İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CONSTRUCTION OF PRE-ENRICHED METAGENOMIC LIBRARIES FOR ISOLATING NOVEL HYDROLASE ENZYMES FOR LIQUID/SUPERCRITICAL CO_2

M.Sc. Thesis by
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SIVI/SUPERKRİTİK CO_2 ORTAMINA UYGUN YENİ HİDROLAZ ENZİMLERİNİN İZOLASYONU İÇİN ÖN-ZENGİNLEŞTİRME UYGULANMIŞ METAGENOMİK KÜTÜPHANE KURULMASI

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FOREWORD

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January 2011

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Food Engineer

TABLE OF CONTENTS

Page

ABBREVIATIONS

App	**:** Appendix
DNA	**:** Deoxyribonucleic acid
dNTP	**:** Deoxyribonucleotide triphosphate
EB	**:** DNA extraction buffer
EDTA	**:** Ethylenediaminetetraacetic acid
LB	**:** Luria Bertani Broth
LCO_2	**:** Liquid carbon dioxide
$SCCO_2$	**:** Supercritical carbon dioxide
PCR	**:** Polymerase Chain Reaction
TAE	**:** Tris-Acetic acid-EDTA
UARR	**:** Universal Amplified Ribosomal Region
Xgal	**:** 5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside

LIST OF TABLES

Page

LIST OF FIGURES

Page

CONSTRUCTION OF PRE-ENRICHED METAGENOMIC LIBRARY FOR ISOLATING NOVEL HYDROLASE ENZYMES FOR LIQUID/ SUPERCRITICAL CO_2

SUMMARY

Liquid CO_2 (LCO_2) is obtained when pressure is increased. When both temperature and pressure exceed the critical point supercritical CO_2 ($SCCO_2$) is formed. LCO_2/ $SCCO_2$ is used in industrial cleaning and in food preservation applications. It is advantageous because it is not toxic and can replace many hazardous chemicals used in cleaning application. However, LCO_2/ $SCCO_2$ is not so effective against hydrophilic dirt. To increase the efficiency of LCO_2/ $SCCO_2$, it can be supplemented with hydrolase enzymes such as lipase and protease. Due to the extreme nature of LCO_2/ $SCCO_2$ such as low pH level and no water content, enzymes to be applied in this system should be active at low pH and in non-aqueous medium. Hydrolase enzymes with such extreme properties can be isolated from acidophiles (low pH) and halophiles (low available water due to high salt content) by metagenomic approach. Metagenomics is defined as the total genome analysis of microbial population present in an environment. A high percentage of microorganisms on earth have not been cultured yet and metagenomics make analysis of those microorganisms possible without any dependence on culturing techniques.Therefore, various novel biotechnologically important products from uncultivable organisms have been isolated by this approach.

In this study, environmental sediment samples were collected from extreme acid mine drainage (AMD) environments. Those samples were subjected to direct DNA isolation, but high quality DNA could not be obtained. To overcome this problem, samples were enriched in an elemental sulfur containing medium (0.1 g of NH_4Cl, 3 g of KH_2PO_4, 0.1 g of $MgCl_2x6H_2O$, 0.14 g of $CaCl_2x2H_2O$, 10 g of UV sterilized sulfur per liter, pH 4.2). Microbial flora enriched in this culture was determined by 16S rDNA PCR method. It was found that flora was dominated by *Acidithiobacillus*, *Sulfobacillus* and uncultered microorganisms. Presence of uncultured bacterium in enrichment culture indicates that this culture may serve as a source for genetic material which have not been discovered or defined yet. Considering this, the enrichment culture was used to construct a fosmid library to screen for lipase and protease enzymes. Firstly, genomic DNA was isolated and ligated into high capacity DNA vectors called fosmids. Ligation products were transferred to phage resistant *Escherichia coli* cells by phage particles and a library that is composed of about 12,000 clones was constructed. 12,000 individuals, each carrying a genome fragment of bigger than 21 kb; generated a library containing microbial genetic information more than 252 Mbp. Screening for protease and lipase enzymes were conducted using skim milk and tributyrin LB agar plates, respectively. Unfortunately, none of the clones showed protease activity but three of the screened clones showed lypolytic acitivity on agar plate. Further studies will be carried out to characterize these fosmid clones carrying potential lipase coding genes.

SIVI/SUPERKRİTİK CO_2 ORTAMINA UYGUN YENİ HİDROLAZ ENZİMLERİNİN İZOLASYONU İÇİN ÖN-ZENGİNLEŞTİRME UYGULANMIŞ METAGENOMİK KÜTÜPHANE KURULMASI

ÖZET

CO_2 normal şartlarda gaz halindedir ve basınç yükseldikçe sıvılaşır (LCO_2). Sıcaklık ve basınç kritik noktayı geçtiği zaman ise superkritik CO_2 ($SCCO_2$) edilir. $LCO_2/SCCO_2$ uygulaması endüstriyel olarak temizleme işlemlerinde ve gıdalarda koruma amaçlı kullanılmaktadır. Toksik olmaması ve temizleme işlemlerinde kullanılan zararlı kimyasalların yerine kullanılabilmesi $LCO_2/SCCO_2$'in avantajlarındandır. Fakat $LCO_2/SCCO_2$ hidrofilik maddelere karşı etkinliği tatmin edici boyutta değildir. Bu sistemin etkinliğinin arttırılabilmesi için lipaz ve proteaz gibi hidrolaz enzimleri ile zenginleştirilmesi gerekmektedir. $LCO_2/SCCO_2$ sisteminin biyolojik olarak ekstrem bir ortam olması sebebiyle kullanılacak enzimlerin düşük pH'da ve düşük su içeriği olan ortamda çalışabilmeleri gerekmektedir. Bu tip enzimler asidofilik (düşük pH) ve halofilik (yüksek tuz konsantrasyonuna bağlı olarak düşük su içeriği) mikroorganizmalardan metagenomiks yaklaşımı ile izole edilebilir. Metagenomiks belirli bir çevrede bulunan mikrobiyal populasyonun toplam genom analizi olarak tanımlanmaktadır. Yeryüzündeki mikroorganizmaların çok büyük bir bölümü henüz kültüre edilememektedir ve metagenomiks kültür tekniklerine bağlı kalmadan bu mikroorganizmaların genomu üzerinde çalışılabilmeyi sağlamaktadır. Bu şekilde, kültüre edilemeyen mikroorganizmalardan endüstriyel açıdan önemli bir çok ürün elde edilebilmiştir.

Bu çalışmada, ekstrem asit maden sahalarından örnekler toplanmıştır. Bu örneklerden direk genomik DNA eldesi yapılmaya çalışılmış fakat yüksek kalitede DNA elde edilememesi sebebiyle örnekler zenginleştirme kültürüne (0.1 g NH_4Cl, 3 g KH_2PO_4, 0.1 g $MgCl_2x6H_2O$, 0.14 g $CaCl_2x2H_2O$, 10 g sülfür, 1 lt, pH 4.2) inoküle edilmiştir. Bu zenginleştirme kültüründe gelişen flora 16S rDNA PCR yöntemi ile belirlenmiş ve florada *Acidithiobacillu*s, *Sulfobacillus* ve kültüre edilememiş bakterilerin baskın olduğu görülmüştür. Zenginleştirme kültürlerinde kültüre edilememiş bakterilerin de bulunması bu kültürün henüz tanımlanmamış ya da keşfedilmemiş genetik materyaller için bir kaynak olabileceğini göstermiştir. Bunu göz önünde bulundurarak, zenginleştirme kültüründen fosmid kütüphanesi kurulmuş ve oluşturulan kütüphane lipaz ve proteaz enzimleri için taranmıştır. İlk olarak, elde edilen yüksek kalitede genomik DNA fosmid adı verilen yüksek kapasiteli DNA vektörlerine aktarılmıştır. Ligasyon ürünü faj ekstraktları aracılığı ile faja dirençli *Escherichia coli* hücrelerine aktarılmış ve yaklaşık 12,000 klon içeren bir kütüphane oluşturulmuştur. 12,000 birey 21 kb'dan büyük bir genom fragmenti taşımaktadır ve kütüphanenin 252 Mbp'den büyük bir genetik bilgiyi içermektedir. Proteaz ve lipaz enzimlerinin taranması için sırası ile yağsız süt tozu ve tributirin içeren LB agar besiyerleri kullanılmıştır. Kolonilerin hiçbiri proteaz aktivitesi göstermezken, taranan kolonilerden üç tanesi agar besiyeri üzerinde lipolitik aktivite göstermiştir. İleriki çalışmalarla bu potansiyel lipaz enzimlerini kodlayan genler karakterize edilecektir.

