	-
A-6	+	+	+	+	-	-
A-7	+	+	+	+	-	-
A-8	+	+	+	w.	-	-
A-9	+	+	+	-	-	-
A-10	+	+	+	+	+	-
A-21	+	+	+	-	-	-
A-24	+	+	+	+	+	-
A-30	+	+	+	+	-	-
A-36a	+	+	+	w.	-	-
A-36b	+	+	+	+	-	-
A-42	+	+	+	w.	-	-
A-44	+	+	+	-	-	-
A-51	+	+	+	-	-	-
A-53	+	+	+	-	-	-
A-56	+	+	+	-	-	-
A-71a	+	+	+	w.	-	-
A-71b	+	+	+	-	-	-
A-76	+	+	+	w.	-	-
A-80	+	+	+	+	-	-
A-89	+	+	+	-	-	-
A-92	+	+	+	w.	-	-
A-94	+	+	+	-	-	-

Table 3.6. (cont.)

Strain name	1% NaCl	2% NaCl	3% NaCl	4% NaCl	5% NaCl	6% NaCl
A-96	+	+	+	w.	-	-
A-97	+	+	+	-	-	-
A-100	+	+	+	-	-	-
A-101	+	+	+	w.	-	-
A-102	+	+	+	+	-	-
A-150	+	+	+	+	-	-
A-241	+	+	+	-	-	-
H-1	+	+	+	-	-	-
H-2	+	+	+	w.	-	-
H-3	+	+	+	-	-	-
H-4	+	+	+	-	-	-
H-5	+	+	+	w.	-	-
H-7	+	+	+	-	-	-
H-8	+	+	+	w.	-	-
H-12	+	+	+	+	-	-
H-16a	+	+	+	+	-	-
H-16b	+	+	+	-	-	-
H-17	+	+	+	w.	-	-
H-19	+	+	+	w.	-	-
H-20	+	+	+	+	-	-
H-21	+	+	+	+	-	-
H-22	+	+	+	w.	-	-
H-28	+	+	+	-	-	-
H-29	+	+	+	-	.-	-
H-33	+	+	+	-	-	-
H-50	+	+	+	-	-	-
H-120	+	+	+	-	-	-
S-1	+	+	+	+	+	+
S-4	+	+	+	+	+	+
S-6	+	+	+	w.	-	-
S-7	+	+	+	w.	-	-
S-8	+	+	+	+	+	+
S-10	+	+	+	+	+	+
S-17	+	+	+	+	+	-
S-19	+	+	+	+	+	-
S-22	+	+	+	+	+	+
S-23	+	+	+	+	+	+
S-24	+	+	+	+	+	+
S-25	+	+	+	w.	-	-

Table 3.6. (cont.)

Strain name	1% NaCl	2% NaCl	3% NaCl	4% NaCl	5% NaCl	6% NaCl
S-26	+	+	+	+	+	+
S-27	+	+	+	+	+	+
S-28	+	+	+	+	+	+
S-32	+	+	+	+	+	+
S-35	+	+	+	+	+	+
S-36	+	+	+	+	+	+
S-37	+	+	+	+	+	+
S-40	+	+	+	+	+	+
S-42	+	+	+	+	+	+
S-43	+	+	+	+	+	+
S-45	+	+	+	+	+	+
S-50	+	+	+	+	+	+
S-51	+	+	+	+	+	+
S-52	+	+	+	+	+	+
S-55	+	+	+	+	+	+
S-56	+	+	+	w.	-	-
S-57	+	+	+	+	+	+
S-62	+	+	+	w.	-	-
S-63	+	+	+	w.	-	-
S-64	+	+	+	w.	-	-

Table 3.7. Differentiation of the isolates according to growth at different NaCl concentrations

Isolates grew at 1%, 2%, 3%,4%, 5%, and 6% NaCl concentrations	S-1, S-4, S-8, S10, S-17, S-23, S-24, S-26, S-27, S-28, S-32, S-35, S-36, S-37, S-40, S-42, S-43, S-45, S-50, S-51, S-52, S-55, S-57
Isolates did not grow only at 6% NaCl concentration	A-2, A-3, A-10, A-24, S-17, S-19
Isolates did not grow at 4%, 5% and 6% NaCl concentrations	A-1, A-94, A-21, A-44, A-36b, A-51, A-56, A-53, A-71b, A-97, A-100, A-241, H-7, H-16b, H-28, H-29, H-33, H-50, H-120, A-89, H-1, H-3, H-4, A-4, A-9
Isolates grew at 1%, 2%, 3% and 4% NaCl concentrations	A-7, A-30,A-36b,A-150, H-12, H-16a, H-20, H-21, A-6, A-80
Isolates grew weakly at 4% NaCl concentration and did not grow at 5%, 6% NaCl concentrations	A-5, A-8, A-36a, A-42, A-71a, A-92, A-76, A-96, A-101, H-2, H-5, H-8, H-17, H-19, H-22, S-6, S-7, S-25, S-56, S-62, S-63, S-64

