Screening for Industrially Important Extracellular Enzymes from Alkalophilic *Bacillus* Genus

By

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To my dear teacher Aysel AYAS

and to my mother Semahat AKBALIK

Sevgili hocam Aysel AYAS'a
ve annem Semahat AKBALIK'a

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ABSTRACT

Alkalophilic *Bacillus* include industrially important species since they can produce many extracellular enzymes which are active and stable at high pH values. These alkaline enzymes (proteases, amylases, xylanases, cellulases, lipases and pectinases) find use in various field of industry such as leather, detergent, paper industries and waste water treatment.

Isolation of diverse bacteria plays an important role in finding novel enzymes with improved characteristics. The aim of this study was therefore to screen for alkaline extracellular enzymes of alkalophilic *Bacillus* isolated from soil, horse feces and leather processing and to characterize these strains by phenotypic tests and by 16S-ITS rDNA based RFLP.

At the end of the study, rod-shaped, endospore forming and Gram positive 116 strains were identified as *Bacillus.* Ten of the 116 strains were found to be obligate alkalophilic. 91 protease, 77 amylase, 18 xylanase, 3 cellulase, 74 pectinolytic enzyme (71 polygalacturonic acid degrading and 72 pectin degrading) and 55 lipase (41 Tween 20 hydrolyzing and 14 Tween 80 hydrolyzing) producing strains were obtained. Isolated and reference strains were classified into 18 groups in respect of the enzymes they produced.

Two enzymes, *Taq* I and *Hae* III were used for 16S-ITS rDNA based RFLP analysis. Both of the enzymes were found to be necessary for the discrimination of the strains. Reference strains were clustered into different groups by both *Taq* I and *Hae* III. *Taq* I digestion revealed 16 genotypic groups while *Hae* III revealed 15 different groups. And comparative analysis of the RFLP profiles of 116 isolates and 5 reference strains resulted in 26 genotypic groups.

ÖZ

Alkalofilik *Bacillus* genusu yüksek pH değerlerinde aktif ve stabil ekstraselüler enzim üreten endüstriyel önemli türler içermektedir. Bu alkali enzimler (proteaz, amilaz, ksilanaz, selülaz, lipaz ve pektinaz) deri, deterjan, kağıt sanayii ve atık su arıtımı gibi endüstrinin çeşitli alanlarında kullanım alanı bulmaktadır.

Farklı bakterilerin izolasyonu daha iyi özellikte yeni enzimlerin bulunmasında önemli rol oynamaktadır. Bu çalışmanın amacı, topraktan, at gübresinden ve deri işlem basamaklarından izole edilen alkalofilik *Bacillus*'un alkali ekstraselüler enzimlerini taramak ve bu izolatları fenotipik testlerle ve 16S-ITS rDNA'ya dayalı RFLP yöntemiyle karakterize etmektir.

Çalışmanın sonucunda, çubuk şekilli, spor üreten ve Gram pozitif 116 izolat *Bacillus* olarak tanımlanmıştır. İzolatlardan 10 tanesinin zorunlu alkalofilik olduğu bulunmuştur. 91 proteaz, 71 amilaz, 18 ksilanaz, 3 selülaz, 74 pektinolitik enzim (71 poligalakturonik asit, 72 pektin degrade eden) ve 55 lipaz (41 Tween 20, 14 Tween 80 hidrolize eden) üreticisi izolat elde edilmiştir. İzole edilen ve referans suşlar enzim üretimlerine göre 18 gruba ayrılmışlardır.

16S-ITS rDNA RFLP için *Taq* I ve *Hae* III olmak üzere iki enzim kullanılmıştır. İki enzimin de izolatların ayırdedilmesinde gerekli olduğu anlaşılmıştır.

Referans suşlar hem *Taq* I hem de *Hae* III tarafından farklı gruplara ayrılmıştır. *Taq* I'le kesim 16 genotipik grup, *Hae* III'le kesim ise 15 farklı grup ortaya çıkarmıştır. Ayrıca 116 izolat ve 5 referans suşun RFLP profillerinin karşılaştırmalı analizi sonucunda 26 genotipik grup olduğu sonucuna varılmıştır.

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ABBREVIATIONS

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

Chapter 1

INTRODUCTION

1. Definition of Alkalophiles

There is no generally agreed definition for alkalophiles because these microorganisms can survive in differing pH spectra and definitions have been made with respect to specific pH ranges (Horikoshi, 1999b).

For example those microorganisms whose optimum rate of growth is observed at least two pH units above neutrality are called true alkalophiles. And microorganisms whose optimum growth pH is around or below neutrality are called alkalotolerants since they can also survive at pH points more than 9 (Kroll, 1990).

On the other hand, Horikoshi (1999b) has used the term alkalophile for microorganisms that grow optimally or very well at pH values above 9.0, but can not grow or only grow slowly at neutral pH values.

According to Krulwich (1989) alkalophiles can be divided into two groups:

1) Alkaline-tolerants showing optimal growth at the 7.0-9.5 but incapable of survival at pHs above 9.5

2) Alkalophilics showing optimal growth at the pH range 10.0 and 12.0

Extreme alkalophiles are further divided among themselves (Krulwich, 1989):

- Facultative alkalophiles showing optimal growth at pH 10.0 or above but having also ability to grow well at the neutral pH.
- Obligate alkalophiles showing optimal growth above pH 10.0 but incapable of growth below pH 8.5-9.0.

1.2. Alkalophilic *Bacillus*

Microbiologists have not as yet produced taxonomic criteria which could enable an unambiguous identification of alkalophilic *Bacillus* (Nielsen *et al.*, 1995).

Bacillus is a rod-shaped, gram positive, spore forming, aerobic or facultatively anaerobic, usually catalase positive, chemoorganotrophic bacterium with a fermentative or respiratory mechanism (Holt *et al.*,1994).

Therefore, if an isolate shows general characteristics of *Bacillus* genus, it is referred as *Bacillus* sp.

1.2.2. Distribution and Isolation

Alkalophilic *Bacillus* can be found mostly in alkaline environments including soda soils, soda lakes and deserts, neutral environments, deep-sea sediments (e.g., Mariana Trench). Animal manure, man-made alkaline environments such as effluents from food, textile, tannery, potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources (Horikoshi 1999, Kumar and Takagi, 1999). Obligate alkalophiles have been isolated even from acidic soils (Krulwich and Guffanti, 1989).

Alkalophilic *Bacillus* is generally isolated by surface plating on a high alkaline medium. The medium described by Horikoshi is the most commonly used general medium for the isolation of alkalophiles. Different types of media have also been used in the past, including nutrient agar, glucose-yeast extract-asparagine(GYA) agar, peptone-yeast extract-glucose (PPYG) medium. Subsequently the isolates are grown on specific media to observe extracellular enzymatic activities (Kumar and Takagi, 1999).

1.2.3. Extracellular Enzymes and Their Industrial Application

Bacillus species, are generally of industrial importance since they have the ability to produce extracellular enzymes (proteases, amylases, cellulases, lipases, pectinases, and xylanases) that are active and stable at high pH values (Martins *et al*., 2001). Studies have led to the discovery of many types of such enzymes with unique properties .

Some areas of application for alkaline-stable enzymes are leather tanning, paper pulp bleaching, production of cyclodextrins and waste treatment (Martins *et al*, 2001).

1.2.3.1. Alkaline Proteases

Proteases are the enzymes which hydrolyse protein substrates. Table 1.1 summarizes the classification of alkaline proteases (Rao *et al*., 1998).

Table 1.1. Classification of Alkaline Proteases.

<table>
<tr><td rowspan="4">Classification based on their catalytic mechanism</td><td rowspan="3">1-Serine proteases
(Ser-His-Asp catalytic triad in the active site)</td><td colspan="2">Serine alkaline proteases</td></tr>
<tr><td rowspan="2">Subtilisins</td><td>subtilisin Carlsberg produced by Bacillus licheniformis</td></tr>
<tr><td>subtilisin Novo or BPN’ from B. amyloliquefaciens</td></tr>
<tr><td colspan="3">2-Metalloproteases
The activity depends on the binding of a divalent metal ion to a His-Glu-Xaa-Xaa-His motif</td></tr>
<tr><td rowspan="2">Classification based on the site of action</td><td colspan="3">1-Exopeptidases: cleavage of the peptide bonds near the amino or carboxy termini</td></tr>
<tr><td colspan="3">2-Endopeptisases: cleavage of the peptide bonds distant from the termini of the substrate</td></tr>
</table>

The first alkaline protease (H-221) from alkalophilic *Bacillus* sp. Strain no.221. has been reported by Horikoshi (1971). The high alkaline protease group, including Savinase, PB92, M-protease, are all produced by alkalophilic bacilli and they have low molecular mass of 26-28 kDa. A salt bridge is characteristic in the structures of high alkaline proteases. For example, in the crystal of M-protease (optimum pH 12,3), there is a salt bridge triad comprising Arg19, Arg265 and Glu269. But this structure is absent in less alkaline subtilisins Carlsberg and BPN’ (Saeki *et al*., 2000).

Most commercial alkaline proteases have been found to be produced by members of the genus *Bacillus* after large screening studies (Rao *et al*., 1998, Kumar and Takagi, 1999). Some industrially important alkaline proteases are summarized in Table 1.2 (Anwar and Saleemuddin, 1998).

Table 1.2. Some Industrially Important Alkaline Proteases.

Species	pH Optimum/Stability	Industrial application(s)	Reference
Bacillus sp.Y. (BYA)	10.0-12.5	Detergent formulations	Shimogaki *et al.*, 1991
Bacillus licheniformis	8.2	Catalyst for N-protected amino acids	Chen *et al*., 1991
Bacillus sp. (AH-101)	12.0-13.0	Dehairing/Leather industry	Takami *et al.,* 1992
Bacillus firmus	8.0	Detergent industry	Moon &Parulekar, 1993
Bacillus sp. (P-001A)	9.5	Production of biomass from natural waste	Atalo&Gashe, 1993
Bacillus sp.*(*B 18)	12.0	-	Fujiwara *et al.*, 1993
Bacillus sp.	12.0	-	Masui *et al.*, 1994
Bacillus sp.	8.5	Dehairing/Leather industry	Loperena *et al*., 1994
Bacillus sp.	9.0-11.0	Detergent formulations	Bossi *et al.*, 1994
Bacillus licheniformis	8.2	Synthesis of biologically active peptides	Chen *et al*., 1995
Bacillus subtilis	8.5	Bating agent in leather industry	Hameed *et al.,* 1996
Bacillus sp.	8.5	-	Sinha *et al.*, 1996
Bacillus subtilis	-	Contact lens cleansing agent	Sanyo *et al.,* 1996
Bacillus brevis	10.5	Detergent formulations	Banerjee et al.,1999
Bacillus sphaericus	10.5	Potential source for detergent formulations and hydrolysis of gelatin layers of used X-ray films	Singh *et al*., 1999
Bacillus sp**.** JB-99	11	Potential source for detergent formulations	Johnvesly and Naik, 2001
Bacillus pseudofirmus AL-89	11	Potential source for the hydrolysis of feather to be used as animal feed supplement	Gessesse *et al*., 2003

Alkaline proteases are applied in various fields of industry:

- Detergent Formulation

Proteases used as detergent additives beside having have high activity and stability at high pH values, they should also be compatible with chelating and oxidizing agents (Rao *et al*.,1998). Detergent-compatible proteases are used in laundry detergents, automatic diswashing detergents, in the cleaning of ultrafiltration and reverse osmosis membranes (Kumar and Takagi,1999, Anwar and Saleemuddin, 1998).

- Leather Industry

Alkaline proteases which have elastolytic and keratinolytic activity have been used in dehairing and bating of hide (Kumar and Takagi, 1999). Enzymatic dehairing is safer than traditional methods in which saturated lime and sodium sulfide must be used (Anwar and Saleemuddin, 1998, Kumar and Takagi, 1999).

The enzyme for bating process is selected depending on its specificity for matrix proteins such as elastin and keratin. The amount of enzyme to be used depends on the type of leather (soft or hard) to be produced (Rao *et al*., 1998).

- Silver Recovery

Used X-ray films and the silver contained in the film can be recyled after proteolytic treatment at high pH. A number of alkaline proteases used in this process have been effective (Singh *et al.*, 1999, Kumar and Takagi, 1999).

- Food Industry

Alkaline proteases are used in the preparation of protein hydrolysates from plants, animals, and dairy products. The protein hydrolysates, from whey protein, soyprotein and casein for example, are mainly used in hypoallergenic infant food formulations. They can also be used for the strengthening of fruit juices, soft drinks,

protein rich therapeutic diets. These protein hydrolysates could also have an important role in blood pressure regulation. Meat tenderization is another potential use of alkaline proteases. It has been reported that an alkaline elastase and thermophilic alkaline protease have the ability to hydrolyze connective tissue proteins and muscle fiber proteins. These properties can be very useful in meat tenderization (Kumar and Takagi, 1999).

- Waste Treatment

Alkaline proteases can solubilize proteins in industrial effluents to recover materials of nutritional value that can be used in animal feed. It has been reported the use of an alkaline protease in processing feathers from poultry slaughterhouses.There are also other reports on the keratinolytic alkaline proteases used in feed technology and for degrading keratinous waste material in household refuse (Kumar and Takagi, 1999).

- Biopolymer Industry

Biopolymers are mostly biodegradable and thus they have found many applications in various industrial fields. For example, using commercial alkaline protease, Proleather, a sucrose-polyester synthesis has been achieved in anhydrous pyridine (Kumar and Takagi, 1999). In another study an alkaline protease from alkalophilic strain *B. pseudofirmus* AL-89 has been used for sucrose ester synthesis in organic solvents (Pedersen *et al*., 2003). Sugar esters are used in the food and cosmetic industry, as antitumoral agents and plant growth inhibitors (Pedersen *et al*., 2003).

1.2.3.2 Starch Hydrolyzing Alkaline Enzymes

General characteristics of starch degrading enzymes and the structure of starch were summarized in Table 1.3 (Starnes, 1990 , Maldonado and Lopez, 1995). Schematic representation of starch degrading enzymes is shown in Figure 1.1.

Table 1.3. Structure of Starch and General Characteristics of Starch Degrading Enzymes.