1. INTRODUCTION

1.1 Metagenomics

1.1.1 Definition and history

At the beginning of the twentieth century, it was believed that microorganisms could not be characterized without being cultured. In the second half of the twentieth century, scientists realized that genomes of microorganisms can be obtained, archived and characterized without using pure culture of the organisms [1]. Today, we know that there are many microorganisms, which are not amenable to culturing [2]. Statistics show that 99 % of the microorganisms present in many environments are not culturable [3]. The percentage of microorganisms in soil that are isolated and proliferated in laboratory conditions is found to be 0.3 % and this value is 0.0001 % for water-associated microorganisms [4]. Despite the fact that we cannot culture all the microorganisms present on the earth, we still know that they exist and this situation brought a new research field out called environmental genomics, or more commonly used metagenomics.

The first definition of metagenomics was made by Handelsman in 1998 as "*Habitat based investigation of mixed microbial populations at the DNA level"* [5]. In addition, some other definitions made by different scientists can be found in the literature. According to Leveau and his friends, metagenomic libraries are databases of bacterial clones, usually *Escherichia coli* carrying DNA fragments that originate from collective genomes of all the organisms present in the particular environment, habitat and assemblage [6]. According to Reinsfeld and his group, metagenomics decribes the functional and sequence based analysis of collective microbial genomes contained in environmental samples and metagenome approach is the culture independent genomic analysis of microbial communities in the environment [7]. The main principle in metagenomics is that "all of the microorganisms are not discovered yet but this does not mean we cannot make use of them, especially of their DNAs". In general, metagenomics analyze complex genomes of microbial niches [3].

In the literature, some other terms defining metagenomic cloning, such as soil DNA library, e(nvironmental)DNA library, microbial population genomics, recombinant environmental genomics, community genomics, whole genome shotgun sequencing, environmental genomics and ecogenomics, can be found [1].

Metagenomics, as can be interpreted from its definition, is in a compact relationship with many other disciplines as shown in the Figure 1.1.

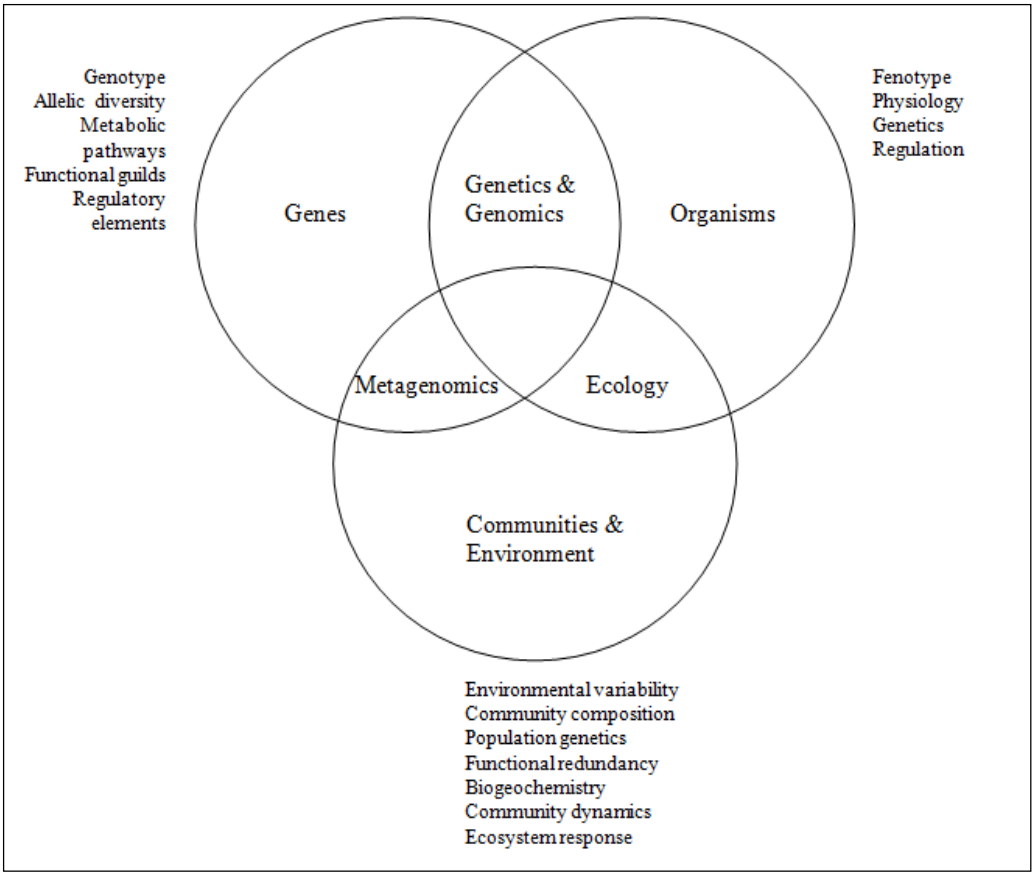

Figure 1.1 : Metagenomics and its relationship with other basic disciplines [Modified from 8].

1.1.2 Applications and tools of metagenomics

The biological sources for a metagenome can be a wide variety of samples such as soil, lake sediments, seawater, air and ancient remnants. In addition, studies on human microbiome are also metagenomic studies in which human body is considered as an environmental system.

The oldest application of metagenomics is the microbial community determination in a given population. The most common method is the 16S rRNA sequencing to

determine the taxa and species of microorganisms. However, this method cannot provide enough information about the functional role of different microbes within the community, genetic information they contain and the physiology, biochemistry or ecological function of these microorganisms [3]. Metagenomics can be applied to understand those mechanisms in an expended way.

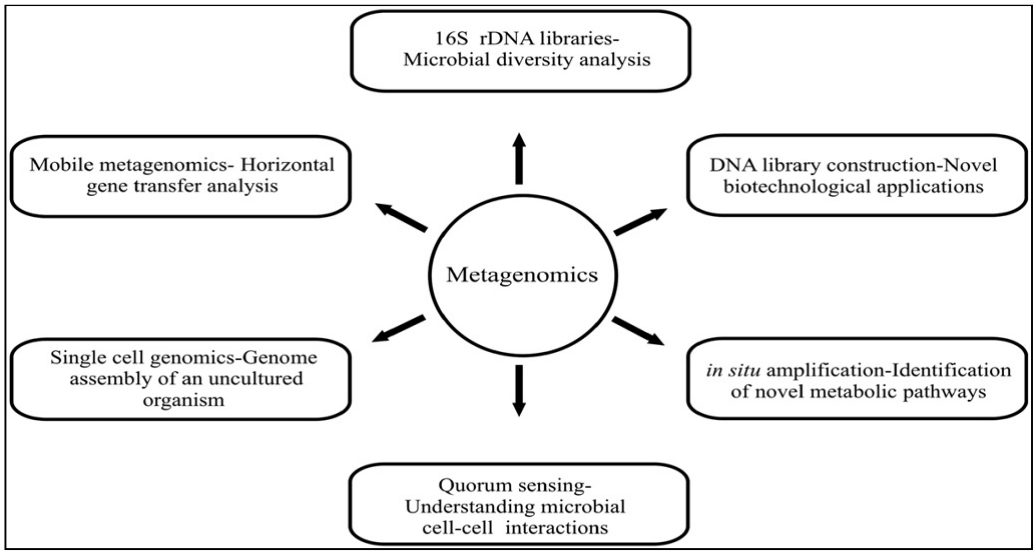

Figure 1.2 : Applications of metagenomics [9].

As summarized in Figure 1.2, there are various applications of metagenomics. However, in the rest part of this text, it will be focused on construction of genomic DNA libraries from soil and sediment samples and on novel biotechnological applications of metagenomics. Comparison of experimental steps in routine approaches and metagenomics approach for isolating microbial genes is shown in Figure 1.3.

General steps in a metagenomic study are DNA isolation from microbial niches, construction of DNA libraries and mining clones and DNA sequence of interest. Those steps will be explained for metagenomic studies mainly focused on soil and sediment samples. In metagenomics, it is a prerequisite to obtain high molecular weight and large quantity of DNA. Widely used experimental steps for DNA isolation and library construction are listed below [9].

Source of the metagenome

Choosing the right environmental samples for targeted genes is a critical step in metagenomic studies. For example, when a gene coding for an enzyme, which may

be a candidate to be used in bioremediation, is targeted, it is reasonable to collect the environmental sample from a polluted area. Samples should be collected carefully to minimize contamination and it is useful to proceed to next steps such as DNA isolation immediately to ensure the quality of DNA.