3.3 Extracellular Enzyme Profiles

Table 3.8 Extracellular Enzyme Profiles

Strain name	Lipase		Pectinase		Xylanase	Cellulase	Amylase
	Substrate T20	Substrate T80	Substrate PGA	Substrate Pectin			
Alicyclobacillus acidocaldarius DSM 446	-	-	-	-	-	-	+
Alicyclobacillus acidoterrestris DSM 2498	-	-	-	-	-	-	-
AGS 2 MIH 332	-	-	-	-	-	-	+
Alicyclobacillus pomorum 3A	-	-	-	-	-	-	-
Alicyclobacillus herbarius CP1	-	-	-	-	-	-	-
MB 1359	+	-	-	-	-	-	+
A-1	+	-	-	-	-	-	+
A-2	+	-	-	-	-	-	-
A-3	+	-	-	-	-	-	-
A-4	-	-	-	-	-	-	+
A-5	+	-	-	-	-	-	-
A-6	-	-	-	-	-	-	+
A-7	-	-	-	-	-	-	+
A-8	+	-	-	-	-	-	-
A-9	-	-	-	-	-	-	+
A-10	+	-	-	-	-	-	-
A-21	+	-	-	-	-	-	+
A-24	+	-	-	-	-	-	-
A-30	-	-	-	-	-	-	+
A-36a	+	-	-	-	-	-	-
A-36b	-	-	-	-	-	-	+
A-42	+	-	-	-	-	-	-
A-44	+	-	-	-	-	-	+
A-51	+	-	-	-	-	-	+
A-53	+	-	-	-	-	-	+
A-56	+	-	-	-	-	-	+
A-71a	+	-	-	-	-	-	-
A-71b	+	-	-	-	-	-	+
A-76	+	-	-	-	-	-	-
A-80	-	-	-	-	-	-	+
A-89	-	-	-	-	-	-	+

PGA:Polygalacturonic acid, T20:Tween 20 T80:Tween 80

Table 3.8. (cont.)

Strain name	Lipase		Pectinase		Xylanase	Cellulase	Amylase
	Substrate T20	Substrate T80	Substrate PGA	Substrate Pectin			
A-92	+	-	-	-	-	-	+
A-94	+	-	-	-	-	-	+
A-96	+	-	-	-	-	-	-
A-97	+	-	-	-	-	-	+
A-100	+	-	-	-	-	-	+
A-101	+	-	-	-	-	-	-
A-102	-	-	-	-	-	-	+
A-150	-	-	-	-	-	-	+
A-241	+	-	-	-	-	-	+
H-1	-	-	-	-	-	-	+
H-2	+	-	-	-	-	-	-
H-3	-	-	-	-	-	-	+
H-4	-	-	-	-	-	-	+
H-5	+	-	-	-	-	-	-
H-7	+	-	-	-	-	-	+
H-8	+	-	-	-	-	-	-
H-12	-	-	-	-	-	-	+
H-16a	-	-	-	-	-	-	+
H-16b	+	-	-	-	-	-	+
H-17	+	-	-	-	-	-	-
H-19	+	-	-	-	-	-	-
H-20	-	-	-	-	-	-	+
H-21	-	-	-	-	-	-	+
H-22	+	-	-	-	-	-	-
H-28	+	-	-	-	-	-	+
H-29	+	-	-	-	-	-	+
H-33	+	-	-	-	-	-	+
H-50	+	-	-	-	-	-	+
H-120	+	-	-	-	-	-	+
S-1	+	-	+	+	-	-	+
S-4	+	-	+	+	-	-	+
S-6	+	-	+	+	-	-	+
S-7	+	-	+	+	-	-	+
S-8	+	-	+	+	-	-	+
S-10	+	-	+	+	-	-	+
S-17	+	-	+	+	-	-	+
S-19	+	-	+	+	-	-	+
S-22	+	-	+	+	-	-	+
S-23	+	-	+	+	-	-	+
S-24	+	-	+	+	-	-	+
S-25	+	-	+	+	-	-	+

Table 3.8. (cont.)

Strain name	Lipase		Pectinase		Xylanase	Cellulase	Amylase
	Substrate T20	Substrate T80	Substrate PGA	Substrate Pectin			
S-26	+	-	+	+	-	-	+
S-27	+	-	+	+	-	-	+
S-28	+	-	+	+	-	-	+
S-32	+	-	+	+	-	-	+
S-35	+	-	+	+	-	-	+
S-36	+	-	+	+	-	-	+
S-37	+	-	+	+	-	-	+
S-40	+	-	+	+	-	-	+
S-42	+	-	+	+	-	-	+
S-43	+	-	+	+	-	-	+
S-45	+	-	+	+	-	-	+
S-50	+	-	+	+	-	-	+
S-51	+	-	+	+	-	-	+
S-52	+	-	+	+	-	-	+
S-55	+	-	+	+	-	-	+
S-56	+	-	+	+	-	-	+
S-57	+	-	+	+	-	-	+
S-62	+	-	+	+	-	-	+
S-63	+	-	+	+	-	-	+
S-64	+	-	+	+	-	-	+

The isolates and reference stains were tested for lipase, pecinase, amylase, xylanase and cellulase activity. Tween 80, Tween 20, polygalactronic acid, pectin, starch, xylan, and carboxymethylcellulose were used as substrates for detecting the activity of these enzymes.