Starch	composed of α-1,4-linked α-D-glucose units with α-1,6-branching points	
Fractions of starch	**amylopectin**	branched polymer containing α-1,4 glycosidic linkages and α-1,6 linked branch points occurring every 17-26 glucose units
	amylose	linear polymer of glucopyranose units linked through α-D-1,4 linkages
Enyzmes involved in the hydrolysis of starch		
Endoamylases (α-amylases)	Cleavage of 1,4- glycosidic bonds Products of hydrolysis : linear and branched oligosaccharides of varying chain lengths with α-configuration on the C1 of the reducing glucose unit produced	
Exoamylases	**β-amylase**	- Cleavage of α-1,4 glycosidic bonds - Gradual removal of maltose units in the β-anomeric form - Inability of hydrolyzing α-1,6 linkages
	glucoamylase	- Cleavage of α-1,4 linkages from the nonreducing end -Products of hydrolysis: D-glucose molecules in the β-configuration -Hydrolysis on the α-1,6 bonds: much more slowly than on α-1,4 bonds
Debranching enzymes	**pullulanases**	- action on α-1,6 linkages in pullulan, starch, amylopectin and related oligosaccharides.
	isoamylase	-hydrolysis of α-1,6 glycosidic linkages in amylopectin -low activity or no activity toward pullulan
Isomerases	Conversion of glucose to fructose.	
Cyclodextrin glucosyltransferases	-degradation of starch by catalyzing cyclization, coupling and disproportianation reactions -products of cyclization reaction: cyclodextrins also known as Schardinger dextrins	

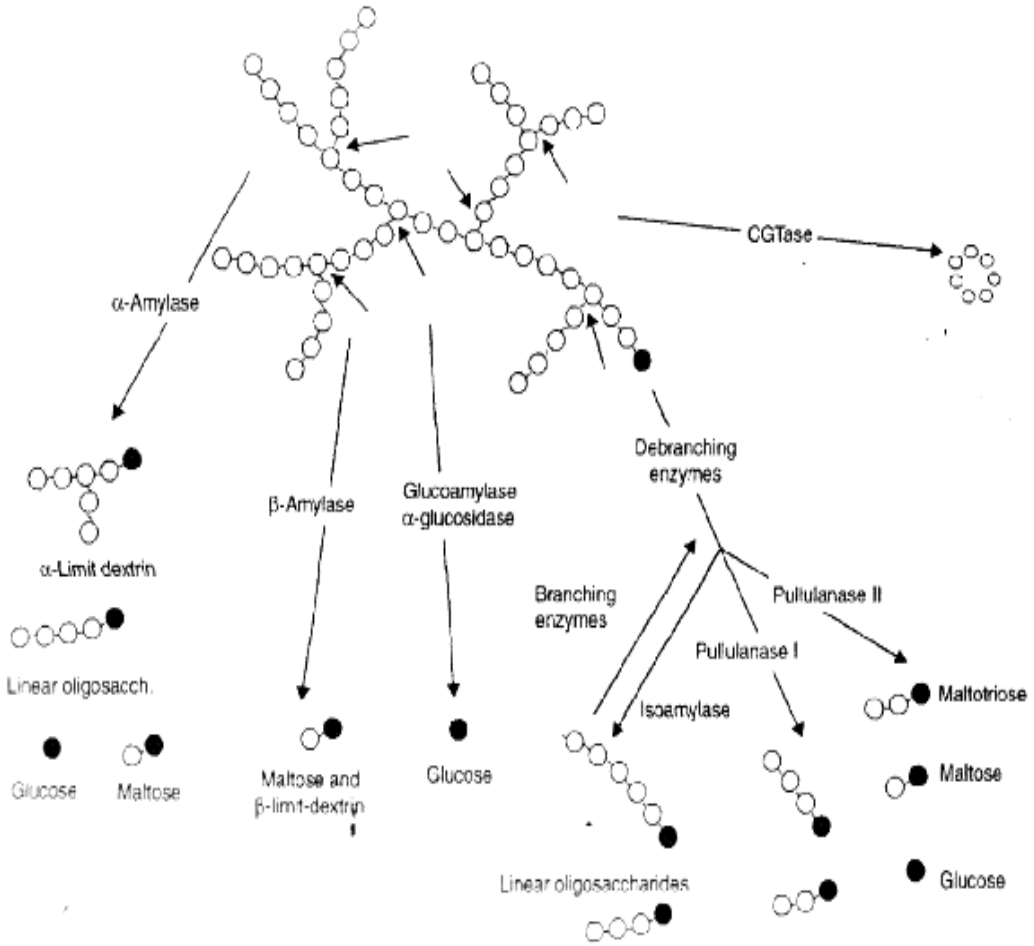

Figure 1.1 Schematic Representation of Starch Degrading Enzymes (Bertoldo and Antranikian, 2002).

Alkaline amylases with optimum pH values higher than 8.0 have potential applications in the starch, textile industries and as detergent additives (Kim *et al*., 1995). Alfa-amylases have been used in powder laundry detergents since 1975. Nowadays, 90% of all liquid detergents contain α-amylase and the demand for automatic dishwashing detergents has been increasing.

An alkaline amylase from an alkalophilic *Bacillus* GM8901 has been the first enzyme which produces predominantly maltotetraose from starch at an extremely high pH (11.0 to 12.0). Maltotetraose has potential uses in food, pharmaceutical and fine chemical industries because of its low sweetness, superior moisture retention, high viscosity and freezing point, and other special properties, compared with convential sugar syrups (Kim et al., 1995).

Some unique debranching enzymes has also been found and characterized such as a high alkaline pullulanase, an alkaline resistant neopullulanase, and an alkaline amylopullulanase. These alkaline amylolytic enzymes have been thought to be suitable as detergent additives (Crabb and Shetty, 1999).

So far, many CGTase producing alkaliphilic *Bacillus* strains have been reported (Kumar and Takagi, 1999). Cyclodextrins are able to form inclusion complexes with various solid, liquid and gaseous compounds. This formation leads to some beneficial effects such as protection of active ingredients against oxidation, elimination of undesirable odors or tastes, stabilization of drugs, colours, flavors and vitamins. Hence they find application in the food, cosmetic and pharmaceutical industries (Starnes, 1990).

1.2.3.3. Alkaline Xylanases

General characteristics of xylan degrading enzymes and the structure of xylan were summarized in Table 1.4 (Beg *et al*., 2001, Ratanakhanokchai *et al*., 1999).

Table 1.4. Structure of Xylan and General Characteristics of Xylan Degrading Enzymes.

Structure of xylan	-complex polysaccharide comprising a backbone of xylose residues linked by β-1,4-glycosidic bonds - major component of hemicellulose of plant cell wall
Common substituent groups in the backbone chain and in the side chain of xylan	- acetyl - arabinosyl - glucuronysyl
Xylan degrading enzymes	- β-1,4-endoxylanase - β-xylosidase - α-L-arabinofuranosidase - α- glucuronidase - acetyl xylan esterase - phenolic acid (ferulic and p-coumaric acid) esterase

Most xylanases isolated so far are optimally active at acidic or neutral pH (Nakamura *et al*.,1993, Gessesse,1998). New alkaline xylanases are therefore needed for example in pulp and paper industry (Nakamura *et al*., 1993).

Prebleaching of kraft pulps is the most effective application of alkaline xylanases (Beg *et al*., 2001). In 1986, Viikari *et al*. introduced the use of xylanases for prebleaching kraft pulp. In this process, a xylanase solution is mixed with washed pulp prior to chemical bleaching (Yang *et al*.,1995). Lignin content in pine and birch Kraft pulp was reduced by 25% and 33% using xylanases (Viikari *et al*.,1994).

Brightening and whitening of the paper pulp is achieved by the removal of lignin and this process is called bleaching (Beg *et al*.,2001). Xylanase pretreatment of pulp results in pores in the xylan coat. This facilitates lignin removal by oxidizing agents in pulp (Subramaniyan and Prema, 2000). Here again alkaline xylanases which are active above pH 9 are needed. This process can also reduce the amount of toxic organic chlorine compounds by up to 20%-40% (Bim and Franko, 2000, Beg *et al*., 2001).

Nakamura *et al*.(1994) have reported xylanases from alkaliphilic *Bacillus* which can be used in biobleaching. Khasin *et al.* (1993) have isolated an alkaline xylanase from *Bacillus stearothermophilus* T-6 that bleaches pulp at pH 9 and 65 °C.

Some properties of alkaline or alkali-stable xylanases are given in Table 1.5.

Table 1.5. Some Properties of Alkaline or Alkali Stable Xylanases.

Source	Production of	pH Optimum/Stability	Temperature Optimum	Reference
Bacillus sp. (V1-4)	One predominant xylanase	8.5	55 °C	Yang *et al*., 1995
Alkalophilic thermophilic *Bacillus* sp.	Xylanases	6-9	75 °C	Bataillon *et al*., 1998
Alkalophilic *Bacillus* sp. strain AR-009	Two xylanases: Xyl A and XylB	XylA: 9 XylB: 10	XylA: 60 °C at pH 9, 70 °C at pH 8; XylB: 75 °C at pH 9, 70 °C at pH 8	Gessesse, 1998
Alkalophilic *Bacillus* sp. strain K-1	Xylan-binding xylanase	5.5/ up to 12	60 °C	Ratanakhanokchai, 1999

1.2.3.4 Alkaline Cellulases

General characteristics of cellulose degrading enzymes and the structure of cellulose were summarized in Table 1.6 (Schülein, 2000, Kim, 1995).

Table 1.6. Cellulose Degrading Enzymes and the Structure of Cellulose.

Structure of cellulose	complex and heterogeneous polymer formed of D-glucose residues linked by β-1,4-glycosidic bonds.
Types of cellulases produced by microorganisms	**1,4-β-D-glucan cellobiohydrolases (CBHs) (E.C.3.2.1.91) (exoglucanases, Avicelases):** cleavage of cellobiosyl units from the nonreducing ends of the cellulase chains
	endo-1,4-β-D-glucan 4-glucanohydrolases (EC 3.2.1.4)(endoglucanases): cleavage of internal cellulosic linkages.
	β-D-glucosidases (e.g. cellobiase): They specifically cleave glucosyl units from the nonreducing ends of cello-oligosaccharides.

The main application of alkaline cellulases is in detergent industry. They remove microfibrils of cotton fabrics allowing the release of stain particles. Bright and soft textiles are obtained. Otherwise the microfibril would reflect the light diffusely and would result in a dull appearance (Schülein, 2000, Shirai *et al.*, 2001). Most of the cellulases are inactive against crystalline cellulose and they do not digest sound fabric (Shirai *et al.*, 2001).

Endo-1,4-β-glucanases are the most common cellulases currently used for detergent additives. An alkaline cellulase which had high durability in detergents has been found in *Bacillus* sp.strain KSM-635. It showed an optimum pH of 9.5 at 45 °C (Shirai *et al.*, 2001).

Three strains of alkalophilic *Bacillus,* KSM-19, KSM-64 and KSM-520 which produced alkaline cellulases suitable for detergent additives have also been isolated. Their optimum pH has been found to be 8.5-9.5 and optimum temperature about 50 °C. Their activities were not inhibited by various components of laundry products such as surfactants, chelating agents and proteases (Shikata *et al.,*1990).

1.2.3.5. Alkaline Pectinolytic Enzymes

Pectinolytic enzymes are able to degrade the complex and colloidal acid polysaccharide called pectin. General characteristics of pectinolytic enzymes and pectic substances are summarized in Table 1.7 (Kashyap *et al*, 2001).

Tablo 1.7. General Characteristics of Pectic Substances and Pectinolytic Enzymes.

<table>
<tr><td>Pectin</td><td colspan="4">Present in the middle lamella and the primary cell walls of young plant cells</td></tr>
<tr><td>Pectic substances</td><td colspan="4">protopectin, pectic acid, pectinic acid and pectin</td></tr>
<tr><td>Criteria for classification of pectinases</td><td colspan="4">- their preferred substrate
- their action on substrate: transelimination or hydrolysis
- their cleavage mechanism: endo-(liquefying or depolymerizing enzymes) or exo-(saccharifying enzymes)</td></tr>
<tr><td rowspan="9">Major groups of pectinases</td><td colspan="4">1. Pectinesterases : Catalysis of deesterification of the methoxyl group of pectin forming pectic acid</td></tr>
<tr><td rowspan="8">2.Depolymerizing enzymes</td><td rowspan="4">2.1 Hydrolyzing glycosidic linkages</td><td rowspan="2">Polymethygalacturonases (PMG)</td><td>EndoPMG</td></tr>
<tr><td>ExoPMG</td></tr>
<tr><td rowspan="2">Polygalacturanases (PG)</td><td>Endo PG</td></tr>
<tr><td>ExoPG</td></tr>
<tr><td rowspan="4">2.2 Cleaving glycosidic linkages by transelimination</td><td rowspan="2">Polymethylgalacturonate lyases (PMGL)</td><td>ExoPMGL</td></tr>
<tr><td>EndoPMGL</td></tr>
<tr><td rowspan="2">Polygalacturonate lyases (PGL)</td><td>ExoPGL</td></tr>
<tr><td>EndoPGL</td></tr>
<tr><td></td><td colspan="4">3. Protopectinase: Solubilization of protopectin forming highly polymerized soluble pectin</td></tr>
</table>

The first publication on alkaline pectinase has been the production of the endo-polygalacturonases by alkalophilic *Bacillus* sp. No.P-4-N (Horikoshi, 1972).

Some properties of alkaline pectinolytic enzymes were summarized in Table 1.8.

Table 1.8. Some Properties of Alkaline Pectinolytic Enzymes.

Source	Type of enzyme	pH optima/ stability	Temperature optima/stability	Other properties	References
Alkalophilic *Bacillus* No. P-4-N	Endo-polygalacturonase	10/-	Stable at 65 °C and up to 70 °C in the presence of Ca^{2+}, completely inactivated at 80-90 °C		Horikoshi, 1972
Bacillus sp. No.RK9	Endo-polygalacturonate lyase	10/-	60 °C	Enzyme production :constitutive	Kelly and Fogarty, 1978
Bacillus sp. Strain KSM-17	Pectate lyase	10,5 in 50mM glycine NaOH, 7,5 in Tris-HCl buffer	60 °C to 65 °C	Protopectinase like activity	Kobayashi, 1999
Alkalophilic *Bacillus* sp. Strain P-4-N	Pectate lyase	11,5	70 °C /stable up to 55 °C without $CaCl_2$, to 60 °C with $CaCl_2$		Hatada *et al*., 2001
Bacillus strain KSM-P576	Exopolygalacturonase	8/ 6-12 at 30° C for 1h	55 °C	Enzyme products: digalacturonic acid from PGA, tri-,tetra-,penta-galacturonic acids	Kobayashi *et al*, 2001

Applications of Alkaline Pectinases:

-Retting and degumming of fiber crops:

The pectin of the bark is decomposed and cellulosic fibers are released during the fermentation process called retting (Kashyap *et al.*, 2001, Hoondal *et al*, 2002). Certain bacteria including *Bacillus* are involved in this process (Kashyap *et al*., 2001). Pulps prepared with pectinolytic enzymes have higher opacity and better printability than pulps prepared by an alkaline process (Hoondal *et al*., 2002).

Another use of alkaline proteases is the degumming of plant fibers such as ramie, sunn hemp, jute, flax and hemp. Degumming process is necessary to use ramie as textile material (Kashyap *et al*, 2001, Zheng *et al*, 2001). Ramie fibers which are natural textiles contain 20-30 % ramie gum. Recently, an alkaline and thermostable polygalacturonase from *Bacillus* sp. MG-cp-2 (Kapoor *et al*., 2001) has been used for the degumming of ramie and sunn hemp bast fibers (Hoondal *et al*., 2002). Three types of treatment, enzymatic, chemical and 'chemical plus enzyme' have been applied. The last type has been found to be most promising for degumming because a complete removal of non-cellulosic gum from the surface fibers has been observed by scanning electron microscopic studies (Kapoor *et al.*, 2001). In another study Kashayp *et al*. (2001) have used an alkali and thermotolerant pectinase from *Bacillus* sp. DT 7 for degumming of buel bast fibers.

-Treatment of pectic wastewater

Removal of pectic substances from food processing units is facilitated by using alkaline pectinase and alkalophilic microorganisms (Hoondal *et al*., 2002). For example, Tanabe *et al*. (1987) have isolated an alkalophilic *Bacillus* sp. (GIR 621), which produces an extracellular endopectate lyase in alkaline media. The enzyme, optimally active at pH 10.0, has been used to remove pectic substances from waste water.

-Oil extraction

Oil is traditionally extracted using hexane which is a potential carcinogen. Recently, use of plant cell-wall-degrading enzymes facilitated the release of oil. Yield of olive oil increases after enzymatic treatment (Kashyap *et al*.,2001).