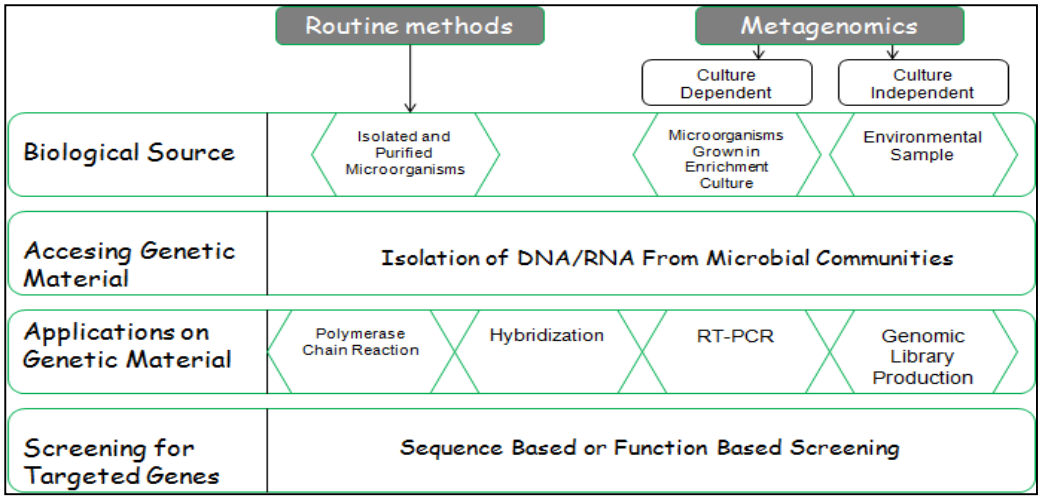

Figure 1.3 : Approaches for isolation of novel genes from microbial sources [Modified from 2]

In constrast to the advantages of metagenomics such as collection of high proportion of genome present in environment and possibility to reach genomes of uncultured microorganisms, one of the major problems in metagenomic library construction is disability in obtaining high quality DNA. Because soil is a complex system, it involves various materials that can degrade or inhibit isolation of DNA [9]. When targeted gene represents a small proportion of total nucleic acid fraction present in the sample, it is possible to overcome this problem by enriching the samples in medium containing specific substances related to targeted genes [2]. Genomic material isolated from enrichment culture is usually more pure and qualified to be used in library production procedures. Some examples for pre-enriched environmental libraries are listed in Table 1.1.

Cell lysis

There are various nucleic cell lysis methods to extract genomic material from cells. Cell lysis is carried out as by using direct or indirect methods. In direct methods, microbial cells in soil can be lysed chemically or by enzymatic reactions. Those are usually gentle methods and penetration into the soil particles is not possible. In mechanical methods such as thermal shocks, bead mill homogenization, microwave heating and ultrasonication, yield of cell lysis increase. Beside the effectiveness of

mechanical methods, shearing of the DNA is the major limiting factor for this application.

Table 1.1 : Examples for biotechnologically important products isolated using pre-enriched metagenomic libraries.

Gene	Vector	Environmental sample	Ref
Glycerol dehydratase	Plasmid	River sediment	10
Diol dehydratase	Plasmid	Sugar beet field	10
Biotin	Plasmid	Forest soil	11
Cellulase	Cosmid	Enriched on dehydrated grasses	12
Carboxylesterase	Plasmid	Mining area	13
Magnetosome islands	Fosmid	Aqueous environments	14
Possible drugs	Fosmid	Plant microbiota	15

Because the efficiency of DNA extraction method depends on the type of soil, there is inconsistency between results of studies on comparison of soil DNA extraction methods [16-19]. However, sodium dodecyl sulphate is the most widely used chemical for direct cell lysis [20].

In indirect lysis procedures, cells are separated from soil matrix with cation-exchange resins or by gradient centrifugation using gradient makers such as Nycodenz®. Indirect lysis methods reveal purer and undamaged DNA but because cells usually adhere to soil particles, extracted DNA usually does not represent whole community.

DNA Extraction

After cell crude is obtained, sample is deproteinased by salting out method or by organic solvents. Salts such as sodium chloride, sodium acetate, ammonium acetate are used for salting out. The main principle in this method is the decreased solubility of proteins in the presence of high salt contents. Phenol, phenol-chloroform and chloroform-isoamylalcohol extractions are widely used organic solvents. Those organic solvents cause a phase formation between aqueous and organic solutions. DNA in the crude lysate remains in the water-based phase while proteins are collected at the interphase. Aqueous phase containing metagenomic DNA can further be purified with isopropanol, ethanol or polyethylene glycol precipitation.

Quantification of the DNA

Once the metagenomic DNA is extracted, its concentration should be determined for further applications. For this purpose, spectrophotometric, electrophoretic and fluorometric analysis can be carried out.

Purification of the DNA

In addition to the concentration, the purity of the DNA is one of the important markers for DNA quality. Metagenomes extracted from soil samples usually contaminated with humic compounds such as humic acid, fulvic acid and humin. Those substances give a brownish colour in solution and interfere with enzymatic reactions such as PCR and cloning. The electrophoretic mobility of humic substances resembles DNA and they co-migrate in the electrical field. For removal of humic substances from DNA solution, agarose gels supplemented with polyvinylpyrolidone (PVP) are used. PVP decreases the mobility of humic compounds and DNA can be further extracted from uncontaminated gel matrix. Some inhibitory materials can be removed by treatment with multivalent cations such as Mg^{2+}, Ca^{2+}, Fe^{3+}, activated charcoal and resin. Gel filtration and electroelution are also useful methods for purification of metagenomic DNA solutions. In addition, to remove humic acids from crude cell extracts, hexadecyltrimethylammonium bromide (CTAB), caesium chloride density gradients, polyvinylpyrrolidone (PVPP), various gel filtration resins and ion-exchange and size exclusion chromatography are also used [21].

Construction of the library

In construction of metagenomic DNA libraries, choosing vector systems is a critical step. Plasmids, bacterial artificial chromosomes (BAC) cosmids, fosmids are widely used vector systems in library production.

Plasmids: Plasmids are used for libraries with small inserts (<10 kb). While expression of the insert is relatively much easier in plasmids, excellent number of clones makes screening studies tedious. Most widely used plasmids are pZErO, pBlueScript, pUC [22-26].

BAC: Those vectors are preferred for very large inserts up to 200 kb. BAC is a modified plasmid that contains an origin of replication derived from the *E. coli F factor*. It is frequently used for large insert cloning experiments. It exists within the cell very much like a cellular chromosome. The size of one insert is about 10 % of bacterial genome. Advantages of BAC are large insert size, controlled replication (2

copy per cell), low chimerism level [27]. Requirement for high amount of sample and being expensive in terms of time and screening are the disadvantages of BACs.

Cosmids and fosmids: Cosmids also have a large insert capacity. They include *cos sequences* originated from lambda phage. *Cos sequences* are essential for DNA to be packaged into the phage capsids. Phage capsids allow foreign genes to be transferred to host cells by transduction. Fosmids can accept inserts about 40 kb in size. Fosmids are vectors based on bacterial F plasmids. They also include *cos sequences* that are essential for being packaged into phage capsids. Advantages of fosmids over BAC and cosmid are that library can be constructed in a few days and fosmids can stay in low copy number while they can be induced to high copy numbers when needed [28]. This property is especially important for clone stability. When large insert vectors are used, the number of clones decreases and more importantly, large gene clusters and operons are not separated. So, genes do not lose their function.

Screening library for targeted genes

Screening the metagenomic library can be sequence driven or function driven. In sequence driven approach, colonies in the library are screened for specific targeted sequences using oligonucleotide probes. In function based approach, members of the library are screened for the required activity. For example, when targeted enzyme is an amylase, colonies are plated on agar medium containing starch, an amylase substrate, and starch hydrolysis activity of the clones are analyzed.

Metagenomics studies have already resulted in discovery of novel biocatalyzers including lipases, agarases, alpha-amylases, beta-glucanase, esterases, amidases, oxidoreductases, dehydratases, chitinases and aldehyde dehydrogenases. In addition to discovery of new enzymes, novel antimicrobial peptides, drug precursors and bioactive components have been identified [29]. Among these products, there are some enzymes showing extremophilic characteristics which have been discovered by metagenomic approach.

1.2 Extremophilic Microorganisms and Industrial Importance

Extremophiles are organisms that can thrive under a variety of extreme environments. They live and proliferate under harsh conditions. They are categorized according to their “extreme” characteristic and they fall into classes such as thermophiles, psychrophiles, piezophiles, alkaliphiles, acidophiles and halophiles. Thermophilic microorganisms are usually found in hot springs and volcanic areas.

They live at a optimum temperature range from 60 to 80 °C. Some hyperthermophiles can also grow higher temperatures than 80 °C [30]. High temperature processes provide increased solubility of substances and decreased contamination risk in biotechnological applications and enzymes isolated from thermophilic microorganisms are advantageous in those biotechological applications. The mostly known example for thermophilic enzymes is *Thermus aquaticus* Taq DNA polymerase which is stable at 90 – 95 °C. Thermophilic proteins have a high structural stability and this is achieved by excluding thermo-sensitive aminoacids, increasing surface charge and increasing hydrophobicity of the protein core [30].