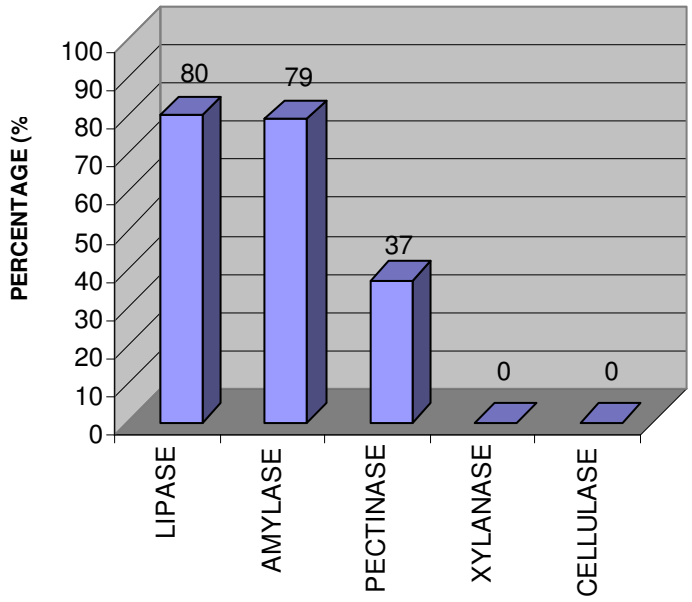


Figure 3.3. Percentages of extracellular enzyme activities observed

Eighty percent of the 86 isolates were able to produce lipase enzyme. None of the isolates were able to hydrolyze Tween 80. The hydrolysis of Tween 20 indicated the presence of lipase activity. Sixty-eight (79%) of the isolates were found to produce amylase enzyme. Pectinase activity was detected in 32 (37%) isolates (Figure 3.3). All pectinase producers were found to hydrolyze polygalacturonic acid and pectin. None of the isolates produced xylanase or cellulase enzyme. *A. acidocaldarius* DSM 446, AGS 2 MIH 332, and MB 1359 were able to produce amylase.

The isolates were further grouped according to the enzymes they produced and 5 enzyme groups were obtained (Table 3.9). The distribution of enzyme groups was shown in Figure (3.4).

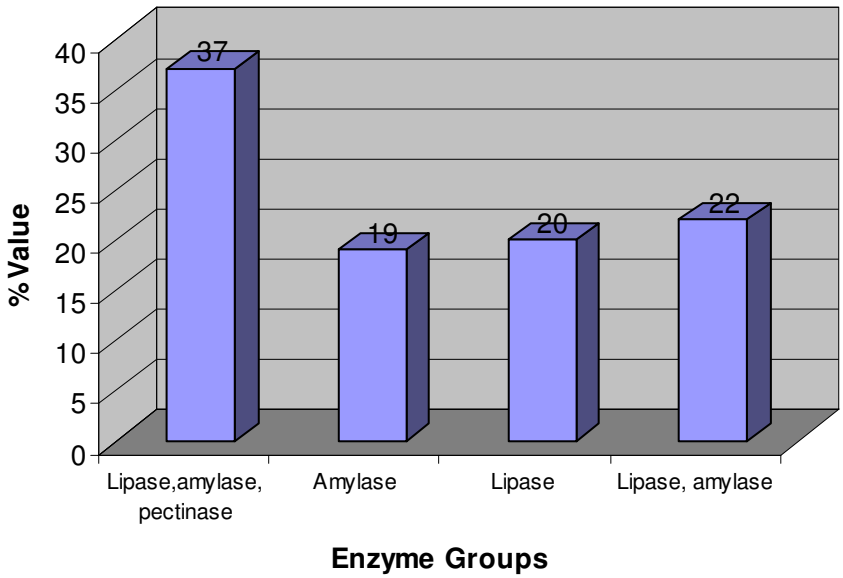


Figure 3.4. Percentages of the enzyme groups obtained

The first group included the isolates showing lipase, amylase and pectinase activity. Lipase, amylase and pectinase producing isolates accounted for 37% of all isolates. (Figure 3.4)

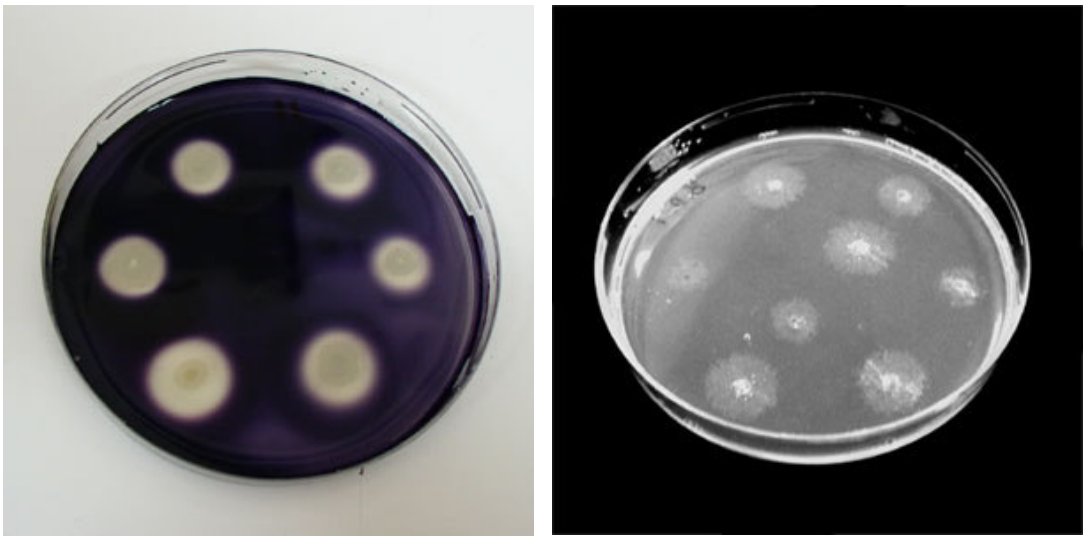
The members of the second group were found to produce amylase only. This group represented 19% of the isolates. (Figure 3.4)