Other important uses of alkaline pectinases are in pulp and paper industry, in preparation of poultry feed and purification of plant viruses (Hoondal *et al*.,2002).

1.2.3.6. Alkaline Lipases

Lipases are serine hydrolases. They catalyze the hydrolysis or formation of ester bonds between glycerol and long-chain fatty acids (Sharma *et al*., 2001). General characteristics of lipases were summarized in Table 1.8 (Sharma *et al*., 2001).

Table 1.9. General Characteristics of Lipases.

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

Most studied alkaline lipases have been thermoalkalophilic lipases (Jeong *et al*.,2001). They are produced mainly by the thermophilic *Bacillus* strains. They show unique thermoactivity and substrate specificity. Therefore they are applied in the enzymatic processing of lipids at high temperatures and in organic solutions. Although they have 90-95% amino acid sequence identity and 30-35% homology with lipases of pathogenic *Staphylococcus* strains, they only display less than 15% sequence homology with those of other *Bacillus* strains (Jeong *et al*., 2001).

The properties of some alkaline lipases were summarized in Table 1.10.

Table 1.10. Properties of Some Alkaline Lipases.

Source	M.W. (kDa)	pH optima	Temp. optima (°C)	Inhibitors	Enhancers	Properties	Temperature Stability ($T_{1/2}$)	Substrate specificity	Reference
Bacillus sp. strain A 30-1 (ATCC 53841)	65	9. 5-10.5	60	Zn^{2+} Fe^{2+}	Cyclodextrin (α,β,γ), Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}	-Stable in the presence of hydrogen peroxide, alkaline protease -EDTA, p-chloromercuribenzoate have no influence on activity	28 h at 50 °C 20 h at 60 °C 16 h at 65 °C	-Triglycerides of C-16:0 to C-22:0 fatty acids -natural fats -oils	Wang *et al.* 1995
Bacillus sp. RS-12		8	50-55				60 m at 65 °C 18 m at 70 °C 15 m at 75 °C	-	Sidhu *et al.*, 1998
Bacillus sp. RSJ-1	37	8.0-9.0	50	Cs^{+}, K^{+}, Co^{+}, Zn^{2+}	Ca^{2+}, Na^{+}, Mg^{2+}, Ba^{2+}	EDTA has no influence on activity	240 m at 55 °C 150 m at 60 °C 90 m at 65 °C 45 m at 70 °C 30 m at 75 °C	-	Sharma *et al.*, 2001

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

Recently, an alkalophilic lipase has also been used in the soaking, washing, dehairing and bating processes of leather (Pandey *et al*., 1999).

1.3. Characterization Methods for Bacteria

1.3.1. Morphological Methods

In identifying bacteria, the morphological features are of importance in that they constitute the first step in characterization. The form, diameter, elevation and margin of colonies, pigment formation can be observed directly. Cellular morphology, Gram status, sporulation and motility of an isolate can be determined by microsope after different staining methods. Phase contrast microscopy is also used to determine spore presence and cellular morphology without staining.

1.3.2. Phenotypic Methods

Phenotypic methods include biotyping, antibiogram, phage typing, serotyping, PAGE/immunoblotting and multilocus enzyme electrophoresis (MLEE).

In biotyping an organism can be identified and classified at the genus and/or species level using various biochemical reagents and parameters.

Antibiogram includes the analysis of the growth of strain in the presence of antibiotic. However it is not very discriminatory and for example antibiogram patterns can change with transformation of plasmids (Busch and Nitschko, 1999).

In phage typing, a particular bacterium is infected by a specific phage. Thus, the isolates can be differentiated based on the infection capability.

Serotyping involves identification of microorganisms according to their reaction to a given antiserum.

Proteins from whole-cell lysate can be separated by SDS-PAGE. Protein patterns are used in classification of strains (Busch and Nitschko, 1999).

1.3.3. Genotypic Methods

Genotypic methods are based on DNA analysis of chromosomal or extrachromosomal (plasmid) genetic material. The main advantages can be summarized as follows:

- They are able to distinguish between two closely related strains: High discriminatory power.
- All strains are typeable since it is always possible to extract DNA from bacteria
- Analytical strategies can be applied to DNA of any source since it is always possible to extract DNA from bacteria.
- The composition of DNA is not affected by cultural conditions or preparation methods.
- Resulting data can be analysed statistically (Farber, 1996).

Genotypic methods include plasmid typing, pulse-field gel electrophoresis, ribotyping, polymerase chain reaction based methods, nucleotide sequencing, DNA-DNA hybridization (Farber,1996, Goodfellow, 2000). Some of these methods were summarized below.

1.3.3.1. Plasmid Typing

Plasmids are self-replicating, extrachromosomal, usually supercoiled genetic elements (Bush and Nitschko, 1999, Farber, 1996). Plasmids generally encodes for products and/or functions which modify the phenotype of the cell.

In plasmid typing, plasmids are isolated from bacterial strains, then their number and size are determined by gel electrophoresis. However different plasmids can be of the same size. Using restriction enzymes, this problem can be solved. Different plasmids will give different fragment patterns (Farber, 1996). The main drawback of the method is the transfer of plasmid between strains and species.

1.3.3.2. Chromosomal DNA Restriction Endonuclease Analysis

In this method, DNA is cut with a frequent-cutting restriction enzyme and the fragments are electrophoresed on agarose gel. Differences in fragment patterns between isolates is referred to restriction fragment length polymorphism (RFLP). Different patterns are due to DNA composition variations. This method is rapid, inexpensive, relatively easy to perform and universally applicable. But interpretation of fragment pattern is not easy since numerous fragments are obtained and they are closely spaced on agarose. In order to obtain interpretable results, several restriction enzymes must be used.

1.3.3.3. Ribotyping

Ribotyping is based on the use of nucleic acid probes to recognize ribosomal genes. In a prokaryotic ribosome there are three types of RNA (23S, 16S and 5S rRNA). The genes coding for rRNA sequences are highly conserved and multiple copies of the rRNA operon exist in most bacteria. Thus, chromosomal fragments containing a ribosomal gene are revealed after hybridization with probes. Resulting hybridization bands (approximately 1 to 15) are compared between isolates. Ribotyping refers to the grouping of bacteria based on this method.

1.3.3.4. PCR-Based Methods

PCR is based on the amplification of DNA by a heat stable DNA polymerase enzyme. Depending on the special primer used, the region of interest is amplified. Reaction includes repeated cycles of high temperature for denaturation of the DNA, oligonucleotide (primer) annealing and an extension step mediated by thermostable DNA polymerase. Amplification cycle is repeated 25-35 times to produce a $>10^6$ fold amplification of the target DNA. The amount of the target DNA is exponentially increased.

1.3.3.4.1. PCR-Ribotyping

In a prokaryotic ribosome genes coding for rRNA are separated by spacer regions which are variable in length or sequence at both the genus and species level (Farber,

1996). Thus, multiple bands are obtained after amplification of spacer regions in different rRNA coding operons for a particular strain.

Besides, spacer region between 16S-23S rRNA or 23S-5S can be amplified and amplification products can be compared on agarose gel. In this method, availability of universal primers is the major advantage.

Sequence variation between ribosomal operons, described especially in the ISR between the 16S and 23S rDNA genes in individual strains (Gürtler and Stanisich, 1996), has been used for bacterial identification (Jensen *et al.*, 1993, Tilsala-Timisjarvi and Alatossava, 1997). Ribosomal internal spacer regions have been found to be more variable than 16S and 23S rDNA between bacterial species (Barry *et al*., 1997). For example, closely related species *B. subtilis* and *B. atrophaeus* have been differentiated by comparing ISRs (Nagpal *et al*., 1998). Flint *et al*. (2001) have shown that ISR sequences have varied in length among the different lactic acid bacterial species and have varied also within some strains of the same species.

1.3.3.4.2. LH-PCR (Length Heterogeneity Analysis of Polymerase Chain Reaction Amplified DNA)

This method is based on the natural length variation within 16S rDNA genes. The variable region is amplified by PCR with fluorescently labeled universal primers that recognize the region in all eubacteria (Tiirola *et al*., 2003).

1.3.3.4.3. PCR-RFLP

In this method PCR amplicons are digested with suitable restriction enzymes. Digested amplicon is run onto an agarose gel and DNA fingerprint results are obtained. 16S, 23S and 16S-23S rRNA spacer regions have been used for locus specific RFLP (Olive and Bean, 1999, Caccamo, 2001).

1.3.3.5. Genomic DNA Based RFLP

PFGE (Pulse Field Gel Electrophoresis) is a very discriminating and reproducible typing method. In this method, intact cells are embedded in agarose plugs to

prevent the shearing of DNA during DNA extraction. Then, these plugs are treated with detergent and enzymes to isolate the DNA. In the following step, the isolated DNA is cut with an infrequently cutting restriction endonuclease which recognizes specific 8-base cutter or 6-base cutter sequences. These enzymes are chosen depending on the G+C content of the bacterial genome. After digestion, very large DNA fragments (10-800 kb) are obtained.

Bacterial plugs are inserted into agarose gel and they are subjected to electrophoresis. In PFGE system the electrical field is alternated at predetermined intervals. At these intervals, called switch time or pulse time, the direction of electrical field is changed. Consequently, the separation of high molecular weight DNA fragments is performed.

Agarose concentration, buffer concentration, pulse times, voltage and electrophoresis run time are important parameters which affect the separation of fragments (Olive and Bean,1999, Busch and Nitschko,1999, Farber, 1996).

1.3.3.6. DNA Sequencing

In this method, the nucleotide composition of a DNA molecule is determined. Generally the 16S rRNA gene or the 16S rRNA are sequenced because they contain variable and conserved regions within bacterial species. Besides, sequencing of whole genome is not practical. Evolutionary trees are constructed based on 16S rRNA.

1.3.3.7 DNA-DNA Hybridization

Denatured complementary strands of DNA can reassociate to form native duplexes under suitable experimental conditions. Nucleic acid fragments are paired according to the similar linear arrangements of the bases along the DNA. Nucleotide sequence similarity between bacterial strains can be detected by measuring the amount of molecular hybrid and its thermal stability (Goodfellow, 2000).

Microbial species are extensively delineated using DNA-DNA relatedness data (Stackebrandt and Goebel, 1994).

1.4. Thesis Objectives

The purpose of this study was,

- Isolation of alkalophilic *Bacillus* strains
- Screening for their extracellular alkaline enzymes: protease, amylase, xylanase, cellulase, lipase and pectinolytic enzymes
- Characterization of these isolates by phenotypic and genotypic methods.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used were listed in Appendix A.

2.1.2. Samples used for isolation

Sample Type	Location	Sample number
Soil	Izmir-Tuzla	10
Horse feces	Izmir-Bostanlı	2
Leather processing	Izmir-Menemen and Ege University	15

2.1.3. Reference strains

1) *Bacillus alcalophilus* NRRL B-14309

2) *Bacillus clausii* NRRL B-23342

3) *Bacillus gibsonii* NRRL B-23346

4) *Bacillus halmapalus* NRRL B-23347

5) *Bacillus pseudofirmus* NRRLB-23349 were kindly provided by Prof. Dr.L.K.Nakamura (Microbiologist Emeritus, Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Departement of Agriculture).

2.2. Methods

2.2.1. Isolation and Preservation of the Isolates

2.2.1.2. Isolation of Alkalophilic *Bacillus*

Ten grams of aseptically taken samples were transfered to 90 ml of 0.85% saline water. Suspensions were shaked for 2 hours in room temperature. Afterwards the samples were incubated for 10 m at 80 °C in waterbath to kill most of the vegetative cells (Mora *et al*., 1998). Following heat treatment, 1ml aliquots from each sample was transferred in 9ml of 0.85% saline water and 6 fold dilutions were prepared. Dilutions were plated on Horikoshi-I agar plates (Appendix B.1) and incubated at 37 °C for 24-72 h. Single colonies showing different morphologies were picked and they were purified using streak plate method.

2.2.1.3. Preservation of Isolates

Glycerol stocks were prepared and stored at -80 °C for long term preservation. Cultures were grown on Horikoshi-I agar plates. They were transferred in Horikoshi-I broth (Appendix B.1) until a turbidity formed. 0.5 ml of each culture broth were transferred into the cyrotubes and 0.5 ml broth containing 40% glycerol were added. Tubes were mixed gently and thoroughly and were stored at -80 °C freezer.

2.2.2. Activation of Isolates

Isolates from glycerol stocks were inoculated on Horikoshi-I agar (Appendix B.1) plates and incubated for 24 h at 37 °C before using for tests.

2.2.3. Phenotypic Identification of Alkalophilic *Bacillus*

2.2.3.1. Gram Staining

Cultures grown on solid media for 24 h were suspended on a microscopic slide with 2-5 µl of distilled water to a faint turbidity. Smear was prepared by spreading the drop with a lamel, it was then dried completely. The underside of the slide was passed three times over the flame of a Bunsen burner to induce adherence.

Following treatments were performed:

- Crystal violet staining reagent (Appendix D.2.1) for 1 m.
- Washing under the tap water for some seconds gently and indirectly
- Staining in iodine mordant (Appendix D.2.2) for 1 m.
- Washing under the tap water
- Washing with 95% ethanol for 6 s
- Washing under the tap water
- Staining in safranin counterstain (Appendix D.2.3) for 30 s
- Washing under the tap water

Finally, the slide was dried and the cells were observed under the light microscope. Purple cells were gram positive and pink coloured cells were gram negative (Murray *et al*., 1994). Cellular morphology was also determined.

2.2.3.2. Examination of Endospores

Isolates grown on Horikoshi-I agar medium (Appendix B.1) for 2-3 days were suspended in 3-5 µl of sterile distilled water on a microscopic slide and covered with a lamel. Endospores were observed as shiny bodies in cells under the phase-contrast microscope.

2.2.3.3. Catalase Test

Three percent hydrogen peroxide solution was poured onto the colonies grown on Horikoshi-I medium (Appendix B.1) for 24-48 h. Formation of air bubbles indicated the presence of catalase enzyme (Smibert and Krieg, 1994).

2.2.3.4. Oxidase Test

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 tamped lightly onto the wet filter paper. Formation of a blue-purple colour was taken as the evidence for oxidase activity (Tarrand *et al*., 1982)

2.2.3.5. Extracellular Enzyme Screening on Solid Agar Media

2.2.3.5.1. Protease Screening

Polypeptone in Horikoshi-I medium (Appendix B.1) was substituted for skimmed milk. Skimmed milk was autoclaved separately for 5 m at 110 °C (twice). Microorganisms were inoculated on solid media. After 1-3 days of incubation, colonies surrounded by a clear zone were determined as protease producers (Horikoshi, 1999b).

2.2.3.5.2 Amylase Screening

0.5% soluble starch was added in modified mineral medium (Appendix B.3)(Fritze *et al*., 1990). After 2 days of incubation at 37 °C, iodine solution (I_2 =1 g, KI= 2 g/ 300 ml)was poured onto the plates (Bragger *et al*., 1989). Amylase producers were surrounded by a clear halo (Horikoshi, 1999b).

2.2.3.5.3 Xylanase Screening

Modified mineral medium (Appendix B.3) containing 0.5% xylan was used (Fritze *et al*., 1990). After 2 days of incubation at 37 °C, the plates were covered with 0.1 aqueous Congo Red for 15 m. Repeated washing with 1 M NaCl followed the staining. Xylanase producers were surrounded by yellow zone on red medium (Gessesse and Gashe, 1997).