Psychrophilic microorganisms thrive under cold temperatures in contrast to thermophiles. They are usually found in deep sea, upper atmosphere or caves and have an optimum growth temperature of about 15 °C. Enzymes obtained from those microorganisms used in laundary applications that can be done at low temperatures. Food industry, pulp and paper industry also get benefit from those cold-adapted enzymes. Psychrophilic adaptation of enzymes to low temperatures is thought to be gained by flexible structure which is achieved by reduced protein core hydrophobicity, reduced charge of surface residues and additional surface loops rich in pyroline and glycine aminoacids and low kinetic energy of molecules is compensated owing to these characteristic features [31].

Piezophiles, previously called barophiles, are organisms that can survive under high pressures. Those usually live in deep oceans under pressures up to about 100 MPa with an optimum of 40 – 60 MPa. While piezophilic microorganisms can survive under those pressures, monomeric proteins can be denatured under pressures higher than 400 MPa. Pressure-resistant proteins are considered to be useful in food processing applications. Because of the difficulties in cultivating piezophiles in laboratory conditions, studies on piezophiles are not so wide and are mainly focused on determination of the effect of pressure on stability and activity of the proteins. The design of biotechnological applications based on piezophiles has still been on progress [32].

Acidophilic and alkaliphilic microorganisms live under extreme pH conditions. Acidophiles have an optimum pH of less than 3.0 while this value is higher than 9.0 for alkaliphiles. Acidophiles are usually found in acidic soils and acid mine drainage environments. Alkaliphiles grow in soda lakes or carbonate rich soils. These

microorganisms usually maintain their cytoplasmic pH very close to neutral values. So, intracellular proteins do not need to adapt to extreme pH values but this is not the same case for extracellular proteins. They have been evolved with some mechanisms to adapt extreme H^+ or OH^- ion concentration outside of the cell [33-34]. In contrast to the acception that intracellular proteins of acidophiles do not need to adapt acidic environment, a study made on acidophilic *Ferroplasma acidiphilum* (pH optimum of 1.7), a chemolithoautrophic archeon hat gains energy by oxidizing iron, revelaed that many intracellular enzymes have an optimum pH much lower than the intracellular pH of 5.6. Main metabolic enzymes such as glycosidase, glycosyltransferase and carboxylase were isolated from this microorganisms and their optimum pH range was between 1.7 and 4.0 [35].

Halophiles are adapted to high salt concentrations and live in hypersaline environments. They can survive by maintaining osmotic balance and they accumulate sodium or potassium salts inside of their cytoplasm [36]. Both intracellular and extracellular proteins from halophiles can maintain their stability and activity in 4 M KCl and >5 M NaCl [37]. It is known that halophilic proteins have more acidic residues and less basic residues compared to mesophilic proteins. In addition, they replace large hydrophobic residues with small and less hydrophobic residues so they require higher salt concentrations for appropriate folding and gaining function [38]. Halophilic enzymes are usually exploitable in organic solvents because of their ability to maintain their activity in low water content mediums.
Classification of extremophiles and some industrially important products of these microorganisms are shown in Table 1.2.

In addition to those mentioned microorganisms, some other extremophiles such as metallophiles, radiophiles and microaerophiles have been defined. Metallophiles, those are resistant to high metal concentrations, are used for ore-bioleaching, bioremediation and biomineralization. Radiophiles, which thrive under high radiation levels, are used in bioremediation of radionuclotide contaminated soils [39]. However, possible biotechnological applications exploiting enzymes obtained from those microorganisms are not clear yet. Among those industrially important extremophilic enzymes, lipases and proteases are of major concern within this study.

Table 1.2 : Major categories of extremophilic microorganisms and applications of biomolecules isolated from them [modified from 30, 39, 40, 41].

Type of the extreme microorganisms	Growth characteristic	Enzymes	Applications
Thermophiles	50 – 110 °C	Protease	Detergents, hydrolysis in food and feed, brewing, baking Starch, cellulose, chitin, pectin processing, textiles
		Amylase Pullulanase Glucoamylase Glucosidase Cellulase Xylanase	Paper bleaching Chitin modification for food and health products
		Lipase Esterase	Detergents, stereo-specific reactions
		DNA polymerase	Genetic engineering
		Dehydrogenase	Oxidation reactions
Psychrophiles	0 – 20 °C	Protease	Detergents, food applications such as cheese maturation and dairy production
		Dehydrogenase	Biosensors
		Amylase	Detergents and bakery
		Cellulase	Detergents, feed and textiles
		Lipases	Detergents, food and cosmetics
		Polyunsaturated fatty acids	Pharmaceuticals
Piezophiles	Pressure up to 130 MPa	To be defined or whole organism can be used	Food processing, antibiotic production and formation of gels and starch granules
Alkaliphiles	pH > 9	Lipase	Food additives, detergents
		Cyclodextrin	Stabilization of volatiles
		Antibiotic	Pharmaceuticals
		Cellulase, protease	Polymer degradation agents
Acidophiles	pH < 2 – 4	Amylase	Starch processing
		Protease	Feed component
		Sulfur oxidation	Desulphurization of coal
		Chalcopyrite concentrate	Valuable metals recovery
Halophiles	3 – 20 % or 2 – 5 M NaCl	Membranes	Cosmetic additives
		Carotene	Food colouring
		Glycerol	Pharmaceuticals
		Protease	Peptide synthesis
		Dehydrogenase	Biocatalysis in organic media

1.3 Lipase and Protease

Lipases are triacylglycerol ester hydrolases (3.1.1.3) and catalyze the hydrolysis of fats and oils with release of fatty acids, diacylglycerols, monoacyglycerols and glycerol. They are required as digestive enzymes for many organisms. They are also involved in the metabolism of intracellular lipids such as biological membranes and considered as present in all organisms. Lipases are widely studied for several reasons. Their catalytic function serves as a model for the regulation of interfacial enzyme catalyzed reactions because lipases have a characteristic helical oligopeptide that behaves like a lid and takes different positions when faced with hydrophobic molecules such as lipid. Lipases are also important in terms of medical concerns because they have important roles in metabolism and are related to diseases such as atherosclerosis and hyperlipidemia [42]. Reactions catalyzed by lipases are shown in Figure 1.4.

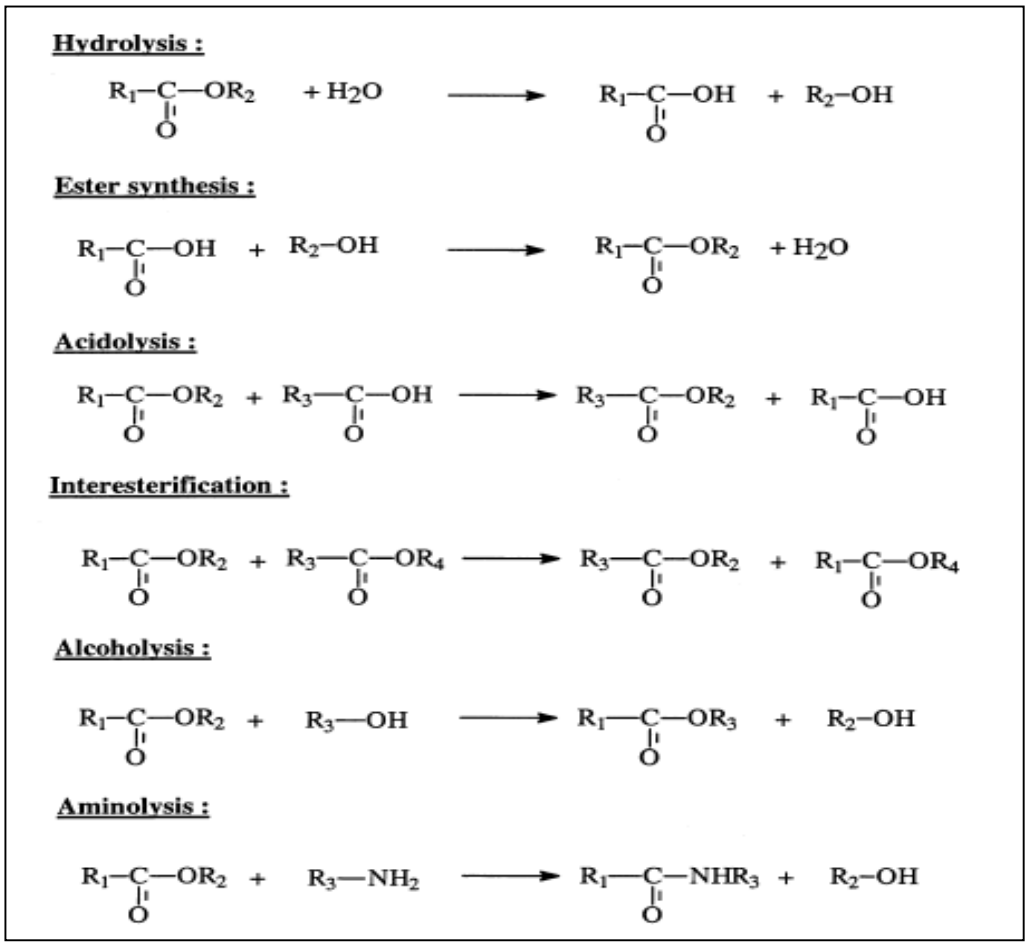

Figure 1.4 : Reactions catalyzed by lipases [42].