Similarly the members of group 3 were able to produce lipase only. This group accounted for 20% of the isolates. (Figure 3.4)

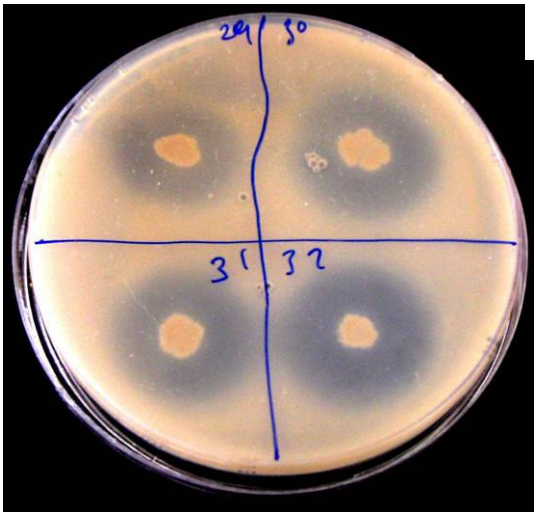
Lipase and amylase producing 19 isolates were grouped together. This group represented 22% of the isolates. (Figure 3.4)

Table 3.9. Enzyme groups, numbers and names of the isolates

Enzyme Groups	Number of Isolates	Name of Isolates
Lipase, pectinase, amylase	32	S-1, S-4, S-7, S-8, S-10, S-17, S-19, S-22, S-23, S-24, S-26, S-27, S-28, S-32, S-35, S-36, S-37, S-40, S-42, S-43, S-45, S-50, S-51, S-52, S-55, S-57, S-6 , S-25, S-56, S-62, S-63, S-64
Amylase	17	A-4, A-6, A-7, A-9, A-30, A-36b, A-80, A-102, A-150, H-1, H-3, H-4, H-12, H-16a, H-20, H-21, A-89
Lipase	18	A-2, A-3, A-5, A-24, A-36a, A-42, A-71a, A-76, A-96 A-96, A-101, H-2, H-5, H-8, H-17, H-19, H-22 ,A-8 ,A-10
Lipase,amylase	19	A-1, A-21, A-71b, A-92, A-94, A-97, A-100, A-241, H-7, H-16b, A-44, A-51, A-53, A-56 ,H-28 ,H-29, H-33 ,H-50 ,H-120
	86	



A B



C

Figure 3.5. Detection of extracellular enzyme activities; A: Amylase, B: Lipase, C: Pectinase

3.4 Genotypic Characterization

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

Amplification of 16S-ITS region mostly yielded one distinct DNA fragment of approximately 2000 bp in length. (Figure 3.6)

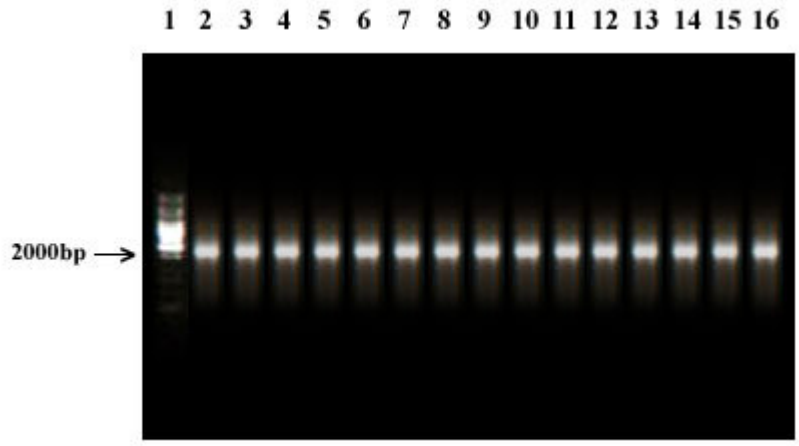


Figure 3.6. Amplification products of 16S-ITS rDNA region from reference strains and a set of isolated strains

Lanes: 1. 1kb DNA ladder; 2. Alicyclobacillus acidocaldarius DSM 446; 3. Alicyclobacillus acidoterrestris DSM 2498; 4. Alicyclobacillus genomic species 2 MIH 332; 5. Alicyclobacillus pomorum 3A; 6. Alicyclobacillus herbarius CP1; 7. MB 1359; 8. Isolate A-10; 9. Isolate A-42; 10. Isolate A-53; 11. Isolate A-89; 12. Isolate H-3; 13. Isolate H-22; 14.Isolate S-1; 15. Isolate S-4

3.4.2 16S-ITS rDNA RFLP Profiles of Isolates and Reference Strains

For the molecular identification of 86 isolated strains 16S-ITS rDNA RFLP profiles were optained with *Taq* I restriction endonuclease. Fist all the digestion products were run in 2% agarose gel to see all different profiles and to group the isolates yielding similar restriction profiles. One digestion product representing each of these groups was then selected and electrophoresed in 2.5% agarose gel with those of 6 reference strains, along with a DNA size marker. (Figure 3.7)