2.2.3.5.4. Cellulase Screening

Cellulase screening was similar to that of xylanase. Here, however 0.5% carboxymethycellulose were used as substrate instead of xylan.

2.2.3.5.5. Pectinolytic Enzyme Screening

The isolates were inoculated on medium described in Appendix B.4. Two different substrates were used: pectin and polygalacturonic acid. After 3 days of incubation at 37 °C , 1% (w/v) cetyltrimethyl ammonium bromide (CTAB) was poured onto the plates and incubated for 10 m at room temperature. Colonies surrounded by a clear zone were taken as pectinolytic enzyme producers. (Kobayashi *et al*.,1999)

2.2.3.5.6. Lipase Screening

The medium described in Appendix B.5 was supplemented with either of two lipid substrates: Tween 20 and Tween 80. After inoculation of isolates, the plates were incubated for 2 days at 37 °C. An opaque halo formed around the colonies were taken as the indication of lipolytic activity (Haba *et al*., 2000).

2.2.3.6. Growth at Different NaCl Concentrations

The growth of microorganisms was observed for 4-5 days on nutrient agar (Appendix B.2) plates containing 5%, 7% or 10% NaCl (pH= 9.9 for medium containing 5% NaCl and 9.7 for media containing 7% and 10% NaCl) at 37 °C.

2.2.3.7. Growth at Different Temperatures

Isolates were inoculated onto the alkaline nutrient agar (Appendix B.2) plates and were then incubated at 45 °C, 50 °C and at 55 °C. The growth was checked for 4-5 days.

2.2.3.8. Growth at pH 7

Nutrient agar plates (pH 7) were used for pH test. The growth was monitored periodically for 5 days.

2.2.4. Genotypic Identification of Alkalophilic *Bacillus*

2.2.4.1. Identification by 16SrDNA-ITS-PCR

2.2.4.1.1. DNA Isolation

Modified method of Ausubel *et al*. (1994) was used for genomic DNA isolation. Isolates were grown on Horikoshi-I agar (Appendix B.1) plates for 24 h at 37 °C. Cells were transferred into 0.5 ml of sterilized distilled water. Tubes were centrifuged for 5 m at 10.000 rpm. The supernatant was discarded and the pellet was suspended in 567 µl of 1×TE buffer (Appendix C.4) . Afterwards, 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added , mixed thoroughly and incubated for 1h at 37 °C. Then 0.1 ml of 5 M NaCl were added and mixed well. After the addition of 80 µl of CTAB/NaCl (Appendix C.8), the tubes were mixed and incubated for 10 m at 65 °C. Following the incubation, 0.7ml of chloroform/isoamyl alcohol (Appendix C.2) were added, mixed thoroughly and centrifuged for 5 m at 10000 rpm. Aqueous, viscous supernatant was removed to a fresh microcentrifuge tube. An equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged for 5 m. The supernatant was again transferred into a fresh microcentrifuge tube and 0,6 volume of isopropanol were added to precipitate the nucleic acids. The tube was shaken back and forth until a white DNA precipitate became visible. It was centrifuged for 5 m, the supernatant was discarded and DNA pellet was washed with 500 µl 70% ethanol. It was centrifuged for 10 m. After this, ethanol was discarded with a micropipette, the pellet was dried and dissolved in 1×TE. The tubes were preserved at -20 °C. Genomic DNA was dissolved by alternating heat-cold shocks (at 80 °C for 10 m and at -20 °C for 20 m).

2.2.4.1.2. Amplification of 16SrDNA-ITS Region

16SrDNA-ITS region of rRNA genes of the isolates and reference strains were amplified using the following DNA oligoprimers (Appendix F):

L1: 5'- CAAGGCATCCACCGT -3' (Mora *et al.,* 1998)
EGE 1 5'- AGAGTTTGATCCTGGCTCAG -3' (Jensen *et al.,* 1993)

First 2 µl of genomic DNA (approx. 500 µg) were transferred into 0.5 ml eppendorf tubes, as the template. PCR mix (Appendix E.1) was prepared and after the addition of *Taq* DNA polymerase (1,25 U), it was mixed gently and centrifuged for 2-3 seconds. Forty-eight microliters 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 2-3 s at 6000 rpm. Finally, they were placed into the wells of a Mini Cycler System (MJ Research INC, USA) using following program:

Step1: 94 °C for 5 m
Step2: 94 °C for 1 m (denaturation)
Step3: 42 °C for 1m (annealing)
Step4: 72 °C for 1m (elongation)
Step5: 72 °C for 10 m (final extension)

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

2.2.4.1.3. Separation of Amplification Products by Agarose Gel Electrophoresis

Amplified fragments were separated in 0.8 % agarose gel. First, 0.96 ml of agarose was dissolved in 120 ml of 1×TAE buffer (Appendix C.4) by boiling(15 µl of ethidium bromide solution (10 mg/ml) were added after cooling the solution to 40 ° C.). The gel was then poured into the gel casting stand and the combs were placed. 1×TAE buffer was carefully added until the buffer was 2-3 mm above the surface of the gel. Five microliters of PCR products were taken and mixed with 1 µl, 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 were loaded into the first well and they were electrophoresed at 40 mA constant voltage until bromophenol blue reached the other end of the gel. DNA fragments were visualized in a photodocumentation system (Vilber-Lourmat, France).

2.2.4.1.4. Chloroform Extraction of Amplification Products

The volume of the samples were adjusted to 100 µl by the addition of 55 µl 1×TE buffer. Two hundred microliter of chloroform were added onto the samples and mixed. Then tubes were centrifuged for 2 m at 6000 rpm. Organic phase was discarded and chloroform extraction was repeated. After that, aqueous phase was transferred into new tubes containing 1/10 sample volume of 3 M sodium acetate and mixed. 2.5 sample volumes of 99% ethanol were added and mixed well. The samples were then centrifuged for 15 m at 7000 rpm. Liquid phase was discarded and DNA pellets were washed with 300 µl, 70% ethanol. After centrifugation for 5 min at 7000 rpm, ethanol was removed. The pellets were dried at room temperature and were then dissolved in 30 µl 1×TE buffer.

2.2.4.1.5. Restriction Enzyme Digestion of 16S-ITS rRNA Gene Fragments

Purified and amplified 16S-ITS ribosomal RNA gene fragments were first electrophoresed in 1% agarose gel to estimate DNA concentration. Eight microliters or 12,5µl of samples were transferred in 1.5 ml eppendorf tubes. Restriction reaction was set up as described in Appendix E.5. Each PCR product was digested by both *Taq* I and *Hae* III restriction enzymes. Because *Taq* I works at 65 °C, samples digested with this enzyme were overlaid with mineral oil to prevent evaporation. Digestion with *Hae* III was carried out at 37 °C. After the digestion the samples were stored at -20 °C.

2.2.4.1.6. Chloroform Extraction of Digestion Products

After the restriction digestion, the volume of the samples were adjusted to 100 µl with 1×TE buffer. Two hundred microliters of chloroform/isoamyl alcohol solution were added onto the samples, mixed and centrifuged for 5 m at 6500 rpm. Aqueous

phase was transferred into new eppendorf tubes containing 0,1 volume of 3 M sodium acetate (pH= 5.2, chilled on ice). They were mixed. Two hundred and fifty microliters of 99% ethanol (chilled on ice) were added and mixed. The samples were then centrifuged for 15 m at 7000 rpm, at 4°C. Liquid phase was removed and pellets were washed with 250 µl of 70% ethanol and centrifuged for 5 m at 7000 rpm, at 4 °C. Ethanol was removed and the pellets were dried at room temperature and were then dissolved in 15 µl 1×TE buffer. Dissolved DNA was kept at -20 °C.

2.2.4.1.7. Electrophoresis of Restriction Fragments

Restriction fragments were first separated in 2 % agarose gel containing 20 µl of ethidium bromide solution (10mg/ml). Electrophoresis was carried out at 60 mA for the first 20 m, then the current was adjusted to 80 mA and the electrophoresis was continued further for 2.5 h.

Some isolates produced similar restriction fragment patterns. These were contained within distinct groups. One digested sample from each group was picked and re-electrophoresed in order to produce concise restriction digestion results for further restriction profile analyses.

Chapter 3

RESULTS AND DISCUSSION

3.1. Isolation of Alkalophilic *Bacillus*

Isolation was carried out using samples of soil, horse feces and leather processing steps on Horikoshi-I agar plates (pH 10.2). Single colonies were purified by repeated streaking. Colony morphologies were determined under the light microscope. Coccus shaped bacteria, actinomycetes and unpure isolates were eliminated. In total 116 rod-shaped isolates were selected for further studies. The source of isolates was demonstrated in Table 3.1.

Table 3.1. Source of the Isolates.

Source	**Name of the Isolates**
Horse faeces	51, 72, 94, 96, 56
Soil	4, 11, 18, 33, 34a, 34b, 39, 40, 42, 43a, 47, 49a, 49b, 52, 62, 70, 74,76, 79a, 79b, 86, 88, 93, 97, 100, 104,106,
Leather processing steps	S-1, S-6, S-12, S-13, S-14, S-15, S-16, S-19, S-20, S-21, S-24, S-25, S-32, S-34, S-38, S-40, S-41, S-44, S-45, S-47, S-48, S-49 , S-51, S-58, S-59, S-62, S-63, S-65, S-68, S-76, S-79,S-81, S-85, S-86, S-90 S-93, S-95, S-98, S-103, S-105, S-111, S-112, D-4, D-7, D-8, D-9, D-12, D-13, D-14, D-19, D-25, D-26,D-29, D-32, D-36, D-37a, D-38 , D-41, D-45, D-51, D-52, D-60, D-61, D-62, D-63, D-65, D-68, D-69, D-77, D-89, D-90, D-93, D-94, D-96, D-97, D-98, D-99

3.2. Phenotypic Characterization of Alkalophilic *Bacillus*

3.2.1 Phase Contrast Microscopy

Isolates were examined under the phase contrast microscope to investigate the presence of endospores. The observation was performed on the second or third day of their growth using Horikoshi-I agar plates. When 0.0005% $MnCl_2$ (Travers, 1987) was included into medium, increase in sporulation rate was observed. All the isolates were found to contain endospores.

3.2.2. Light Microscopy

All the isolated strains were Gram stained. They were always observed as purple cells under the light microscope and therefore, they were all Gram positive.

3.2.3. Catalase and Oxidase Tests

Isolates were catalase positive except D-19. Oxidase presence was determined in isolates except 33, 79b, S-38, S-40, D-63, D-77, D-93 and D-94. Weak oxidase production was also observed in 37, 39, 40, 43a, 47, 54, S-41, S-45, S-48, D-62, D-87 and D-89.

3.2.4. Physiological Tests

Growth at pH 7 was observed to determine whether the isolates were obligate alkalophilic (Table 3.2). Ten isolates (D-19, D-36, D-37a, D-41, D-77, D-89, D-90, D-93, D-94, S-76) could not grow at this pH value.

According to the study of Nielsen et *al*. (1995) *B. pseudofirmus* and *B. alcalophilus* have been strictly alkalophilic whereas the growth of reference strains used in this study was observed on the third day. They have observed that *B. halmapalus* has grown at pH 7 while no growth was observed at this pH value in this present study.

Growth of the isolates were also observed at 5% , 7%, 10% NaCl concentrations and at 45 ºC, 50 ºC and 55 ºC respectively on alkaline nutrient agar plates (Table 3.3).

Some isolates gave variable reactions for physiological tests, as observed for example for S-6 at 10% NaCl and for D-51 at 55 ºC (Table 3.3).

Type of the medium used for temperature tests was found to affect the temperature tolerance. Twelve isolates (S-68, S-81, S-85, S-87, S-89, S-90, D-4, D-7, S-95, S-98, S-111, S-112) could grow at 50 ºC, but not at 55 ºC, on alkaline nutrient agar plates (pH 10.3). On the other hand, a good growth was observed for all the twelve isolates at 55 ºC on Horikoshi-I agar plates (pH 10.2).

Table 3.2. Growth at pH 7.

Strain No.	Day of Growth			Strain No.	Day of Growth			Strain no	Day of Growth		
	1.	2.	3-5.		1.	2.	3-5.		1.	2.	3-5.
4	+	+	+	**S-19**	+	+	+	**D-7**	+	+	+
11	+	+	+	**S-20**	+	+	+	**D-8**	+	+	+
18	+	+	+	**S-21**	+	+	+	**D-9**	-	+	+
33	w	w.	+	**S-24**	+	+	+	**D-11**	+	+	+
34a	+	+	+	**S-25**	+	+	+	**D-12**	+	+	+
34b	+	+	+	**S-32**	+	+	+	**D-13**	+	+	+
39	+	+	+	**S-34**	-	-	+	**D-14**	+	+	+
40	w.	w.	+	**S-38**	-	-	+	**D-19**	-	-	-
42	+	+	+	**S-40**	-	-	+	**D-24**	+	+	+
43a	+	+	+	**S-41**	-	-	+	**D-25**	+	+	+
47	+	+	+	**S-44**	w.	w.	+	**D-26**	-	-	+
49a	+	+	+	**S-45**	w.	w.	+	**D-29**	+	+	+
49b	+	+	+	**S-47**	-	-	+	**D-32**	-	+	+
51	+	+	+	**S-48**	-	-	+	**D-36**	-	-	-
52	+	+	+	**S-49**	-	-	+	**D-37a**	-	-	-
56	+	+	+	**S-51**	w.	w.	+	**D-38**	-	-	+
62	+	+	+	**S-58**	+	+	+	**D-41**	-	-	-
70	+	+	+	**S-59**	+	+	+	**D-45**	-	-	+
72	+	+	+	**S-60**	+	+	+	**D-51**	+	+	+
74	+	+	+	**S-61**	+	+	+	**D-52**	+	+	+
76	v.w.	+	+	**S-62**	+	+	+	**D-60**	-	-	+
79a	+	+	+	**S-63**	+	+	+	**D-61**	-	-	+
79b	+	+	+	**S-65**	+	+	+	**D-62**	-	-	+
86	+	+	+	**S-68**	+	+	+	**D-63**	-	-	+
88	+	+	+	**S-76**	-	-	-	**D-65**	+	+	+
93	+	+	+	**S-79**	-	-	+	**D-68**	+	+	+
94	+	+	+	**S-81**	+	+	+	**D-69**	+	+	+
96	+	+	+	**S-85**	+	+	+	**D-77**	-	-	-
97	+	+	+	**S-86**	+	+	+	**D-79**	+	+	+
100	+	+	+	**S-87**	+	+	+	**D-82**	+	+	+
104	+	+	+	**S-90**	+	+	+	**D-89**	-	-	-
106	+	+	+	**S-93**	+	+	+	**D-90**	-	-	-
S-1	+	+	+	**S-95**	+	+	+	**D-93**	-	-	-
S-6	+	+	+	**S-98**	+	+	+	**D-94**	-	-	-
S-12	+	+	+	**S-103**	-	w.	+	**D-96**	+	+	+
S-13	+	+	+	**S-105**	+	+	+	**D-97**	-	-	+
S-14	+	+	+	**S-111**	+	+	+	**D-98**	-	-	+
S-15	+	+	+	**S-112**	+	+	+	**D-99**	-	w.	+
S-16	+	+	+	**D-4**	+	+	+				

Table 3.3. Physiological Test Results.