Lipases are widely used in many industrial applications. Fat hydrolysis in milk, analysis of fatty acid compositions in fats and oil, blood triglyceride assays, synthesis of esters, transesterification of natural oil and removal of stains are some of the examples for lipase exploitation in industry.

Proteases catalyze the breakdown of peptide bonds. They are ubiquitous because they are found in every organism. Proteases are categorized according to their catalytic mechanisms: serine (EC.3.4.21), cycteine or sulfhydryl (EC.3.4.22), aspartic (EC.3.4.23) and metalloproteases (EC.3.4.24). Proteases are important enzymes in maintaining hemeostatis because they are involved in cell growth, cell death, blood clotting and immune defense. Furthermore, pathogenic microorganisms and some viruses used proteases for infection and life cycles. For this reason, they are targeted molecules in drug design. In addition to their metabolic importance, proteases have attracted biotechnological area and account for 40 % of total enzyme sales in various industries. Proteases have been exploited by detergent, food, pharmaceutical, leather, diagnostic and leather industries. Detergent industry, the major user of proteases, widely utilizes alkaline proteases which are active at high pH values. Alkaline proteases usually have a serine in active site or they are metalloproteases [43-45].

1.4 Liquid and Super Critical Carbondioxide

CO_2 is in gas form under standard pressure and temperature (STP). Liquid CO_2 (LCO_2) is obtained when pressure is increased and when temperature and pressure exceed the critical point, which is about 30 °C and 80 bar, supercritical CO_2 ($SCCO_2$) is formed as shown in the Figure 1.5. Some important characteristics of LCO_2/$SCCO_2$ make it attractive in biotechnological researches. LCO_2/$SCCO_2$ is inert, non-toxic and not flammable. It works under low temperatures and prevents oxidation. In addition, LCO_2/$SCCO_2$ has high diffusivities and when applied to a surface it can evaporate with no residual substance at the end of the process. It is known that LCO_2/$SCCO_2$ has good cleaning activity against hydrophobic dirt. High pressure dry cleaning using compressed CO_2 has been applied at industrial scale [47-49]. When it is used for cleaning, disinfection and sterilization purposes, the amount of water used is decreased. LCO_2/$SCCO_2$ can also replace hazardous organic

substances such as formaldehyde, glutaraldehyde and ethylene oxide which are highly toxic and suspected to be mutagenic.

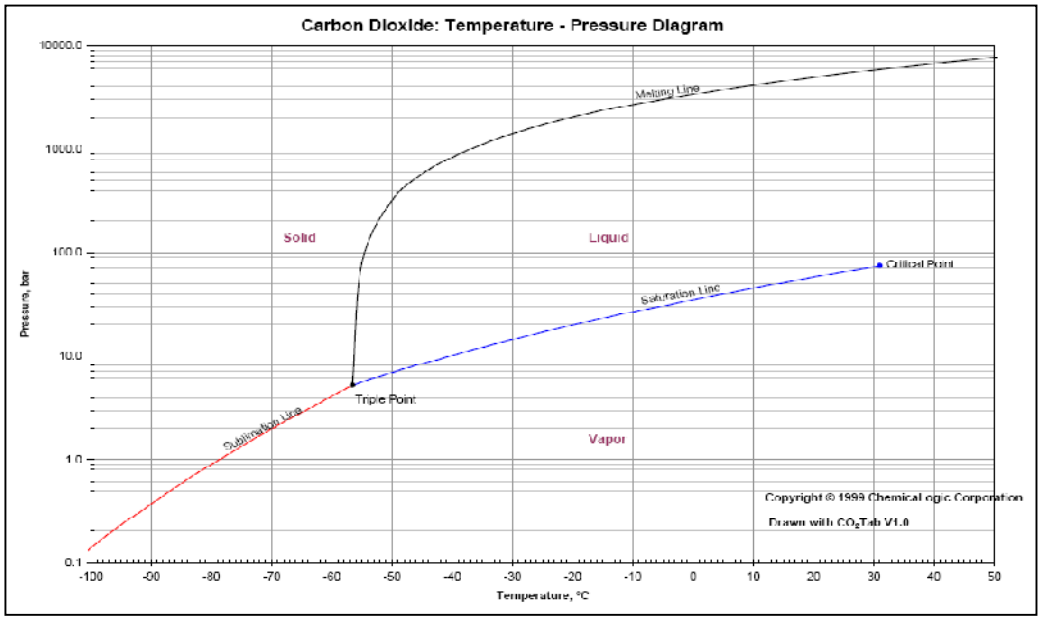

Figure 1.5 : Phase diagram of CO_2 [46].

The antimicrobial effect of $LCO_2/SCCO_2$ has been showed in many studies. Those studies are mainly focused on inactivation of microorganisms in liquid or solid culture mediums and in food products [50-54]. In addition, compressed CO_2 has been evaluated for its disinfection property on fabrics used in medical applications [55]. $LCO_2/SCCO_2$ is also prospected to be advantageous in cleaning, sterilization and disinfection of thermo-labile medical devices such as endoscopes and implants. However the performance of $LCO_2/SCCO_2$ on hydrophilic substances is not so satisfactory. To enhance this property, $LCO_2/SCCO_2$ can be enriched with extreme hydrolase enzymes such as protease and lipase that can exhibit activity at low pH and at low water activity. While there is only a few study in the literature about lipase utilization in $SCCO_2$, the activity of lipases isolated from *Pseudomonas fluorescens, Rhizopus javanicus, R. niveus* and *Candida rugosa* were not so promising when used in $SCCO_2$ [56].

1.5 Aim of The Research

As mentioned above, liquid or supercritical CO_2 is effective in terms of cleaning applications and its activity can be enhanced by using extremophilic enzymes which provides great advantages in biotechnological processes. LCO_2 or $SCCO_2$ is an

extreme medium with no water content and acidic pH. So, enzymes applicable in LCO_2 or $SCCO_2$ should be active both in low water content and acidic pH. Enzymes having these extreme characteristics can be isolated from halophiles and acidophiles. Halophilic enzymes are adapted to high salt content, thus reduced water activity and usually active in many processes based on organic solvents. In addition, acidophilic enzymes have evolved to maintain their activity under low pH conditions.

The aim of this study was to construct genomic libraries of acidophilic environmental samples and to screen those libraries for lipase and protease enzymes. Those enzymes will further be candidates to be used in LCO_2 or $SCCO_2$ cleaning/disinfection system.

2. MATERIALS & METHODS

2.1 Materials

2.1.1 Bacterial strains

E. coli TOP10 strains; F- mcrAΔ (mrr-hsdRMS-mcrBC) φ 80lacZ ΔM15 ΔlacX74 recA1 araD139Δ (araleu) 7697 galU galK rpsL (StrR) endA1 nupG (One Shot TOP10Electrocompetent cells, Catalog #C4040-10, Invitrogen) strain was used in cloning 16S ribosomal genes. EPI300^{TM} - T1^{R} *E. coli* strain.

F- mcrA Δ(*mrr-hsd*RMS-*mcr*BC) (StrR) φ80d*lac*Z ΔM15 Δ*lac*X74 *rec*A1*end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G *trf*A *ton*A *dhfr* (EPI300™-T1R Phage T1-resistant *E. coli* Plating strain, Epicentre) also was used in genomic library production.

2.1.2 Cloning vectors

2 different vectors were used in cloning studies. pCR®2.1.-TOPO® vector (given in Appendix A) was used in cloning 16S ribosomal genes (Catalog #K4560-40, Invitrogen). This vector is linearized with single 3' - thymidine (T) end and covalently bound to Topoisomerase I enzyme. The second vector is CopyControl PCC1FOS^{TM} Fosmid Vector (given in Appendix B) which is linearized at the unique *Eco* 72 I site and dephosphorylated. It has chloramphenicol resistance gene as antibiotic selectable marker and *ori*V – high copy replication origin that is activated when induced.