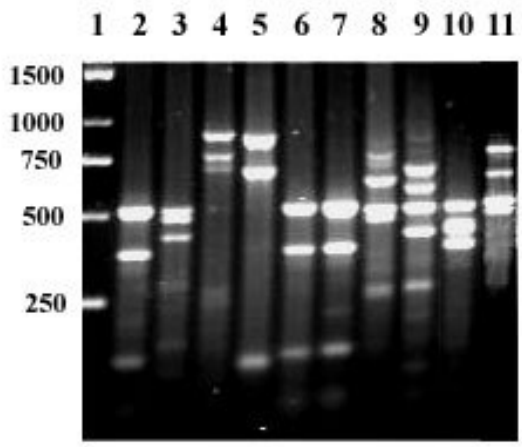


Figure 3.7. 16S-ITS rDNA RFLP profiles of *Taq* I digested isolates and reference strains. Lanes: 1. 1kb DNA ladder; 2. *Alicyclobacillus acidocaldarius* DSM 446; 3. *Alicyclobacillus pomorum* 3A; 4. *Alicyclobacillus herbarius* CP1; 5. *Alicyclobacillus acidoterrestris* DSM 2498; 6. MB 1359; 7. Isolates H33 (G-2); 8. Isolate H22 (G-3); 9. Isolate S1 (G-1); 10. Isolate A89 (G-4)

The image of the gel was further analyzed by using Bio RD++ computer programme. The dendrogram of the representative isolates and the reference strains were shown in Figure (3.7). Fragment sizes obtained were given in Table (3.10). *Taq* I restriction analysis of 16S-ITS rDNA fragments of strains and 6 reference strains yielded 10 distinct genotypic groups.

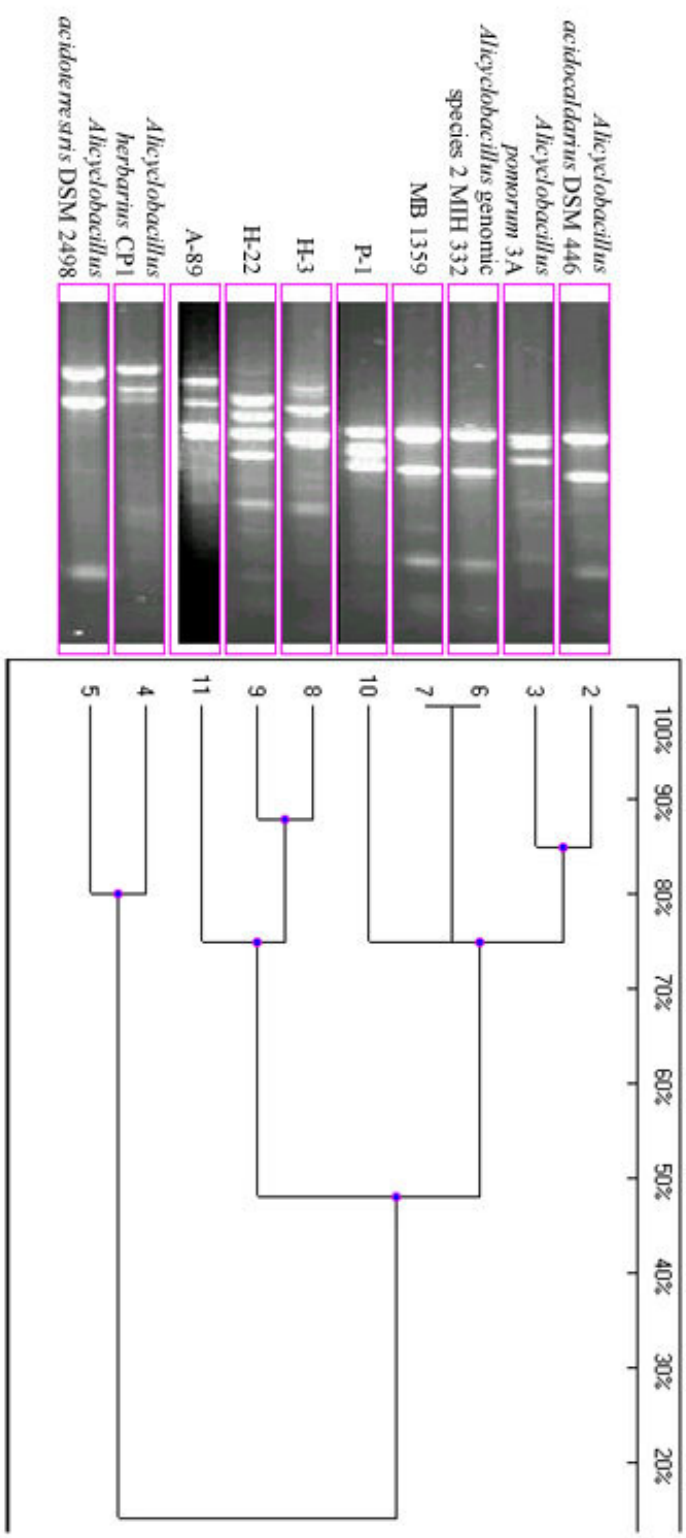


Figure 3.8. *Taq* I-RFLP Dendrogram of the representative isolates and the reference strains