Strain no	Growth at 5%NaCl	Growth at 7%NaCl	Growth at 10%NaCl	Growth at 45 ºC	Growth at 50 ºC	Growth at 55 ºC
4	+	-	-	+	-	-
11	+	+	+	-	-	-
18	+	+	+	+	+	-
33	+	+	+	-	-	-
34a	+	-	-	+	-	-
34b	+	+	-	+	-	-
39	+	-	-	+	+	-
40	+	+	-	-	-	-
42	+	-	-	+	-	-
43a	+	-	-	+	-	-
47	+	-	-	+	-	-
49a	+	-	-	+	-	-
49b	+	+	-	+	-	-
51	+	+	-	+	+	-
52	+	+	-	+	-	-
56	+	+	+	+	+	+
62	+	+	-	+	-	-
70	+	+	-	+	-	-
72	+	+	-	+	+	-
74	+	-	-	+	-	-
76	+	+	+	-	-	-
79a	+	-	-	+	-	-
79b	+	+	-	+	-	-
86	+	+	-	+	-	-
88	+	+	-	+	-	-
93	+	-	-	+	+	-
94	+	-	-	+	+	-
96	+	-	-	+	-	-
97	+	+	-	+	-	-
100	+	+	-	-	-	-
104	+	+	-	+	+	-
106	+	+	-	+	-	-
S-1	+	+	+	+	+	+
S-6	+	+	+/-	+	+	-
S-12	+	+	+	+	+	-
S-13	+	+	+	+	+	-
S-14	+	+	+	+	+	-
S-15	+	+	+	+	+	-
S-16	+	+	+	+	+	-
S-19	+	+	+	+	+	-
S-20	+	+	+	+	+	-
S-21	+	+	+/-	+	+	-

(Cont. on next page)

Table 3.3. (cont.)

Strain no	Growth at 5%NaCl	Growth at 7%NaCl	Growth at 10%NaCl	Growth at 45 ºC	Growth at 50 ºC	Growth at 55 ºC
S-24	-	-	-	+	-	-
S-25	+	+	+/-	+	+	-
S-32	+	-	-	-	-	-
S-34	+	+	-	-	-	-
S-38	+	+	+	+	-	-
S-40	+	+	+	+	-	-
S-41	+	+	+	+	-	-
S-44	+	+	+	+	+	-
S-45	+	+	+	+	+	-
S-47	+	+	+	-	-	-
S-48	+	+	+	+	-	-
S-49	+	+	+	+	-	-
S-51	+	+	+	+	+	-
S-58	+	+	+	+	+	+
S-59	+	+	+	+	+	-
S-60	+	+	-	+	+	-
S-61	+	+	-	+	+	-
S-62	+	+	+	+	+	+
S-63	+	+	+	+	+	-
S-65	+	+	+	+	+	+
S-68	+	+	+	+	+	-
S-76	+	+	-	-	-	-
S-79	+	+	+	-	-	-
S-81	+	+	+/-	+	+	-
S-85	+	+	+	+	+	-
S-86	-	-	-	+	-	-
S-87	+	+	-	+	+	+
S-90	+	+	-	+	+	-
S-93	+	+	+	+	+	-
S-95	+	+	+	+	+	-
S-98	+	+	-	+	+	-
S-103	+	+	+	+	+	-
S-105	+	+	+	-	-	-
S-111	+	+	-	+	+	-
S-112	+	+	-	+	+	-
D-4	+	+	+	+	+	-
D-7	+	+	+	+	+	-
D-8	+	+	-	+	+	-
D-9	+	+	+	-	-	-
D-11	+	+	+	+	+	-
D-12	+	+	+/-	+	+	-
D-13	+	+	+/-	+	+	-
D-14	+	+	+	+	+	-

(Cont. on next page)

Table 3.3. (cont.)

Strain no	Growth at 5%NaCl	Growth at 7%NaCl	Growth at 10%NaCl	Growth at 45 ºC	Growth at 50 ºC	Growth at 55 ºC
D-19	+	+	-	-	-	-
D-24	+	+	+	+	+	+
D-25	+	+	+/-	+	+	-
D-26	+	+	+	-	-	-
D-29	+	+	+	+	+	-
D-32	+	+	+	-	-	-
D-36	+	+	-	-	-	-
D-37a	+	+	-	-	-	-
D-38	+	+	-	-	-	-
D-41	+	+	-	-	-	-
D-45	+	+	-	-	-	-
D-51	+	+	+	+	+	-
D-52	+	+	+	+	+	-
D-60	+	+	-	+	-	-
D-61	+	+	-	-	-	-
D-62	+	+	-	-	-	-
D-63	+	+	-	-	-	-
D-65	+	+	+	+	+	-
D-68	+	+	+	+	+	-
D-69	+	+	+	+	+	+
D-77	+	+	-	-	-	-
D-79	+	+	+	+	+	-
D-82	+	+	+	+	+	-
D-89	+	+	+	-	-	-
D-90	+	+	+	-	-	-
D-93	-	-	-	-	-	-
D-94	+	+	-	-	-	-
D-96	+	+	-	+	+	-
D-97	+	+	-	-	-	-
D-98	+	+	+	-	-	-
D-99	+	+	+	-	-	-

3.2.5. Extracellular Enzyme Screening

The enzyme screening was performed on solid agar media and was based on the observation of zones around the colonies (Figure 3.1). Isolates producing different enzyme groups were thus identified (Table 3.4).

3.2.5.1. Optimization of Conditions for Amylase and Xylanase Zone Detection

Clear zones around amylase producing colonies were determined after an incubation period of about 15-20 m following the addition of iodine solution. The reason of incubation time was the increase in diameter of zones with the evaporation of solution. Clear zones which did not appear at the beginning could be observed better after 15-20 min. incubation.

Incubation time of isolated strains on mineral medium was optimized for xylanase screening. When plates were incubated for 3 days, clear zones covered all the plate and made impossible to determine xylanase producers. Two days of incubation was found to be suitable for the zone observation.

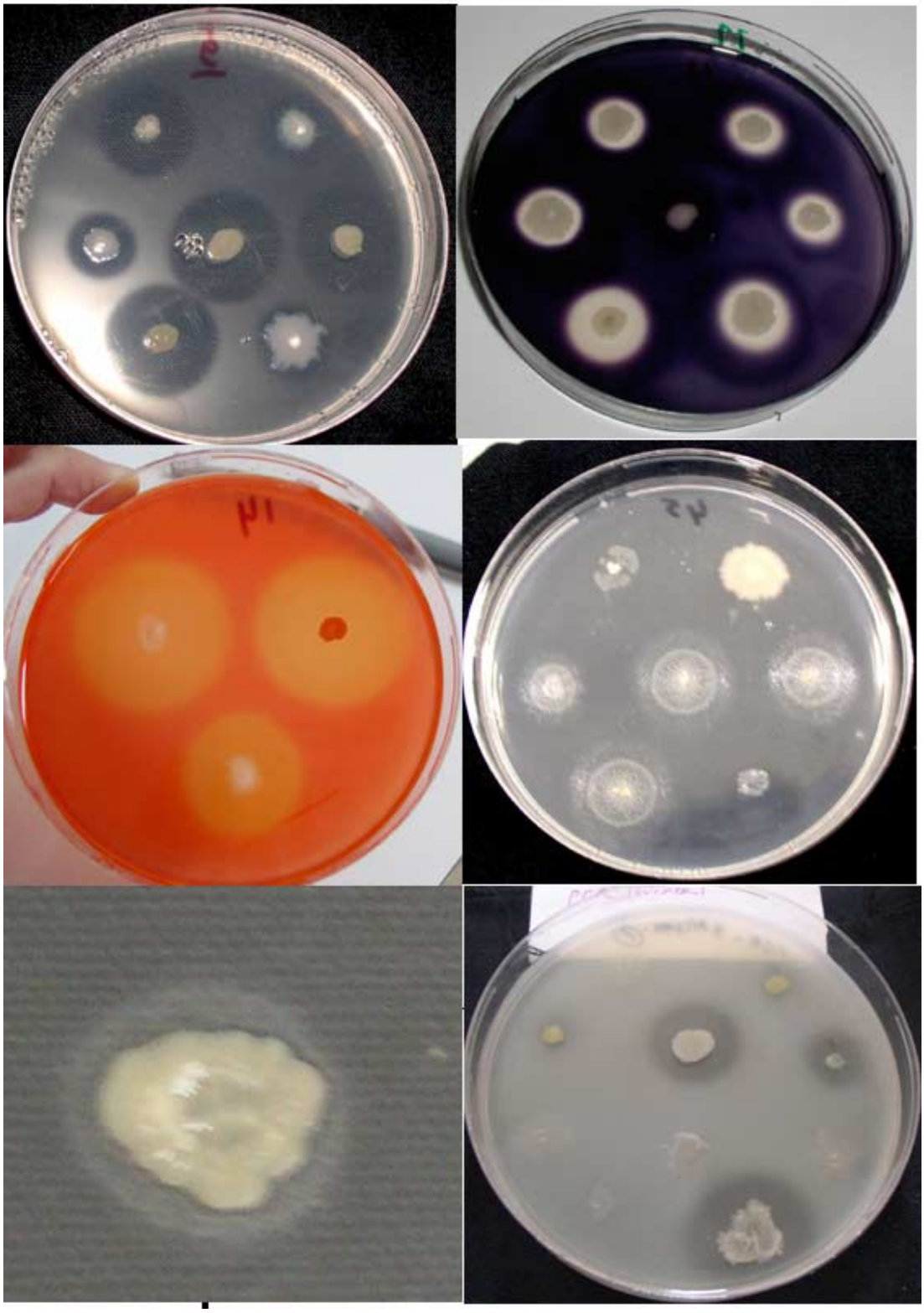

Figure 3.1. Extracellular Enzyme Screening.

1.Protease; 2. Amylase; 3. Xylanase (same observation for cellulase); 4. Lipase (Substrate T20); 5. Lipase (Substrate T80); 6. Pectinase

3.2.5.2. Extracellular Enzyme Groups

Isolated strains were classified into 18 groups in respect of the enzymes they produced (Table 3.5.). For example while some isolates produced only one type of enzyme (E-1 in Table 3.5.), another group could produce four different enzyme (E-15 in Table 3.5).

Table 3.4. Extracellular Enzyme Profiles.

PGA: polygalacturonic acid, T20: Tween20, T80: Tween 80,

Strain no	Protease	Amylase	Xylanase	Cellulase	Pectinolytic Enzyme		Lipase	
					Substrate PGA	Substrate Pectin	Substrate T 20	Substrate T80
4	+	-	-	-	-	-	+	+
11	+	+	-	-	+	+	-	-
18	+	+	-	-	+	+	-	-
33	+	+	+	+	-	+	+	-
34a	+	-	-	-	-	-	+	+
34b	+	-	-	-	-	-	-	-
39	-	+	-	-	-	-	-	-
40	+	+	-	-	-	-	-	-
42	-	+	-	-	-	-	-	-
43a	-	+	-	-	-	-	-	-
47	-	+	-	-	-	-	-	-
49a	+	-	-	-	-	-	+	-
49b	+	-	-	-	-	-	+	+
51	+	+	-	-	+	+	-	-
52	+	-	-	-	-	-	+	+
56	+	-	-	-	+	+	-	-
62	+	-	-	-	-	-	+	+
70	+	-	-	-	-	-	+	+
72	+	+	-	-	+	+	-	-
74	+	-	-	-	-	-	+	+
76	+	+	-	-	-	-	-	-
79a	+	-	-	-	-	-	-	-
79b	-	+	-	-	-	-	-	-
86	+	-	-	-	-	-	+	+
88	+	-	-	-	-	-	+	+
93	+	-	-	-	-	-	+	+
94	+	-	-	-	-	-	+	+
96	-	+	-	-	-	-	-	-
97	+	-	-	-	-	-	+	+

(Cont. on next page)

Table 3.4. (cont.)

Strain no	Protease	Amylase	Xylanase	Cellulase	Pectinolytic Enzyme		Lipase	
					Substrate PGA	Substrate Pectin	Substrate T 20	Substrate T80
100	+	-	-	-	-	-	+	+
104	+	+	-	-	+	+	-	-
106	+	-	-	-	-	-	+	+
S-1	+	+	-	-	+	+	-	-
S-6	+	+	-	-	+	+	-	-
S-12	+	+	-	-	+	+	-	-
S-13	+	+	-	-	+	+	-	-
S-14	+	+	-	-	+	+	-	-
S-15	+	+	-	-	+	+	-	-
S-16	+	+	-	-	+	+	-	-
S-19	+	+	-	-	+	+	-	-
S-20	+	+	-	-	+	+	-	-
S-21	+	+	-	-	+	+	-	-
S-24	+	+	+	+	-	-	+	-
S-25	+	+	-	-	+	+	-	-
S-32	+	-	-	-	-	-	+	-
S-34	-	-	+	-	+	-	+	-
S-38	+	+	-	-	-	-	-	-
S-40	+	+	-	-	-	-	-	-
S-41	+	+	-	-	-	-	-	-
S-44	+	+	-	-	+	+	-	-
S-45	+	+	-	-	+	+	-	-
S-47	-	+	-	-	-	-	-	-
S-48	+	+	-	-	-	-	-	-
S-49	+	+	-	-	-	-	-	-
S-51	+	+	-	-	+	+	-	-
S-58	+	+	-	-	+	+	-	-
S-59	+	+	-	-	+	+	-	-
S-60	+	-	-	-	+	-	+	-
S-61	+	-	-	-	-	-	+	-
S-62	+	+	-	-	+	+	-	-
S-63	+	+	-	-	+	+	-	-
S-65	+	+	-	-	+	+	-	-
S-68	+	+	-	-	+	+	-	-
S-76	+	-	-	-	-	-	+	-
S-79	+	+	-	-	-	-	-	-
S-81	+	+	-	-	+	+	-	-
S-85	+	+	-	-	+	+	-	-
S-86	+	+	+	+	-	-	+	-
S-87	+	+	-	-	+	+	-	-

(Cont. on next page)

Table 3.4. (cont.)

Strain no	Protease	Amylase	Xylanase	Cellulase	Pectinolytic Enzyme		Lipase	
					Substrate PGA	Substrate Pectin	Substrate T 20	Substrate T80
S-90	+	+	-	-	+	+	-	-
S-93	+	+	-	-	+	+	-	-
S-95	+	+	-	-	+	+	-	-
S-98	+	+	-	-	+	+	-	-
S-103	-	+	-	-	+	+	+	-
S-105	+	-	-	-	-	+	-	-
S-111	+	+	-	-	+	+	-	-
S-112	+	+	-	-	+	+	-	-
D-4	+	+	-	-	+	+	-	-
D-7	+	+	-	-	+	+	-	-
D-8	+	+	-	-	+	+	-	-
D-9	+	+	-	-	-	-	+	-
D-11	+	+	-	-	+	+	-	-
D-12	+	+	-	-	+	+	-	-
D-13	+	+	-	-	+	+	-	-
D-14	+	+	-	-	+	+	-	-
D-19	+	+	-	-	-	+	-	-
D-24	+	+	-	-	+	+	-	-
D-25	+	+	-	-	+	+	-	-
D-26	+	+	-	-	-	-	-	-
D-29	+	+	-	-	+	+	-	-
D-32	+	+	-	-	-	-	+	-
D-36	-	-	+	-	+	+	+	-
D-37a	-	-	+	-	+	+	+	-
D-38	-	+	-	-	+	+	-	-
D-41	-	-	+	-	+	+	+	-
D-45	-	-	+	-	+	+	+	-
D-51	+	-	-	-	+	+	-	-
D-52	+	+	-	-	+	+	-	-
D-60	-	-	+	-	+	+	+	-
D-61	-	-	+	-	+	+	+	-
D-62	-	-	+	-	+	+	+	-
D-63	-	+	-	-	+	+	-	-
D-65	+	+	-	-	+	+	-	-
D-68	+	+	-	-	+	+	+	-
D-69	+	+	-	-	+	+	-	-
D-77	-	-	+	-	+	+	+	-
D-79	+	+	-	-	+	+	-	-
D-82	+	+	-	-	+	+	-	-
D-89	-	-	+	-	+	+	+	-
D-90	-	-	+	-	+	+	+	-
D-93	-	+	+	-	-	-	+	-

(Cont. on next page)

Table 3.4. (cont.)