2.1.3 Enzymes

The used enzymes are listed below.

Taq DNA polymerase

Taq DNA polymerase is a standard enzyme for the amplification of DNA fragments up to 3 kb by polymerase chain reaction (Catalog # 04738241001, Roche).

Proteinase K

This enzyme is an endolytic protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic and hydrophobic amino acids (Catalog #EO0491, Fermentas). It is classified as serine protease. It was used in genomic DNA isolation from cultures.

Not I

Not I is a restriction endonuclease that specifically recognizes the sequence shown below (Catalog #ER0591, Fermentas). This enzyme cuts the PCC1FOS vector from position 2 and 643 which includes the cloning site.

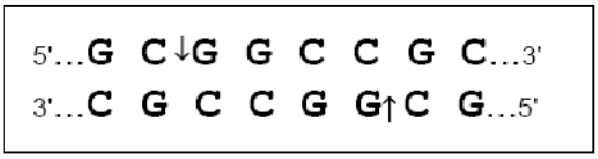

Figure 2.1 : Not I restriction site.

2.1.4 DNA molecular weight markers

DNA molecular weight standards were purchased from Fermantas.

2.1.5 Oligonucleotides

Oligonucleotides, listed in the following, were synthesized by Genova (Alpha DNA) using Applied Biosystems 308A DNA synthesizer.

pA – F 5'- AGAGTTTGATCCTGGCTCAG 3'
pH – R 5'- AAGGAGGTGATCCAGCCGCA 3'
M13 – F 5'- GTAAAACGACGGCCAG 3'
M13 – R 5'- CAGGAAACAGCTATGAC 3'

2.1.6 Culture medium

LB (Luria-Bertani)

10 g tryptone (Merck), 5 g yeast extract (Labo) and 5 g NaCl (Carlo Erba) were dissolved in distilled water up to 1 lt and the pH is adjusted to 7.0 with 10 M NaOH. Medium was sterilized by autoclaving for 15 min at 121 °C.

LB Agar Medium

10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar (Merck) were dissolved in 1lt

distilled water and the pH is adjusted to 7.0 with10 M NaOH. Medium was sterilized by autoclaving for 15 min at 121 °C. For protease acitivity screening, 1 % skim milk (Sigma Aldrich) was added to the LB agar mixture and autoclaved. After sterilization appropriate amount of 500X induction solution and chloramphenicol (Applichem) were added to the warmed solution and poured onto plates. For lipase activity screening, 1 % tributyrin (Sigma Aldrich) and 1 % gum arabic (Fluka) were added to the LB agar mixture and autoclaved. After sterilization, mixture was cooled, supplemented with 0.0001 % rhodamine B (Sigma Aldrich) and sonicated during 2 min with 10 sec/10sec pulse on/off periods to yield a homogenous emulsion. Mixture gaining a milky appearance after sonication indicates tributyrin miscelles getting small. After sonication, appropriate amount of 500X induction solution and chloramphenicol were added to the warmed solution and poured onto plates.

SOC Medium

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

35S Medium

0.1 g NH_4Cl (Merck), 3 g KH_2PO_4 (Merck), 0.1 g $MgCl_2x6H_2O$ (Merck), 0.14 $CaCl_2x2H_2O$ (Merck) were dissolved in distilled water up to 1 lt and pH was adjusted to 4.2 with ½ diluted HCl. The solution was autoclaved at 121 °C for 15 min. 10 g of sulphur (Merck) was soaked with 70 % EtOH and sterilized under UV radiation until EtOH is evaporated completely. Sterilized sulphur was aseptically layered on to the liquid media.

2.1.7 Stock solutions

Ampicilline stock

1 g of ampicilline sodium salt was dissolved in 10 ml distilled water, filter sterilized and stored at -20 °C.

Chloramphenicol stock

125 mg of chloramphenicol was dissolved in 10 ml EtOH, filter sterilized and stored at -20 °C.

X-gal stock

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

Glycerol stock

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

2.1.8 Buffer solutions

Sodium Acetate Buffer

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

50X TAE Buffer

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

Phosphate Buffered Saline

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were dissolved in 800 ml distilled water and the pH was adjusted to 7.4. Distilled water was added to adjust the volume to 1 lt.

Phage Dilution Buffer

Phage dilution buffer was prepared to have a 10 mM Tris-HCl (pH 8.3), 100 mM NaCl and 10 mM $MgCl_2$.

DNA Extraction Buffers

DNA extraction buffer for soil samples (EB I) were composed of 100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1 % CTAB (w/v). All ingredients were mixed at 65 °C and stored at room temperature. DNA extraction buffer for culture samples (EB II) were composed of 50 ml 0.5 M EDTA (pH 8.0), 5 ml 5M NaCl, 25 ml 20 % SDS (w/v), 20 ml 1 % CTAB (w/v). All ingredients were mixed at 65 °C and stored at room temperature.

Alkaline Lysis Buffers

STET solution was prepared with 8 % sucrose, 5 % Triton X – 100, 50 mM EDTA (pH 8.0) and 50 mM Tris-HCl (pH 8.0). Resuspension buffer was composed of 50 mM glucose, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA. Lysis buffer was freshly prepared by dissolving 1 % SDS (w/v) in 200 mM NaOH. To prepare the neutralizing solution, 29.5 g potassium acetate was dissolved in 88.5 ml distilled water and 11.5 ml glacial acetic acid solution was added. STET solution and neutralizing solution were stored at room temperature while resuspension buffer was stored at 4 °C to prevent contamination.

2.1.9 Laboratory equipments

Laboratory equipments are listed in Appendix C.

12.2 Methods

2.2.1 Sample collection

Sediment samples from acid mine drainage were kindly provided by Assist. Prof. Dr. Nurgül Çelik Balcı from Geological Engineering department, ITU. Samples were aseptically collected into sterile bags or tubes and stored at -20 °C.

2.2.2 Genomic DNA isolation from environmental sample

Direct DNA isolation from sediment samples was carried out according to Zhou et. al., 1999 and the procedure is explained below [57]. Soil samples of 5 g were mixed with 13.5 g EB I and 100 µl proteinase K (10 mg/ml) and incubated at 37°C by horizontal shaking at 225 rpm for 30 min. After the shaking treatment, 1.5 ml of 20% SDS was added, and the samples were incubated in a 65 °C water bath for 2 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 6,000 *g* for 10 min at room temperature and transferred into clean tubes. The soil pellets were treated with 4.5 ml of the EB I and 0.5 ml of 20% SDS, vortexed for 10 s, incubated at 65 °C for 10 min, and centrifuged as before. This extra step repeated twice. Supernatants from the three cycles of extractions were combined in the same clean tube and mixed with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol). The sample was mixed throughly for 5 min and centrifuged at 10,000 rpm for 5 min. Aqueous phase is formed on the up side of the liquid and it is transferred to a clean tube. About 0.6 volume of isopropanol was adde

to the solution and incubated at room temperature for 1 h. The pellet of crude nucleic acids (that are precipitated by isopropanol) was obtained by centrifugation at 14,000 *g* for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionized water.

In addition to the manual DNA isolation method, as a commercial kit Fast DNA Spin Kit for Soil (Catalog # 6560-200, MPBio) were also used. DNA isolation protocol is described below. About 1 g of soil sample was treated with 500 µl PBS, centrifuged at 14000 rpm for 10 min and supernatant was discarded. This washing step repeated twice. Up to 500 mg of washed sample added to Lysing Matrix E tube. 978 µl of sodium phophate buffer and 128 µl MT buffer was added to the sample in the tube. Mixture was homogenized in FastPrep Instrument for 40 seconds at a speed of 6. Sample was centrifuged at 14000 g for 5 min. Supernatant was transferred to a clean tube and protein precipitation solution was added and mixed by shaking the tube 10 times. Sample was centrifuged at 14000 g for 5 min. Supernatant is taken to a clean 2 ml tube. Binding matrix solution was resuspended and 1 ml of it was added to the supernatant. Solution was mixed by inverting by hand for 2 min to allow binding the DNA. Tube is placed on a rack for 3 min to allow settling the silica matrix. 500 µl of the upper solution was discarded and the remaining mixture was resuspended. 600 µl of the mixture was transferred to SPIN Filter, centrifuged at 14000 g for 1 min, catch tube was empitied, the remaining mixture was added and centrifuged before. 500 µl of SEWS-M (+EtOH) was added to the filter and was centrifuged at 14000 g for 1 min. Catch tubes was changed. A second centrifugation for 2 min was applied to dry the matrix completely. SPIN Filter was air dried for 5 min. Binding matrix above the filter was resuspended gently in 50 µl DNase-Pyrogen-Free Water. Tube was incubated at 55 °C for 5 min to increase the DNA yield. A clean tube was placed under spin filter and centrifuged at 14000 g for 1 min. DNA solution in the tube was stored at – 20 °C. To increase the DNA amount and concentration, 4 isolation processes were carried out simultaneously and the solutions were mixed before filtering step to detain the DNA in the same filter.