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

Strains	Fragment sizes (bp)
Alicyclobacillus acidocaldarius DSM 446	81, 382, 500, 525
Alicyclobacillus pomorum 3A	431, 492, 525
Alicyclobacillus herbarius CP1	764, 901
Alicyclobacillus acidoterrestris DSM 2498	88, 698, 885
Alicyclobacillus genomic species 2 MIH 332	108, 396, 516, 542
MB 1359	122, 410, 508, 533
Isolate H-33	287, 500, 533, 651
Isolate H-22	299, 453, 533, 598, 686
Isolate S-1	417, 453, 484, 533
Isolate A-89	533, 560, 686, 823

Table 3.11. Groups obtained by *Taq* I digestion of 16S-ITS ribosomal DNA region

Group no	Name of isolates
G 1	S-1, S-4, S-6, S-7, S-8, S-10, S-17, S-19, S-22, S-23, S-24, S-25, S-26, S-27, S-28, S-32, S-35, S-36, S-37, S-40, S-42, S-43, S-45, S-50, S-51, S-52, S-55, S-56, S-57, S-62, S-63, S-64
G 2	A-1, A-21, A-71b, A-92, A-94, A-97, A-100, A-241, H-7, H-16b, H-28,H-29, H-33, H-50, H-120, A-44, A-51, A-53, A-56, A-2, A-10, A-24
G 3	H-22, A-3, A-5, A-8, A-36a, A-42, A-71a, A-76, A-96, A-101, H-2, H-5, H-8, H-17, H-19
G 4	A-4, A-6, A-7, A-9, A-30, A-36b, A-80, A-89, A-102, A-150, H-1, H-3, H-4, H-12, H-16a, H-20, H-21

3.4.3 Analysis of the Genotypic and Phenotypic Groups

Strains included in G-1 showed the same extracellular enzyme profiles (lipase, pectinase and amylase). Moreover their physiological properties were also similar. Seven strains (S-6, S-7, S-25, S-56, S-62, S-63 and S-64) differed from the others in respect of pH and NaCl tolerance. These strains were not able to grow at pH 3, and 5% or 6% NaCl concentrations. And also they could grow weakly at 4% NaCl concentration. Strains S-6 and S-7 were found to grow weakly at 70^0C althought the others showed normal growth at this temperature. Strains of G-2 (except A-2, A-10, A-24) were in the same enzyme group. They also showed the same physiological properties (no growth at 37^0C, at 4%, 5%, 6% NaCl concentrations, at pH 2 and pH 7).

All the strains of G-3 (except A-3, A-71a, A-8, H-2, H-5, H-8) were able to hydrolyze Tween 20 and had the same physiological characteristics suh as no growth at 70^0C or at 5% and 6% NaCI concentrations or at only pH 7. Thus these strains thougth to be similar. Strains A-71a and A-8 had the same physiological features except their growth temperature range. Thus these two strains might be the members of the same species. And also H-2, H-5 and H-8 showed the same physiological features (growth at 37^0C, 45^0C, 65^0C, 70^0C, no growth at 5% and 6% NaCl concentrations, at pH 7). Hence H-2, H-5 and H-8 were also thougth to be the strains of the same species.

All the strains in G-4 were found to produce amylase. The strains were classified into two main groups in respect of their physiological features. The first group included strains A-89, H-1, H-3, H-4, A-4, A-9. The strains (except A-89) belonging to the first group exhibited the same physiological properties such as no growth at 37^0C , at 4%, 5 % and 6% NaCl concentrations at pH 6 and pH 7. It was concluded that the isolates in the first group were the members of same species. A-89 was thougth to be a strain of these species.

The members of the second group showed the same physiological properties. They were found to grow at 37^0C, 45^0C, 65^0C and 70^0C. They were not able to grow at 5% and 6% NaCl concentrations. Except A-6, A-7 and A-102 all the members could not grow only at pH 6 and pH 7. Therefore the members of the second group were also thought to be the members of the same species, except A-6, A-7 and A-102. The latter three might be the strains of the species in the second group.

3.4.4 Partial Sequence Analysis of 16S rRNA gene

Sequencing results were obtained in a SEQ4×4 personal sequencing system (Amersham Pharmacia Biotech). The results were submitted to GenBank; so far the following accession numbers were obtained: AY601903 (isolate H-22 of G-3, 679 nucleotides), AY606276 (isolate S-1 of G-1, 330 nucleotides).

CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, 86 thermoacidophilic strains were isolated from olive, olive husk and soil contaminated with alpechin collected within different olive oil mills in Ayvalık. The strains were screened for the presence of 5 extracellular enzyme activities. These were; lipases, pectinases, amylases, xylanases and cellulases. For enzyme screening Tween 20, Tween 80, polygalacturonic acid, pectin, starch, xylan and carboxymethylcellulose were used as substrates. In total 69 lipase (Tween 20 as substrate), 32 pectinase and 68 amylase activities were detected. All of the isolates were Gram(+), endospore forming rods, thus they were identified as *Bacillus* sp.

Taq I was used for 16S-ITS rDNA RFLP. The isolated strains were clustered into four groups by *Taq* I restriction profiles of 16S-ITS rDNA.

One representative isolate among the members of each of the 16S-ITS RFLP homology groups was chosen and used for 16S rRNA gene partial sequence analysis. Sequencing results were submitted to GenBank. So far the indicated accession numbers were obtained: AY601903 (isolate H-22 of G-3, 679 nucleotids), AY606276 (isolate S-1 of G-1, 330 nucleotids)

Lipases, pectinases and amylases produced by the isolated strains can be studied in respect of enzyme activity, purification and production.

The genes coding for these enzymes can also be cloned to obtain recombinant thermoacidophilic enzymes.