Strain no	Protease	Amylase	Xylanase	Cellulase	Pectinolytic Enzyme		Lipase	
					Substrate PGA	Substrate Pectin	Substrate T 20	Substrate T80
D-96	+	+	-	-	+	+	-	-
D-97	-	-	+	-	+	+	+	-
D-98	-	-	+	-	+	+	+	-
D-99	+	-	-	-	-	-	-	-

Isolated strains within each enzyme groups were further classified according to their physiological characteristics (for example growth at different NaCl concentrations, at different temperatures), the presence of pigments (P groups in Table 3.5).

Table 3.5. Grouping of the Isolates on the Basis of Extracellular Enzyme Production and Physiological Characteristics. Phys.: Physiological

Enzyme Group No.	Enzymes produced by isolates	Isolates	Growth at							Phys. Group No.
			pH 7	5% NaCl	7% NaCl	10% NaCl	45 ºC	50 ºC	55 ºC	
E-1	Protease	79a	+	+	-	-	+	-	-	P-1
		D-99	+	+	+	+	-	-	-	P-2
		34b	+	+	+	-	+	-	-	P-3
E-2	Amylase	39, 42 ,43a, 79b, 96	+	+	-	-	+	-	-	P-4
		S-47 (pale brown pigment)	+	+	+	+	-	-	-	P-5
E-3	Protease, amylase	40 (dark yellow pigment)	w.	+	+	-	+	-	-	P-6
		S-40, S-41, S-48, S-49	+	+	+	+	+	-	-	P-7
		S-38 (yellow pigment)	+	+	+	+	+	-	-	P-8
		76, S-79, D-26, *B. pseudofirmus*	+	+	+	+	-	-	-	P-9
		B.halmapalus	-	+	-	-	-	-	-	P-10
E-4	Protease, P.E. (pectin)	S-105	+	+	+	+	-	-	-	P-11
E-5	Xylanase, lipase (T20)	D-93	-	-	-	-	-	-	-	P-12
E-6	Protease, lipase (T20)	49a	+	+	-	-	+	-	-	P-13
		S-32	+	+	-	-	-	-	-	P-14
		S-61	+	+	+	-	+	+	-	P-15
		S-76	-	+	+	-	-	-	-	P-16

Table 3.5. (cont.)

Enzyme Group No.	Enzymes produced by isolates	Isolates	Growth at							Phys. Group No.
			pH 7	5% NaCl	7% NaCl	10% NaCl	45 ºC	50 ºC	55 ºC	
E-7	**Protease, amylase, lipase (T2O)**	D-9, D-32	+	+	+	+	-	-	-	**P-17**
E-8	**Protease, lipase (T20 and T80)**	4, 34a, 74,	+	+	-	-	+	-	-	**P-18**
		49b, 52, 62, 70, 86, 88, 97, 106	+	+	+	-	+	-	-	**P-19**
		93, 94	+	+	-	-	+	+	-	**P-20**
		100	+	+	+	-	-	-	-	**P-21**
E-9	**Protease, amylase, P.E. (pectin)**	D-19	-	+	+	-	-	-	-	**P-22**
E-10	**Protease, P.E. (PGA), lipase(T20)**	S-60	+	+	+	-	+	+	-	**P-23**
E-11	**Xylanase, lipase (T20), P.E. (PGA)**	S-34	+	+	+	-	-	-	-	**P-24**
E-12	**Protease, P.E. (pectin, PGA)**	56,	+	+	+	+	+	+	+	**P-25**
		D-51	+	+	+	+	+	+	-	**P-26**
		B. gibsonii	+	-	-	-	-	-	-	**P-27**
E-13	**Amylase, P.E. (pectin, PGA)**	D-38, D-63	+	+	+	-	-	-	-	**P-28**

(Cont. on next page)

Table 3.5. (cont.)

EnzymeGroup No.	Enzymes produced by isolates	Isolates	Growth at							Phys. Group No
			pH 7	5% NaCl	7% NaCl	10% NaCl	45 ºC	50 ºC	55 ºC	
E-14	**Amylase,lipase (T2O), P.E.(pectin, PGA)**	S-103	w.	+	+	+	+	+	-	**P-29**
E-15	**Xylanase, lipase(T20), P.E. (pectin, PGA)**	D-36, D-37a, D-41, D-77, D-94,	-	+	+	-	-	-	-	**P-30**
		D-45, D-60, D-61, D-62, D-97	+	+	+	-	-	-	-	**P-31**
		D-98	+	+	+	+	-	-	-	**P-32**
		D-89, D-90	-	+	+	+	-	-	-	**P-33**
E-16	**Protease, amylase, P.E. (pectin, PGA)**	*B. clausii* 18, S-12, S-13, S-14, S-15, S-16, S-19, S-20, S-59, S-63, S-68, S-85, S-93, S-95, D-4, D-7, D-11, D-14, D-29, D-52, D-68, D-65, D-79, D-82	+	+	+	+	+	+	-	**P-34**

(Cont. on next page)

Table 3.5. (cont.)

EnzymeGroup No.	Enzymes produced by isolates	Isolates	Growth at .							Phys. Group No.
			pH 7	5% NaCl	7% NaCl	10% NaCl	45 ºC	50 ºC	55 ºC	
E-16	**Protease, amylase, P.E. (PGA, pectin)**	51, 104, 72, S-90, S-98, S-111, S-112, D-8, D-96	+	+	+	-	+	+	-	**P-35**
		S-87	+	+	+	-	+	+	+	**P-36**
		S-1, S-58, S-62, S-65, D-24, D-69	+	+	+	+	+	+	+	**P-37**
		S-6, S-21, S-25, S-81, D-12, D-13, D-25	+	+	+	+/-	+	+	-	**P-38**
		S-44, S-45, S-51 (pale brown pigment)	+	+	+	-	+	+	-	**P-39**
		11 (yellow pigment)	+	+	+	+	-	-	-	**P-40**
		B. alcalophilus	+	-	-	-	-	-	-	**P-41**
E-17	**Protease, amylase, xylanase, cellulase, lipase(T20)**	S-24, S-86	+	-	-	-	+	-	-	**P-42**
E-18	**Protease, amylase, xylanase, cellulase, P.E.(pectin), lipase(T20)**	33	+	+	+	+	-	-	-	**P-43**

(Cont. on next page)

3.3. Genotypic Characterization

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

Amplification of 16S-ITS region mostly produced one distinct DNA fragment of approximately 2000 bp in length (Figure 3.2).

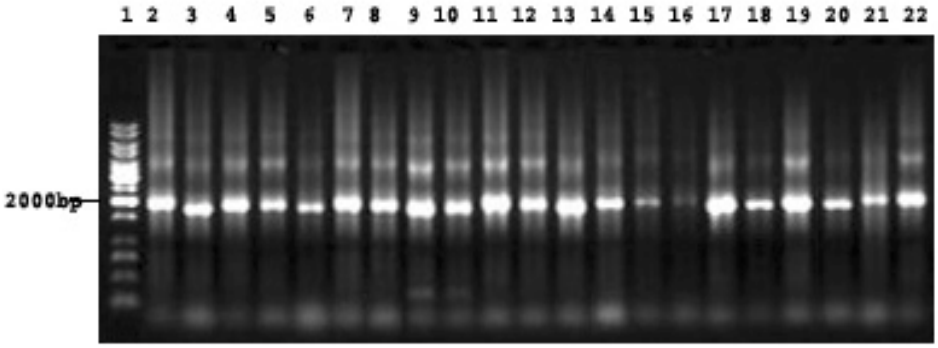

Figure 3.2. Amplification of 16S-ITS Region from a Set of Isolated Strains.
Lanes 1. Direct Load™ Wide Range DNA Marker; 2. S-112; 3. S-51; 4. S-90; 5. S-81; 6.D-98; 7. D-96; 8. S-98; 9. 70; 10. 34a; 11. S-15; 12. S-25; 13. S-58; 14. S-12; 15. S-24; 16. D-37a; 17. S-13; 18. S-40; 19. S-85; 20. S-38; 21. D-65; 22. S-62

3.3.2. 16S-ITS rDNA Based RFLP Profiling

For the identification of 116 isolated strains according to their restriction profiles two restriction enzymes, *Taq* I and *Hae* III were used. First, all the samples were run in 2 % agarose gel to see all different profiles and to make groups within the digestion products by each of the enzymes. After this, digestion product from each of the groups was selected and were resolved in 2.5 % agarose gel (Figure 3.3, Figure 3.4).

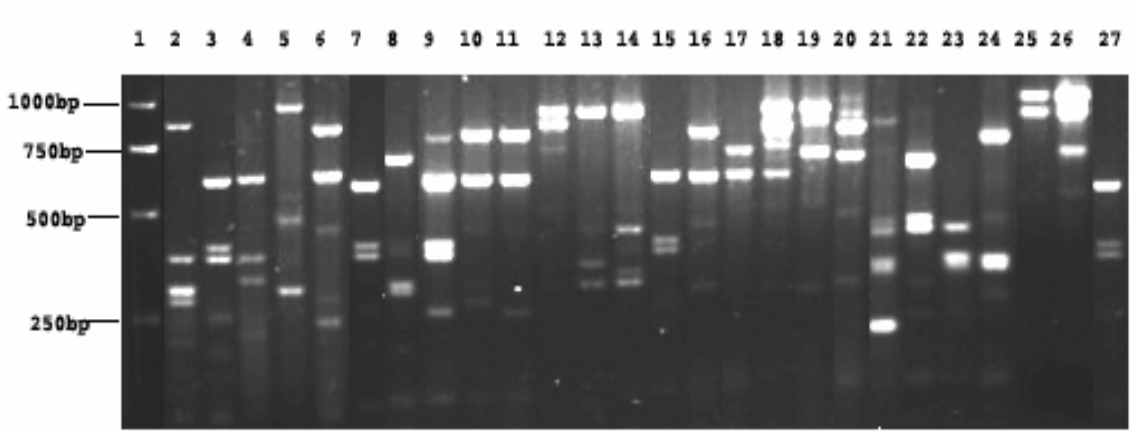

Figure 3.3 *Taq* I Digests of 16S-ITS rDNA Fragments of Representative Isolates and Reference Strains (2,5% agarose gel). Lanes 1. 1kb DNA ladder Gene Ruler™ 2. *B. alcalophilus,* 3. *B.. clausii,* 4. *B. gibsonii,* 5. *B. halmapalus*, 6. *B. pseudofirmus,* 7. S-6, 8. D-36, 9. S-47, 10. D-19, 11. S-38, 12. 79a, 13. 40, 14. 11, 15. S-79, 16. D-32, 17. S-76, 18. 106, 19. 42, 20. S-24, 21. 33, 22. S- 103, 23. D-38, 24. D-63, 25. S-60, 26. S-105, 27. S-95

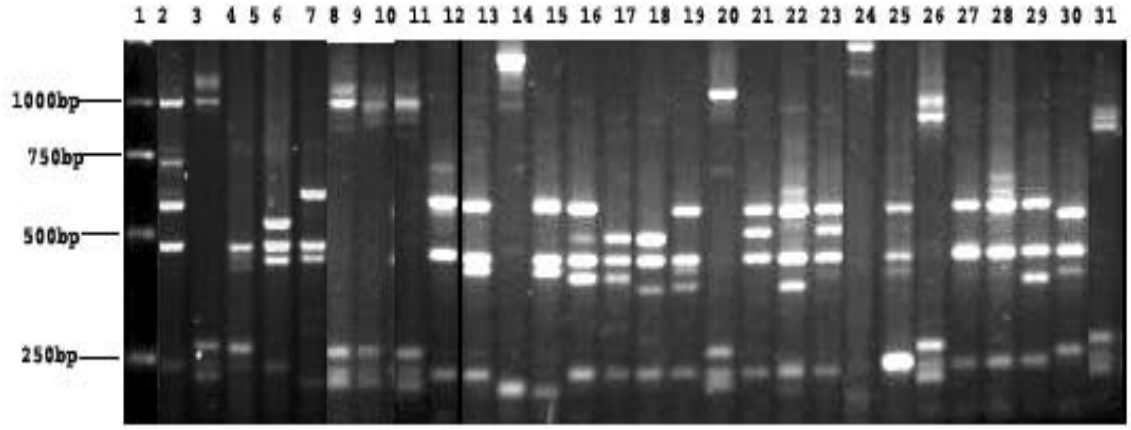

Figure 3.4. *Hae* III Digests of 16S-ITS rDNA Fragments of Representative Isolates and Reference Strains (2,5% agarose gel). Lanes 1. 1kb DNA ladder Gene Ruler™ 2. *B. alcalophilus,* 3. *B. clausii,* 4. *B. gibsonii,* 5. *B. halmapalus*, 6. *B. pseudofirmus,* 7. S-65, 8. D-79, 9. S-6, 10. D-36, 11. S-47, 12. D-19, 13. S-38, 14. 79a, 15. 40, 16. 11, 17. S-79, 18. D-32, 19. S-76, 20. 106, 21. 42, 22. S-24, 23. 33, 24. S-103, 25. D-38, 26. D-63, 27. S-60, 28. S-105, 29. S-95

Resulting gel profiles were analyzed by the program BIO-ID++. Dendrogram of representative strains were carried out using 13% homology coefficient (Figure 3.3 and 3.4). Fragment sizes were given in Table 3.6.

Hae III restriction analysis of 16S-ITS rDNA fragments of strains and 5 reference strains revealed 15 distinct groups (Table 3.8). On the other hand, 16 groups were obtained by *Taq* I digested restriction profiles (Table 3.9).