2.2.3 Pre-enrichment of sediment samples

DNA isolation from sediment samples were not successful enough to produce a high quality metagenomic library. To overcome this problem, sediment samples were inoculated into specific media to enrich the microbial flora.

Acid mine drainage are usually composed of sulfur oxidizing bacteria, especially *Thiobacillus* species, whose optimum growth temperature varies about 25 to 35 °C. To increase the viable cell number in acid mine drainage sample, 35S media was used. 10 g of sediment sample was inoculated into 100 ml liquid media and incubated at 30 °C for 30 days by horizontal shaking at 225 rpm.

2.2.4 Determination of microbial population enriched in 35S medium

2.2.4.1 Genomic DNA isolation from enrichment culture

Genomic DNA from enrichment culture was isolated using MoBio UltraClean™ Microbial DNA Isolation Kit (Catalog #12224-50). 1.8 ml of the 35S culture was taken into a 2 ml tube, centrifuged at 2000 rpm for 2 min to pellet the sulphur. Supernatant was taken to a new 2 ml tube, centrifuged at 10000 rpm for 5 min. Supernatant was discarded, cell pellet was resuspended with 300 µl MicroBead solution. Resuspended cells were transferred to MicroBead tube. 50 µl of MD1 solution was added to MicroBead tube. Samples were vortexed for 10 min at maximum speed using the special aparatus for microbead tubes. MicroBead tube was centrifuged for 30 sec at 10000 g. Supernatant was transferred to a 2 ml collection tube. 100 µl of MD2 solution was added, vortexed for 5 sec and incubated at 4 °C for 5 min. Tube was centrifuged at 10000g for 1 min. Supernatant was transferred to a new collection tube. 900 µl of MD3 solution was added, vortexed for 5 sec. 700 µl of the solution was transferred to spin filters and centrifuged for 30 sec. Filtrate, collected in collection tube, was discarded and remaining solution added to same filter, centrifuged again and filtrate was discarded. 300 µl of MD4 solution was added to filter and centrifuges at 10000 g for 30 sec. Filtrate was discarded. Empty filter was centrifuged at 10000 g for 1 min again to dry. Spin filter tube was replaced in a new collection tube. 50 µl of MD5 solution was added and centrifuged at 10000 g for 30 sec. DNA solution in the collection tube was stored at - 20 °C.

2.2.4.2 Universal amplified ribosomal region PCR

To determine the species present in the enrichment culture is important in terms of getting access to genomic sequences and targeted enzyme properties. The microbial flora enriched in 35S medium is determined by 16s rDNA UARR PCR method. The genomic DNA is isolated from culture with MoBio DNA Isolation Kit was used as

template DNA. Approximately 1500 bp long part of the 16S rDNA region was amplified by PCR using forward pA- F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse pH-R (5'-AAGGAGGTGATCCAGCCGCA-3') primers. Reaction components and PCR conditions are shown in Table 2.1 and Table 2.2 respectively.

Table 2.1 : Chemicals used for UARR PCR.

Chemical	Amount
10 X PCR Buffer	5 µl
10 mM dNTP	1 µl
Forward Primer pA-F	1 µl
Reverse Primer pH-R	1 µl
Taq Polymerase	1 µl
25 mM $MgCl_2$	6 µl
Template DNA	1 µl
Water	34 µl
Total Volume	50 µl

Table 2.2 : UARR PCR Conditions.

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

2.2.4.3 Agarose gel electrophoresis and gel extraction

Presence of PCR products were confirmed by 1 % agarose gel electrophoresis. 0.4 g of agarose was boiled in 40 ml of TAE buffer in a microwave oven for 30 sec. After that, agarose solution was poured onto horizontal gel system and let to solidify. DNA samples were mixed with 5X Amesco DNA Loading Dye & Buffer and loaded into wells. PCR products were electrophoresed under 8V/cm and visualized by UV

Transilluminator. After the presence of DNA band about 1500 bp long, all of the PCR reaction loaded into the gel to purify the PCR product. After electrophoresis, corresponding band was cut and transferred to a 2 ml tube. Extraction was performed by QIAGEN – QIAquick Gel Extraction Kit (Catalog # 28604, Qiagen) as described below. Weight of the gel slice was determined. 3 volumes of Buffer QG was added to 1 volume of gel. 100 mg gel was considered as approximately 100 µl. Sample was incubated at 50 °C until gel was completely molten. 1 gel volume of isopropanol was added to the sample and mixed by inverting the tube several times. Samples were transferred to MinElute column and cetrifuged at 13000 rpm for 1 min. 500 µl of Buffer QG was added to the column and centrifuged at 13000 rpm for min. Filtrate was discarded. 750 µl of Buffer PE was added to the column and centrifuged at 13000 rpm for min. Filtrate was discarded. MinElute column was centrifuged again at 13000 rpm for 1 min to dry completely. Column was placed on a clean tube and 10 µl of EB Buffer was added to center of the membrane. Assembly was centrifuged at 13000 rpm for 1 min after 1 min incubation to increase the dissolved DNA amount. DNA solution was stored at – 20 °C until usage.

2.2.4.4 Cloning and transformation of PCR products

TOPO TA® Cloning Kit was used for cloning PCR products. Chemicals used in cloning reaction are given in Table 2.3.

Table 2.3 : Chemicals used in clonnig reaction.

Chemicals	Amount
Salt solution	1 µl
TOPO TA Vector	1 µl
PCR Product	2 µl
Water	µl

All chemicals were mixed in a 1.5 ml tube and incubated at room temperature for 15 min. 2 µl of the cloning reaction was added into a vial of (50 µl) One Shot Electrocompetent *E. coli* and mixed gently avoiding formation of bubles. Mixture was transfered to electroporation cuvette and 1800 V was applied. After electrotransformation, 250 µl SOC medium was added and the mixture was transfered to a sterile 2 ml tube. Mixture was incubated at 37 °C for 20 min to allow

the antibiotic resistance genes to be expressed. Cells were spread into LB agar plates containing 100 µg/ml ampicilline and 40 µl of X-gal stock solution. Agar plates were incubated at 37 °C for 16 hours.

2.2.4.5 Blue/White screening and plasmid isolation

White colonies grown on LB agar plates were selected and transfered to 15 ml tubes containing 3 ml of LB broth and 3 µl of amplicilline stock solution. Cells were incubated at 37 °C for 16 hours. Roche High Pure Plasmid Isolation Kit was used for plasmid isolation (Catalog #1754785). LB culture was centrifuged at 6000 rpm for 1 min to pellet the cells. Supernatant was discarded. Cell pellet was resuspended with 250 µl of Suspension+Rnase Buffer. 250 µl of Lysis Buffer was added to the mixture. Tubes were inverted 6 – 8 times and incubated for 5 min at RT. 350 µl of pre-chilled Binding Buffer was added and mixture was incubated on ice for 5 min after mixing gently. Samples were centrifuged at 13000 g for 10 min. Collection tubes were assembled under High Pure Filter tubes and supernatant from step 5 was transferred to the filter. Samples were centrifuged at 13000 g for 10 min. Liquid in the collection tube was discarded. Filter was washed with 500 µl of Wash Buffer I and with 700 µl of Wash Buffer II, respectively. Sample was centrifuged at 13000 g for 1 min after each wash. Empty filter tube was centrifuged at 13000 for 2 min to dry completely. Filter tube was transferred to a clean 1.5 ml tube. 100 µl of Elution Buffer was added to the filter tube and centrifuged at 13000 g for 1 min. Plasmids were stored at – 20 °C until usage.

2.2.4.6 Sequence PCR and sequence PCR purification

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

After completion of sequence PCR, products were purified according to the procedure below. PCR reaction were transferred to a 1.5 ml tube. 1 µl sodium acetate (3 M, pH 5.2) and 25 µl ice-cold 95 % EtOH were added. Tubes were incubated on ice for 15 min. At the end of the incubation, samples were centrifuged at 14000 rpm for 15 min. Supernatant was discarded. Pellets were washed with ice-cold 70 % EtOH, centrifuged at 14000 rpm for 15 min. Supernatant was discarded. Excess

EtOH was evaporated at 95 °C. Pellets were dissolved in 20 µl formamide. DNA was denatured at 95 °C for 3 min. Tubes were wrapped with aluminum foil and stored at 4 °C until loading into sequence analyzer. Sequence analysis was performed by ABI 3100 Avant automized sequencer.