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APPENDIX A

CHEMICALS

Table A.1. Chemicals Used in Experiments

No	**Chemical**	Code
1	Agar-Agar	Merck 1.01613
2	D-Glucose	Applichem A3666
3	Yeast Extract	Merck 1.03753
4	Glycerol	Applichem A2926
5	NaCl	LabM MC27
6	K_2HPO_4	Applichem A2942
7	$MgSO_4$ x $7H_2O$	Merck 1.05886
8	Xylan from birchwood	Sigma X-0502
9	Pectin from citrus peel	Fluka 76280
10	Polygalacturonic acid	Fluka 81325
11	H_2SO_4	Merck 1.0735
12	Thermo Sequenase Cy 5.5 Dye Therminator Cycle Sequencing Kit	Amersham Biosciences
13	Carboxymethylcellulose sodium salt	Fluka Biochemica 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	Merck1.05443
24	Congo Red	Sigma C6767
25	Potassium Iodide	Sigma P8256

26	DNA isolation kit	Applichem A3421
27	N,N,N`,N`-Tetramethyl-p-phenyl enediamine	Sigma T3134
28	Tween80	Applichem A1390
29	Tween 20	Applichem A1389
30	Tris Base	Sigma T6066
31	EDTA	Applichem A2937
32	Isopropanol	Applichem A3928
33	Proteinase K	Applichem A3830
34	Tryptone	Oxoid 1.0123
35	Ethidium bromide	Applichem A1151
36	Ethanol	Applichem A3678
37	Taq DNA polymerase	MBI, Fermentas EP0401
38	dNTP set	MBI, Fermantas R0181
39	Taq I	Fermantas ER0671
40	Primers Ege 1, Ege 2, L1	Promega
41	Proteinase K	Applichem A3830
42	Chloroform	Applichem A3633
43	Isoamyl alcohol	Applichem A2610
44	Bromophenol blue	Merck 1.08122
45	Boric acid	Applichem A2940
46	Standard agarose	Applichem A2114

APPENDIX B

MEDIA

B.1 Media Used for Isolation (*Bacillus acidocaldarius* Medium) (Broth)

Solution A

Yeast extract	1	g
$(NH_4)_2SO_4$	0.2	g.
$MgSO_4$ x 7H2O	0.5	g.
$CaCl_2$ x 2H2O	0.25	g.
KH_2PO_4	0.6	g.
Distilled water	500	ml.

Adjust pH to 3.7 with 1 M H_2SO_4

Solution B

Glucose	1	g.
Distilled water	500	ml.

Solution A and Solution B were seperately sterilized at 121ºC for 15 min. They were cooled to 50ºC and then combined.

***Bacillus acidocaldarius* Medium (Agar)**

Solution A

Yeast extract	1	g
$(NH_4)_2SO_4$	0.2	g.
$MgSO_4$ x 7H2O	0.5	g.
$CaCl_2$ x 2H2O	0.25	g.
KH_2PO_4	0.6	g.

Distilled water	500	ml.

Adjust pH to 3.7 with 1 M H_2SO_4

Solution B

Glucose	1	g.
Agar	20	g.
Distilled water	500	ml.

Solution A and B were autoclaved seperately at 121 ºC for 15 min. They were cooled to 50º C and then combined.

YSG Agar

	g/l
Yeast extract	2
Soluble starch	2
Glucose	1
Agar	20

The pH was adjusted to pH 3.7 with 1 M H_2SO_4. Medium was sterilised by autoclaving at 121ºC for 15 min.

B.2 Media Used for Extracellular Enzyme Screening

B.2.1 Media used for Pectinase Screening

Medium I

	g/l
Ammonium sulfate	1
Na_2HPO_4	6
KH_2PO_4	3
Polygalacturonic acid	5
Agar	20

Ingredients (except agar) were dissolved in distilled water. The pH of the medium was adjusted to 4.0 with 1M H_2SO_4. Agar was added. Medium was sterilised by autoclaving at 121 ºC for 15 min.

Medium II

	g/l
Yeast extract	1
Ammonium sulfate	2
Na_2 HPO_4	6
KH_2 PO_4	3
Pectin from citrus peel	5
Agar	20

Ingredients (except agar) were dissolved in distilled water. The pH was adjusted to 4,0 with 1 M H_2 SO_4. Agar was added. Medium was sterilised by autoclaving at 121º C for 15 min.

B.2.2 Media Used for Lipase Screening

Medium I

	g/l
Nutrient broth	13
$CaCl_2$ x 2H2O	0.1
Tween 80	10ml
Agar	20

Ingredients (except Tween 80 and agar) were dissolved in deionized water. The pH of the medium was adjusted to 4.0 with 1 M H_2SO_4. Agar was added. Medium was sterilised by autoclaving at 121 ºC for 15 minutes. Tween 80 was autoclaved seperately and added to the medium.

Medium II

	g/l
Nutrient Broth	13
$CaCl_2$ x 2H2O	0.1
Tween 20	10 ml
Agar	20

Ingredients (except Tween 20 and agar) were dissolved in deionizied water. The pH of the medium was adjusted to 4.0 with 1 M H_2SO_4. Agar was added. Medium was sterilised by autoclaving at 121° C for 15 min. Tween 20 was autoclaved seperately and added to the medium.