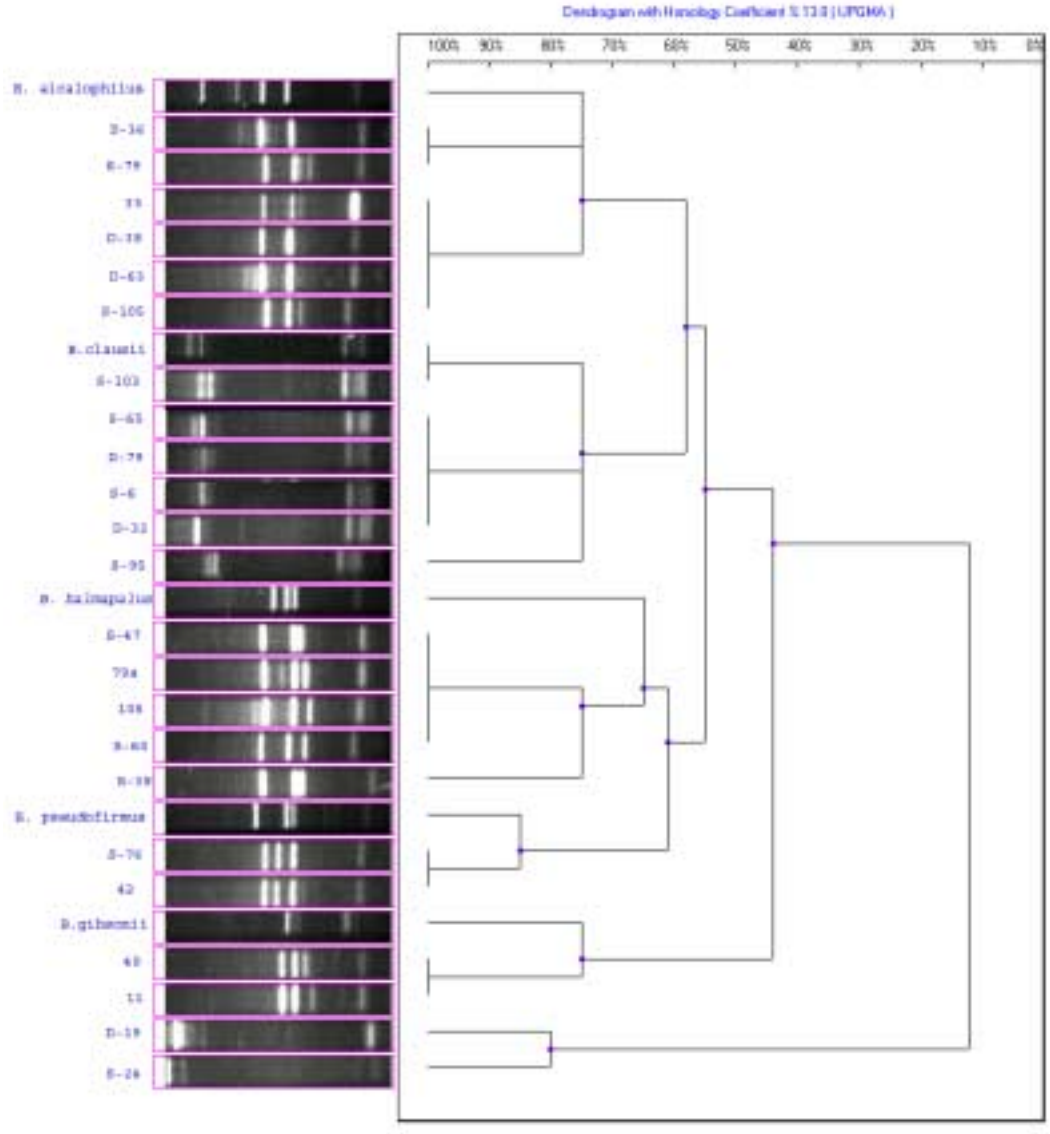

Figure 3.5 Dendrogram of Representative *Hae* III RFLP Profiles of Isolated and Reference Strain Groups.

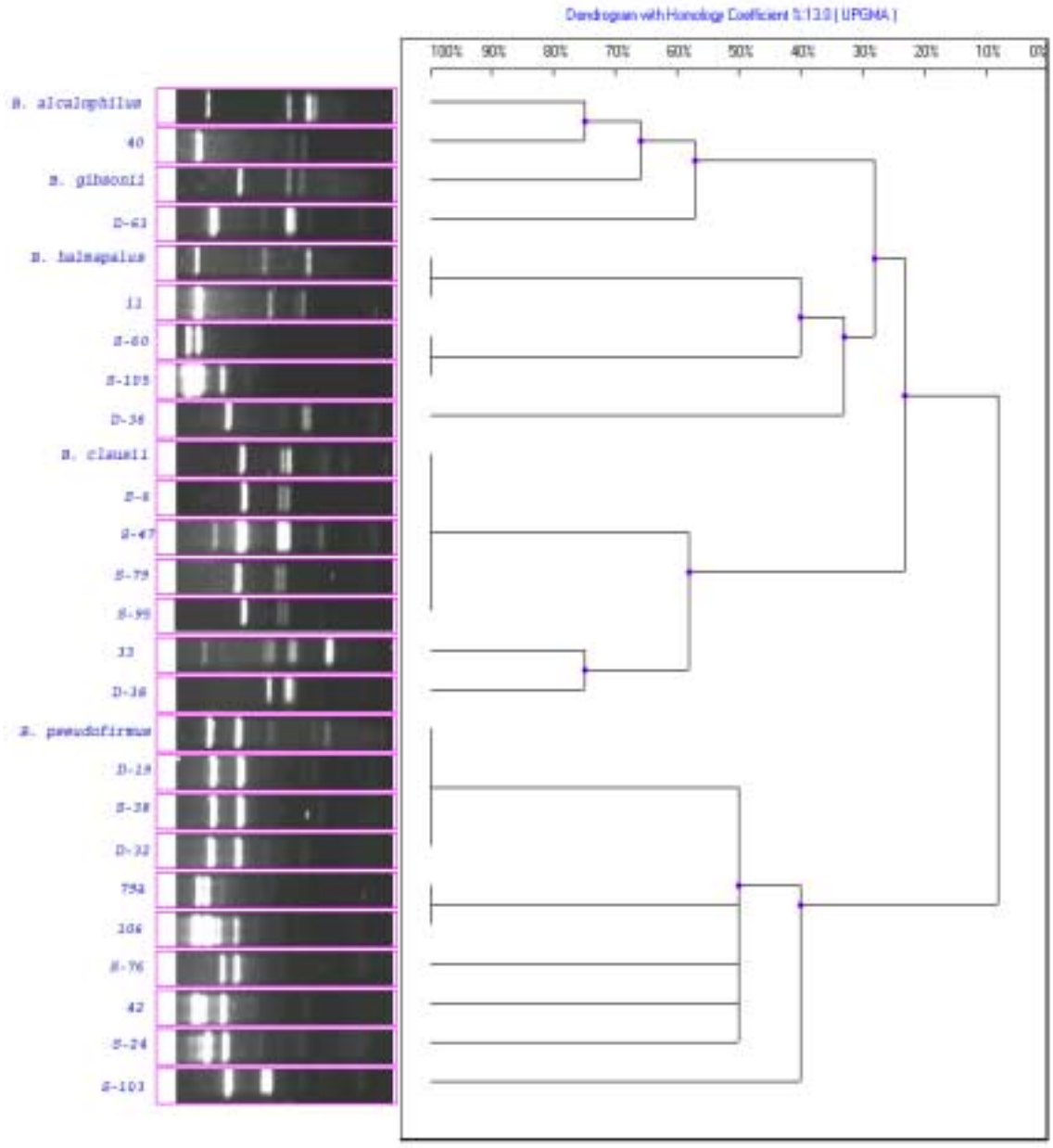

Figure 3.6 Dendrogram of Representative *Taq* I RFLP Profiles of Isolated and Reference Strain Groups.

L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	
1.000	0.986	1.000	0.466	0.529	0.609	0.986	0.986	0.986	0.567	0.583	1.211	0.575	0.575	
0.750	0.575	0.270	0.447	0.473	0.473	0.260	0.260	0.260	0.447	0.441	0.188	0.434	0.434	
0.500	0.466	0.239	0.428	0.434	0.441	0.216	0.216	0.216	0.216	0.416		0.410	0.398	
0.250	0.285	0.222	0.270			0.199	0.199	0.199		0.222		0.182	0.216	
	0.239													
L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	L28	L29
0.486	0.486	0.567	1.026	0.567	0.567	0.575	1.250	0.567	0.917	0.567	0.583	0.575	0.559	0.876
0.434	0.441	0.441	0.260	0.507	0.441	0.507	0.239	0.447	0.270	0.447	0.453	0.460	0.466	0.285
0.398	0.375	0.222	0.216	0.441	0.381	0.441	0.165	0.250	0.239	0.250	0.244	0.404	0.265	0.260
0.216	0.216		0.199	0.222	0.222	0.222			0.216			0.250		0.233

Table 3.6. Fragment Sizes of Hae III Digests of 16S-ITS rDNA Fragments of Representative Isolates and Reference Strains. L1. DNA M.W., L2. *B. alcalophilus,* L 3. *B. clausii,* L4. *B. gibsonii,* L5. *B. halmapalus*, L 6. *B. pseudofirmus,* L7. S-65, L8. D-79, L9. S-6, L10. D-36, L11. S-47, L12. D-19, L13. S-38, L14. 79a, L15. 40, L16. 11, L17. S-79, L18. D-32, L19. S-76, L20. 106, L21. 42, L22. S-24, L23. 33, L24. S-103, L25. D-38, L26. D-63, L27. S-60, L28. S-105, L29. S-95

L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14
1.000	0.862	0.614	0.614	0.984	0.847	0.585	0.712	0.614	0.818	0.818	0.969	0.969	0.969
0.750	0.382	0.407	0.382	0.485	0.624	0.414	0.328	0.414	0.604	0.624	0.877	0.363	0.455
0.500	0.305	0.382	0.328	0.311		0.388	0.311	0.395				0.328	0.328
0.250	0.289		0.213										
	0.194												
L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	
0.634	0.862	0.750	0.984	0.969	0.847	0.477	0.712	0.462	0.818	1.059	1.059	0.594	
0.427	0.624	0.644	0.892	0.750	0.712	0.455	0.485	0.388	0.376	0.969	0.938	0.414	
0.407						0.376	0.462	0.370				0.395	
						0.357							
						0.237							

Table 3.7 Fragment Sizes of *Taq* I Digests of 16S-ITS rRNA Genes of Representative Isolates and Reference Strains. L1. DNA M.W. marker, L2. *B. alcalophilus,* L3. *B. clausii,* L4. *B. gibsonii,* L5. *B. halmapalus*, L 6. *B. pseudofirmus,* L7. S-6, L8. D-36, L9. S-47, L10. D-19, L11. S-38, L12. 79a, L13. 40, L 14. 11, L15. S-79, L16. D-32, L17. S-76, L18. 106, L19. 42, L20. S-24, L21. 33, L22. S-103, L23. D-38, L24. D-63, L25. S-60, L26. S-105, L27. S-95

Table 3.8. Groups Obtained by *Hae* III Restriction Analysis of 16S-ITS Ribosomal DNA Fragment.
Representative strains used in dendrogram were emphasized by bold characters.

***Hae* III Groups**	**Isolates**
H 1	***Bacillus alcalophilus***
H 2	**D-36, S-79,** D-37a, D-41, D-45, D-61, D-77, D-90, D-94, D-97, D-98, S-34,D-93, D-62, D-60, D-89
H 3	**33, D-38, D-63, S-105,** D-99
H 4	***Bacillus clausii,* S-103**, 56
H 5	**S-65, D-79, S-6, D-32,** S-68, D-68, D-69, S-12, S-16, S-19, S-21, S-25, S-20, S-63, S-81, S-85, D-25, D-29, D-52, S-87, 72, 51, S-98, S-90, D-11, D-82, D-26, D-9, 104, D-96, D-8, S-111, D-51, D-12, S-62, D-4, D-14, S-1, S-15, S-112, D-65, D-7, D-13, 18
H 6	**S-95,** S-13, S-14, S-58, S-59, S-93, D-24
H 7	***Bacillus halmapalus***
H 8	**S-47, 79a, 106, S-60,** 76, S-32, 4, 34a, 49b, 52, 62, 70, 74, 86, 88, 97, 100, 106, 93, 94, 34b, S-61
H 9	**S-38,** S-40, S-41, S-44, S-45, S-48, S-49, S-51
H 10	***Bacillus pseudofirmus***
H 11	**S-76, 42,** 49a, 43a, 96, 47, 79b, 39
H 12	***Bacillus gibsonii***
H 13	**40, 11**
H 14	**D-19**
H 15	**S-24,** S-86

Table 3.9. Groups Obtained by *Taq* I Restriction Analysis of 16S-ITS Ribosomal DNA Fragment.

Representative strains used in dendrogram were emphasized by bold characters.

***Taq* I Groups**	**Isolates**
T 1	***Bacillus alcalophilus***
T 2	**40**
T 3	***Bacillus gibsonii***
T 4	**D-63**
T 5	***Bacillus halmapalus*, 11**
T 6	**S-60, S-105,** S-61, D-99
T 7	**D-36,** D-37a, D-41, D-45, D-61, D-77, D-90, D-94, D-97, D-98, S-34, D-93, D-62, D-60, D-89
T 8	***B. clausii*, S-6, S-47, S-79, S-95,** S-12, S-16, S-19, S-21, S-25, S-20, S-63, S-81, S-85,D-25, D-29, D-52, S-87, 72, 51, S-98, S-90, D-11, D-82, S-13, S-14, S-58, S-59, S-93, D-24, 104 , D-96, D-8, S-111, D-51, D-12, S-62, D-4, D-14, S-1, S-15, S-112, D-65, D-7, D-13, 18,S-65, S-68, D-68,D-69, D-79, 76
T-9	**33**
T 10	**D-38**
T 11	***Bacillus pseudofirmus*, D-19, S-38, D-32,** S-40, S-41, S-44, S-45, S-48, S-49, S-51, D-26, D-9
T 12	**79a, 106, ,** S-32, 4, 34a, 49b, 52, 62, 70, 74, 86, 88, 97, 100, 93, 94, 34b, 49a
T 13	**S-76**
T 14	**42,** 43a, 96, 47, 79b, 39
T 15	**S-24,** S-86
T 16	**S-103,** 56

The use of two restriction enzymes enabled the revelation of diversity among isolated and reference strains. When the results of digestion were combined 26 distinct genotypes were obtained (Table 3.10). The results indicated that some isolate groups were differentiated by one restriction enzyme, while some others were differentiated by the other enzyme. Thus both of the enzymes were necessary for 16SrDNA-ITS Region RFLP.

Genotypic groups were formed on the basis of 2 different criteria:

- Strains differentiated by both *Taq* I and *Hae* III (G-1-13 in Table 3.10)
- In the second case, groups contained strains differentiated by only one of the enzymes. For example, in some cases a strain or a group of strain were differentiated by *Taq* I but the same strain or the group of strains were clustered with other strains by *Hae* III into a large group (G-14-21). Some groups of the strains were on the other hand better identified by *Hae* III (G-22-26).

In this study , 16S rDNA and ITS regions were used as a single amplicon for RFLP analysis. No report was found in literature on genotypic characterization of alkalophiles using this method. Similar study has been performed in this institute for genotypic characterization of thermophilic bacilli and lactic acid bacteria (Yavuz, 2003, Bulut, 2003). 17 different genotypic groups have been obtained after 16S rDNA-ITS Region RFLP of 112 isolated strains by *Taq* I and *Hae* III (Yavuz, 2003). On the other hand Bulut (2003) has used the same method for further identification; isolates which couldn't have been identified phenotypically, could have been identified by using 16S rRNA-ITS Region RFLP. In other studies, 16S rDNA and ITS regions were used separately for RFLP analysis (Caccamo *et a*l., 2001, Blanc *et al*., 1997, Kuisiene *et al.,* 2002).

Table 3.10. Genotypic Grouping Based on the Combination of *Taq* I and *Hae* III Digestion Groups.