B.2.3 Media Used for Amylase Screening

Medium I

	g/l
Yeast exract	1
$MgSO_4$ x 7 H2O	0.1
K_2HPO_4	7
$KH_2 PO_4$	2
$(NH_4)_2$ SO4	1
NaCl	5
Starch	5
Agar	20

All Ingredients (except agar) were dissolved in deionized water. The pH of the medium was adjusted to 4.0 with 1 M H_2SO_4. Agar was added. Medium was sterilised by autoclaving at 121° C for 15 min.

Medium II

	g/l
Yeast extract	1
Soluble starch	5
Agar	20

Ingredients (except agar) were dissolved in deionized water. The pH of the medium was adjusted to 4.0 with 1 M H_2SO_4. Agar was added. Medium was sterilised by autoclaving at 121° C for 15 min.

B.2.4 Media used for Xylanase Screening

	g/l
Yeast extract	1
Birchwood xylan	5
Agar	20

Ingredients (except agar) were dissolved in deionized water. The pH of the medium was adjusted to 4.0 with 1 M H_2SO_4.Agar was added. 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	20

Ingredients (except agar) were dissolved in deionized water. The pH of the medium was adjusted to 4.0 with 1 M H_2SO_4. Agar was added. Medium was sterilised by autoclaving at 121° C for 15 min.

B.3 Media Used for Growth Tests at Different Sodium Chloride Concentrations

Bacillus acidocaldarius medium (Agar) was used for growth tests at different sodium chloride concentrations. The only difference was addition of 1%, 2%, 3%, 4%, 5% or 6% NaCl to Solution A of BAM Agar.

B.4 Media Used for Growth Tests at Different pH

TYG Agar

	g/l
Tryptone	1
Yeast extract	1
Glucose	1
Agar	20

Ingredients (except agar) were dissolved in distilled water. The pH of the medium was adjusted to 2, 3, 5 or 6 with 1 M H_2SO_4. Agar was added. Medium was sterilised by autoclaving at 121° C for 15 min.

APPENDIX C

PCR-RFLP RECIPIES

C.1 PCR Mixture

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

C.2 6X Gel Loading Buffer (20ml)

10xTBE	2ml
Glycerol	6ml
Deionized water	12ml

Bromophenol blue was added until a sufficient color of the solution is obtained.

C.3 dNTP (10X)

Ten µl of each 100mM dATP, dCTP, dGTP and dTTP in seperate vials were taken. They were mixed in 0.2 ml PCR tubes and 460 µl steril deionized water was added. They were mixed gently and 2mM concentrations of each was obtained and stored at -20º C.

C.4 Restriction Enzyme Mixture

Restriction enzyme buffer	5µl
Sterile deionized water	35

DNA	10µl
Restriction Enzyme	0.5 µl (5U)

C.5 Oligonucleotide Primers

L1: 5'- CAAGGCATCCACCGT -3'

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

EGE 1: 5'-AGAGTTTGATCCTGGCTCAG -3'

Five hundred and ninety micrograms 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. Hence 100 µl, 10 picomole/µl working solution was obtained. Stock and working solutions were stored at -20ºC.

EGE 2: 5'-CTACGGCTACCTTGTTACGA -3'

Five hundred and ninety micrograms primer EGE 2 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 100µl, 10 picomole/µl working solution was obtained. Stock and working solutions were stored at -20ºC.

APPENDIX D

STAINS AND INDICATORS

D.1 Solutions for Gram Staining

D.1.1 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.1.2 Iodine Solution

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Iodine and potassium iodide were grinded.Water was added slowly and the solution was stirred until the iodine was dissolved.The solution was stored in amber bottle

D.1.3 Safranin Solution

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

APPENDIX E

BUFFERS AND STOCK SOLUTIONS

E.1 50XTAE

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

E.2 1XTAE

Twenty 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 10XTBE

One hundred and eight grams Tris Base and 55 g boric acid were weighed. They were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was added. The volume was brought to 1 L with deionized water.

E.4 1M Tris-HCI pH 8

Tris base (121.1 g) was dissolved in 800 ml of deionized water. pH was adjusted to 8 with concentrated HCI. Volume was brought to 1L with deionized water.

E.5 1XTBE

Hundred milliliters 10XTBE was taken and the volume was brought to 1 liter with deionized water to obtain 1 liter 1X TBE buffer.

E.6 0.5 M EDTA pH 8.0

EDTA (186.12g) 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.7 1XTE

10mM Tris, pH 8, 1mM EDTA

E.8 Ethidium Bromide Stock Solution (10 mg/ml)

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

E.9 Chloroform-Isoamyl Alcohol Solution

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

E.10 Phenol

Liquified phenol was taken from the freezer and it was melted at 68ºC in a waterbath. Then equal volume of buffer (0.5 M Tris.Cl pH 8 at room temperature) was added to the phenol. The mixture was stirred for 15 minutes. After the two phases have separated, the aqueous (upper) phase was removed by using a seperation funnel. Equal volume of 0.1 M Tris.Cl pH 8 was added to the phenol. The mixture was again stirred for 15 minutes. The aqueous phase was again removed. The extractions were repeated until the pH of the phenolic phase was >7.8. The pH was measured with a pH paper. Then it was divided into aliquots and stored at -20ºC. Before use, hydroxyquinoline and β-mercaptoethanol were added to final concentration of 0.1% and 0.2% respectively. The phenol solution can be stored in this form at 4ºC.

E.11 CTAB/NaCl Solution

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