Genotypic Groups	Strains differentiated by both Taq I and Hae III	Taq I Digestion Group	Hae III Digestion Group
G-1	S-24, S-86	**T-15**	**H-14**
G-2	*B. alcalophilus*	**T-1**	**H-1**
G-3	*B. gibsonii*	**T-3**	**H-12**
G-4	S-105, D-99	**T-6**	**H-3**
G-5	S-60, S-61	**T-6**	**H-8**
G-6	*B. clausii*	**T-8**	**H-4**
G-7	S-47, 76	**T-8**	**H-8**
G-8	S-12, S-16, S-19, S-21, S-25, S-20, S-63, S-81, S-85, D-25, D-29, D-52, S-87, 72, 51, S-98, S-90, D-11, D-82, 104, D-96, D-8, S-111, D-51, D-12, S-62, D-4, D-14, S-1, S-15, S-112, D-65, D-7, D-13, 18, S-65, S-68, D-68,D-69, D-79, S-6	**T-8**	**H-5**
G-9	S-79	**T-8**	**H-2**
G-10	D-9, D-26, D-32	**T-11**	**H-5**
G-11	**79a, 106,** S-32, 4, 34a, 49b, 52, 62, 70, 74, 86, 88, 97, 100, 93, 94, 34b	**T-12**	**H-8**
G-12	49a	**T-12**	**H-11**
G-13	11	**T-5**	**H-13**
	Strains differentiated by Taq I but clustered into a large group with other strains by Hae III		
G-14	D-36, D-37a, D-41, D-45, D-61, D-77, D-90, D-94, D-97, D-98, S-34, D-93, D-62, D-60, D-89	**T-7**	**H-2**
G-15	S-103, 56	**T-16**	**H-4**
G-16	D-63	**T-4**	**H-3**

Table 3.5. (cont.)

G-17	33	**T-9**	**H-3**
G-18	D-38	**T-10**	**H-3**
G-19	S-76	**T-13**	**H-11**
G-20	39, 42, 43a, 47, 79b, 96	**T-14**	**H-11**
G-21	40	**T-2**	**H-13**
	Strains differentiated by Hae III but clustered into a large group with other strains by Taq I		
G-22	D-19	**T-11**	**H-14**
G-23	S-38, S-40, S-41, S-44, S-45, S-48	**T-11**	**H-9**
G-24	*Bacillus pseudofirmus*	**T-11**	**H-10**
G-25	S-95, S-13, S-14, S-58, S-59, S-93, D-24	**T-8**	**H-6**
G-26	*B.halmapalus*	**T-5**	**H-7**

3.3.3. Analysis of the Genotypic and Phenotypic Groups

A correlative analysis was performed in order to understand whether there was a relation between genotypic (G groups in Table 3.10) and enzymatic-physiological groups (E and P groups in Table 3.5).

3.3.3.1. Analysis of the Genotypic and Phenotypic Groups of Reference Strains

B. alcalophilus, B. gibsonii, B. pseudofirmus, B. halmapalus and B. clausii were all clustered into different genotypic groups (G-2, G-3, G-24, G-26, G-6 in Table 3.10 respectively).

They were also differed from each other in terms of their enzyme production and physiological characters (P-41, P-27, P-9, P-10, P-34 in Table 3.5 respectively).

3.3.3.2. Analysis of the Genotypic and Phenotypic Groups of Isolated Strains

Most isolates into same genotypic group showed common phenotypic profiles. On the other hand, some of them differed from each other in terms of physiological characteristics (for example salt and temperature tolerance) or enzyme production. However these strains might belong to the same species. Nielsen *et al.* (1995) has demonstrated that a strain of *B. clausii* has tolerated 8% NaCl whereas other members have tolerated about 10% NaCl. On the other hand, while some strains of *B.gibsonii* have hydrolyzed Tween 40 and Tween 60, the type strain has not.

Three genotypic groups, G-6, G-8 and G-25 (Table 3.10) displayed approximately 75% similarity level by *Hae* III and yielded identical RFLP profiles by *Taq* I. Strains included in these groups were in the same enzyme group (E-16 in Table 3.5: protease, amylase, pectinolytic activity) and showed similar physiological characteristics (growth at 5%, 7% NaCl and at 45 ºC and 50 ºC).

Strains included in the group G-14 (except strains S-34 and D-93) showed the same extracellular enzyme profiles (E-15 in Table 3.5: xylanase, lipase (hydrolysis of T20), pectinolytic enzyme production (degradation of pectin and PGA)). Moreover, their physiological properties were also similar (growth at 5% and 7% NaCl, no growth at 45 ºC, 50 ºC and 55 ºC). Some strains could tolerate 10% NaCl (P-32, P-33 in Table 3.5). On the other hand, some couldn't grow at pH 7 (P-30, P-31 in Table 3.5). Strain S-34 (P-31 in Table 3.5) showed similar characteristics with other isolates except the degradation of pectin. However these 13 strains could be members of the same species although their salt and pH tolerance profiles varied. On the other hand, D-93 in the same genotypic group could be correlated only by some aspects with other strains in this group (no growth at pH 7 and production of xylanase and lipase (T20 hydrolysis).

Strains of G-1 showed the same physiological and extracellular enzyme profiles. Hence these strains were thought to be the same.

Five of the genotypic groups G-19, G-21, G-22, G-12, G-13 and G-17 in Table 3.10 had only one member strain. Each of the members also displayed different physiological characteristics (P-16, P-6, P-22, P-13, P-40, P-43 in Table 3.5).

Strains of G-11 (except 34b, 79a and S-32) produced the same enzyme profiles (E-8 in Table 3.5 : protease, lipase (hydrolysis of both Tween 20 and Tween 80)) and quite similar physiological characteristics (growth at pH 7 and at 5% NaCl, no growth at

10% NaCl and at 55 ºC). However, their growth at 7% NaCl at 45 ºC and 50 ºC varied (P-18, P-19, P-20, P-21). Three strains in the same genotypic group, S-32, 34b and 79a, showed similar properties to previous strains except the hydrolysis of lipase (Strain S-32 was not able to hydrolyze Tween 80, on the other hand 34b and 49a couldn't hydrolyze both Tween 20 and Tween 80).

Strains 39, 42, 43a, 47, 79b and 96 of G-20 formed a distinct phenotypic group (P-4 in Table 3.5) in respect of their enzyme production and physiological features (production of amylase, growth at pH 7, at 5% NaCl and at 45 ºC). Hence, these 6 strains were thought to be the same.

Five strains of G-23 were in the same enzyme group (E-3 in Table 3.5: protease, amylase) and had the same physiological characteristics (P-7 and P-8 in Table 3.5: growth at pH 7, at 5%, 7% and 10% NaCl and at 45 ºC, no growth at 50 ºC and 55 ºC). These strains could be the same. On the other hand, strains S-44, S-45 and S-51 were in another enzyme group (E-16 in Table 3.5 : protease, amylase, pectinolytic enzyme production) and had the same physiological properties (P-39 in Table 3.5: growth at pH 7, at 5%, 7% NaCl and at 45 ºC and 50 ºC, no growth at 10% NaCl and at 55 ºC). Thus these last strains were also thought to be the same.

Strain S-105 and D-99 of G-4 were phenotypically similar in many respects (P-11 and P-2 in Table 3.5). Both of the strains had the same physiological properties (growth at pH 7 and at 5%, 7%, 10% NaCl, no growth at 45 ºC, 50 ºC and at 55 ºC). Their enzyme production profiles was also similar except the degradation of pectin by S-105. Therefore, these two strains might be the members of the same species.

Strains D-9, D-26 and D-32 of G-10 were also similar in terms of their enzyme production (protease and amylase production) except that D-26 could degrade Tween 20. All the three isolates had the same physiological properties (growth at pH 7 and at 5%, 7%, 10% NaCl, no growth at 45 ºC, 50 ºC and at 55 ºC). Strains D-9, D-26 and D-32 might thus be the strains of the same species.

Strains D-38 and D-63 were clustered into different genotypic groups (G-16 and G-18 in Table 3.10 respectively) even the enzyme production and physiological properties were the same. Thus, it could be suggested that these strains were distinct. Similarly, although strain S-79, 76 , D-26 and *B. pseudofirmus* were the same in terms of their enzyme production and physiological properties, they were clustered into different genotypic groups (G-9, G-7, G-24 in Table 3.10 respectively).

Strains S-103 and 56 of G-15 were clustered in different enzyme groups (E-14 and E-12 in Table 3.5 respectively). On the other hand, similar physiological features were observed (growth at 5%, 7% and 10% NaCl and at 45 ºC and 50 ºC).

Strains of G-7 had similar enzymatic and physiological profiles (S-47: amylase production, 76: amylase and protease production) and carried physiologically same properties (growth at pH 7, at 5%, 7% and 10% NaCl and no growth at 45 ºC, 50 ºC and at 55 ºC). These two strains could be the members of the same species.

In conclusion 116 isolated and 5 reference strains were clustered into 26 genotypic groups.

An important observation demonstrated the influence of growth media on temperature tolerance. Twelve strains could grow on Horikoshi-I agar but not on nutrient agar at 55 ºC. It could be assumed that media content was important in adaptation to high temperatures. For instance Mg^{2+} ions in Horikoshi-I could have stabilized the enzyme structures.

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, alkalophilic bacteria were isolated from samples of soil, horse feces and leather processing. They were first characterized phenotypically (cell morphology, endospore formation, gram behaviour, physiological tests such as growth at pH 7, at different NaCl concentrations and at different temperatures). Extracellular enzyme screening was performed on solid agar media. Extracellular enzyme producing strains were selected and further characterized by 16S-ITS rDNA based RFLP profiling.

In conclusion, 116 rod-shaped bacteria were subjected to phenotypic and genotypic characterization. All the isolated strains were found to contain endospores and were Gram positive. Thus they were identified as *Bacillus.* All the strains were catalase positive except D-19. Most of the isolates contained oxidase. Ten isolated strains could not grow at pH 7, they were thus obligate alkalophilic.

Extracellular enzyme screening resulted in 91 protease, 77 amylase, 18 xylanase, 3 cellulase, 74 pectinolytic enzyme (71 polygalacturonic acid degrading and 72 pectin degrading) and 55 lipase (41 Tween 20 hydrolyzing and 14 Tween 80 hydrolyzing) producing strains. They were classified into 18 groups in respect of the enzyme production profile (E groups in Table 3.5). These groups were further classified in terms of physiological characteristics of strains (P groups in Table 3.5).

In restriction analysis of 16S-ITS rDNA fragments of isolated strains and reference strains two restriction enzymes, *Taq* I and *Hae* III, were used. Both of the enzymes were found to be necessary for discrimination. *Taq* I revealed 16 genotypic groups while *Hae* III revealed 15 different groups. Reference strains were clustered into different genotypic groups by both *Taq* I and *Hae* III . In total the results of two enzymes were combined and 26 distinct genotypic groups (reference strains included) were obtained.

Extracellular enzyme characterization will be essential to identify enzymes with the highest activity, to determine substrate specifities, molecular weight and optimum conditions for production..

If necessary, enzyme characteristics may be changed by protein engineering to improve their properties. For example, site directed mutagenesis may be used to design novel enzymes with unique specificities, increased resistance to harsh environmental conditions.

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APPENDIX A

CHEMICALS USED

Table A.1 Chemicals Used in Experiments (cont. on next page)

NO	CHEMICAL	CODE
1	Agar	Merck 1.01613
2	Bacteriological pepton	Oxoid LP037
3	D-Glucose	AppliChem A3666
4	Yeast Extract	Merck 1.03753
5	Skimmed milk	LabM MC27
6	Glycerol	AppliChem A2926
7	NaCl	AppliChem A2942
8	K_2HPO_4	AppliChem A2945
9	$MgSO_4.7H_2O$	Merck 1.05886
10	Tween80	Applichem A1390
11	Ammonium sulfate	Applichem A3485
12	Nutrient broth	Merck 1.05443
13	Sodium carbonate	Merck 1.06392
14	KH_2PO_4	Merck 1.04871
15	Disodium hydrogen phosphate	Applichem A2943
16	Pectin from citrus peel	Fluka 76280
17	Polygalacturonic acid	Fluka 81325
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	N,N,N',N'- Tetramethyl-p-phenylenediamine	Sigma T3134
24	Congo Red	Sigma C6767

Table A.1 (cont.)

NO	CHEMICAL	CODE
25	Potassium Iodide	Sigma P8256
26	Tris Base	Sigma T6066
27	EDTA	AppliChem A2937
28	Isopropanol	AppliChem A3928
29	Proteinase K	AppliChem A3830
30	Ethidium bromide	AppliChem A1151
31	Ethanol	AppliChem A3678
32	Taq DNA polymerase	Promega M1865
33	Primers: Ege 1 and L1	Promega
34	dNTP set	MBI, Fermentas, R0181
35	Standard agarose (low electroendoosmosis)	AppliChem A2114
36	Chloroform	AppliChem A3633
37	Isoamyl alcohol	AppliChem A2610
38	Bromophenol blue	Merck 1.08122
39	Boric acid	AppliChem A2940

APPENDIX B

RECIPIES FOR MEDIA USED IN EXPERIMENTS

B.1 HORIKOSHI BROTH AND AGAR

HORIKOSHI-I BROTH

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

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

HORIKOSHI-I AGAR (pH=10.2)

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

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

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

	g/l
Nutrient Broth	8
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved at 121 °C for 15 m. $Na_2 CO_3$ was sterilized separately and added into medium.

B.3 MINERAL MEDIUM USED IN CELLULASE, XYLANASE AND AMYLASE SCREENING (pH= 10)

	g/l
Xylan/ Carboxymethyl cellulose/ Starch	5
K_2HPO_4	7
KH_2PO_4	2
$MgSO_4$.7 H20	0.1
$(NH_4)_2SO_4$	1
NaCl	5
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved for 15 m at 121 °C. $Na_2 CO_3$ was sterilized separately and added into medium.

B.4 MEDIUM USED FOR PECTINOLYTIC ENZYME SCREENING

	g/l
$(NH_4)_2SO_4$	2
KH_2PO_4	3
Na_2HPO_4	6
Yeast extract	1
Polygalacturonic acid/ pectin	5
Agar	13
Na_2CO_3	10
Deionized water	1000.0 ml

All ingredients were dissolved in deionized water. Medium was autoclaved for 15 m at 121 °C. Na_2CO_3 was sterilized separately and added into medium. (pH of medium containing pectin: 9.5; pH of medium containing polygalacturonic acid: 9.1)

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

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

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

APPENDIX C

BUFFERS AND STOCK SOLUTIONS

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

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

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

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

C.3 50 X TAE

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

C.4 1XTAE

Twenty milliliters of 50X TAE buffer was taken and the volume was adjusted to 1000 ml with deionized water to obtain 1 liter 1X TAE buffer.

C.5 1X TE BUFFER

10 mM Tris pH 8.0
1 mM EDTA pH 8.0

C.6 10 X TBE

One hundred and eight grams Tris Base and 55 g boric acid were weighed. They were dissolved in nearly 800 ml of deionized water and 40 ml 0.5 M EDTA pH 8.0 was

added. The volume was brought to 1000 ml with deionized water.

C.7 1X TBE

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

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

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

APPENDIX D

STAINS AND INDICATORS

D.1. BROMTYMOL BLUE SOLUTION

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

D.2. SOLUTIONS FOR GRAM STAINING

D.2.1. CRYSTAL VIOLET STAINING REAGENT

Solution A

Crystal violet	2g
Ethanol (95%)	20ml

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

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

D.2.2. IODINE SOLUTION

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

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

D.2.3. SAFRANIN SOLUTION

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

APPENDIX E

PCR RECIPIES

E.1. PCR MIX

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

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

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

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

E.3. dNTP (10X)

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

E.4. RESTRICTION ENZYME MIX

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

APPENDIX F

OLIGONUCLEOTIDE PRIMERS

L1: 5’- CAAGGCATCCACCGT -3’

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

EGE 1 5’- AGAGTTTGATCCTGGCTCAG -3’

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

APPENDIX G

RECOGNITION SITES OF RESTRICTION ENZYMES

G1. *Taq* I

5’- T▼CG A -3’

5’- A GC ▲T -3’

G2. *Hae* III

5’-GG▼CC-3’

5’-CC▲GG-3’