Table 2.4 : Sequence PCR components.

Chemicals	**Amount**
ABI-RR100 Dye	2 µl
ABI 5X PCR Buffer	2 µl
M13 Forward/Reverse Primer	3.2 pmole (1 µl)
Template	1 µl
Water	4 µl

Table 2.5 : Sequence PCR conditions.

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

2.2.4.7 Phylogenetic analysis

Sequence results obtained from sequence analysis were compared in NCBI database using Basic Local Alignment Search Tool (BLAST). Results with the best similarity were accepted.

2.2.5 Genomic library construction

2.2.5.1 High molecular weight DNA extraction

High High molecular weight and high quality genomic DNA isolation from enrichment culture was carried out according to Zeng et al., 2008 with some modifications [58]. The procedure is described below. The culture media was centrifuged at 2000 rpm for 2 min to pellet the sulfur. Supernatant was transferred to a clean tube and centrifuged at 10000 rpm for 10 min. Cell pellet was washed with

PBS buffer and pelleted by centrifugation as before. Pellet of the cell (about0.1 g) was re-suspended in 2 ml of EB II and incubated in boiling water for 8 min with inversions every 2 min. Then, the suspension was further incubated at 65 °C for 30 min followed by incubation at 75 °C for 30 min with gentle inversions every 10 min. After that incubation period, an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added and mixed gently for 5 min. Mixture was centrifuged at 10000 rpm for 5 min and the aqueous phase formed at the up side of the tube transferred to a clean tube. DNA in the aqueous phase was precipitated by adding 2.5 volumes of ice-cold ethanol and incubating at -20 °C overnight. After overnight incubation, sample was centrifuged at 14000 rpm for 15 min and washed with 70 % ethanol twice. DNA pellet was left to dry for 15 min at room temperature and dissolved in distilled water.

2.2.5.2 Genomic library production

For the construction of genomic library of the microorganisms grown in 35S media, CopyControl™ Fosmid Library Production Kit (Epicentre, Catalog # CCFOS110) was used. The overall steps in fosmid library production is shown in Figure 2.2.

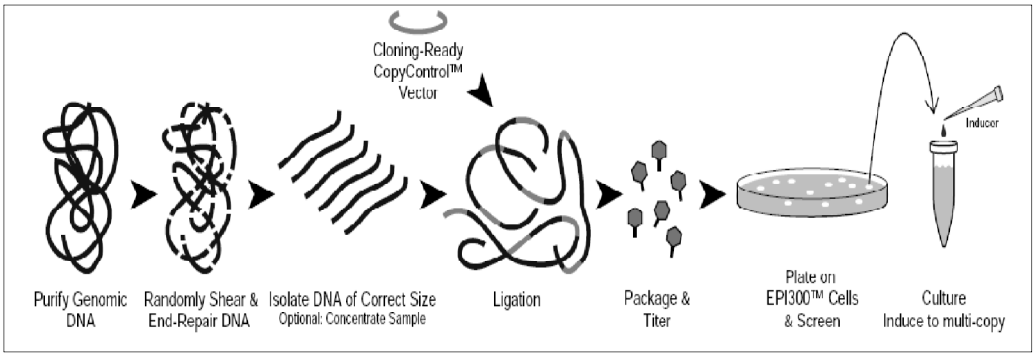

Figure 2.2 : Overview of steps in fosmid library production [28].

Fosmid library production kit allows an insert size of approximately 40 kb. When it was confirmed that the genomic DNA is big enough, end repair reaction was performed. In the end-repair reaction, phosphate groups were added to the free DNA ends. End-repair of the purified genomic DNA was carried out using components listed in Table 2.6. Reaction components were mixed on ice and incubated at RT for 45 min. At the end of the incubation period, sample was hold at 70 °C to inactivate the enzyme. End repaired DNA was loaded to a 20 cm 1 % low melting point agarose gel and electrophoresed with 3V/cm during 8 hours. Long-time

electrophoresis ensures that high molecular weight DNA fragments separate better on the gel.

Table 2.6 : Components used in end-repair reaction

Chemical	Amount
Sterile water	2 µl
10X End Repair Buffer	8 µl
2.5 mM dNTP	8 µl
10 mM ATP	8 µl
DNA	50 µl
End Repair Enzyme Mix	4 µl
Total volume	80 µl

To avoid DNA damage cause of UV light, DNA to be cloned was only mixed with loading dye and not visualized in trans-illuminator. Only a small aliquot of it was mixed with DNA dye, visualized by UV light and according to its position, corresponding site on the gel that includes main DNA portion, which is about 25 - 40 kb, were cut out of the gel at the end of electrophoresis process. Gel extraction was performed using an enzyme based procedure because other methods that include spin filters usually damage DNA. In this procedure the advantage earned by low melting point agarose usage was used. After the gel slice was taken, its weight was determined. Gel was incubated on a heat block at 70 °C until molten. GELase 50X buffer was warmed to 45 °C. Appropriate volume of warmed buffer and GELase enzyme (1U per 100 µl agarose) was added to the molten agarose. Reaction was incubated at 45 °C for 1 hour. After incubation period, the reaction was transferred to 70 °C for 10 min to inactivate the GELase enzyme. Solution was aliquoted into 500 µl volume and chilled on ice for 5 min. Tubes were centrifuged at 10000 g for 20 min to pellet any insoluble components. Supernatant was taken into a clean tube, 1/10 volume of sodium acetate and 2.5 volumes of ice-cold absolute ethanol were added. Mixture was incubated on ice for 1 hour. It was centrifuged at the end of 1 hour at 14000 rpm for 15 min. DNA pellet was washed with 70 % ethanol twice. DNA was left to dry for 15 min and dissolved in 8 µl sterile water. DNA was stored at – 20 °C until usage. A small aliquot (1 µl) of the DNA solution was loaded into agarose gel and concentration of the DNA was determined. After determination of

the concentration, DNA and fosmid vector were ligated by ligation reaction whose components are shown in Table 2.7.

Table 2.7 : Chemicals used in ligation reaction.

Chemical	Amount
10X Ligation Buffer	1 µl
10 mM ATP	1 µl
CopyControl pCC1FOS vector	1 µl
DNA	6 µl
Fast-Link DNA Ligase	1 µl
Total Volume	10 µl

Ligation was performed at RT for 4 hours. Reaction was stopped by incubating at 70 °C for 10 min. Immediately after ligation reaction, packaging the fosmid clones was performed. For this reaction, 25 µl of MaxPlax Lambda Packaging Extracts, which were stored at – 70 °C, was thawed on ice and mixed with ligation reaction product without formation of bubbles. Mixture was incubated at 30 °C for 2 hours. A second 25 µl of MaxPlax Lambda Packaging Extracts was thawed and added to the mixture and incubation was continued for 2 more hours. At the end of incubation, 940 µl phage dilution buffer and 25 µl chloroform were added and stored at 4 °C.

EPI 300 T1 $^{(R)}$ *E. coli* cells were spread on LB plates containing no antibiotic before library production and incubated at 37 °C overnight. Before the day of packaging reaction, one single colony was transferred to 50 ml of LB Broth containing 10 mM $MgSO_4$ and 0.2 % maltose and incubated at 37 °C overnight. On the day of packaging reaction, 5 ml culture was inoculated into the same medium and incubated at 37 °C until OD_{600} reached at 0.8 ~ 1.0. This culture was further used for plating the cells. For plating the cells, 10 µl of this reaction was mixed with 90 µl of *E. coli* culture and incubated at 37 °C for 1 hour. Mixture (100 µl) was spread out onto LB agar plates containing 12.5 µg/ml chloramphenicol were incubated at 37 °C overnight. At the end of the incubation period, grown colonies were observed. To confirm the construction of the library and to check the average insert size, 15 colonies were selected and transferred to 3 ml of LB broth containing 3 µl chloramphenicol stock solution. Cells were grown overnight at 37 °C. Fosmid isolation from culture was carried out using the alkaline lysis method according to

Sambrook, [59]. 1.5 ml of the culture was centrifuged at 14000 rpm for 2 min to pellet the cells. Cells were washed with 0.25 volume of STET and centrifuged again. Cell pellet was resuspended with 100 µl of resuspension buffer. 200 µl of freshly prepared lysis buffer was added and the tubes were inverted 5 times gently. 150 µl of ice-cold neutralizing solution was added to the mixture and incubated on ice for 5 min. Cell debris was pelleted by centrifugation at 14000 rpm for 5 min. The supernatant was transferred to a clean tube, an equal volume of P:C:I (25:24:1) was added. Sample was mixed by vortexing and centrifuged at 14000 rpm for 2 min. Aqueous phase was transferred to a clean tube and two volumes of ethanol was added. Mixture was incubated at RT for 2 min, centrifuged at 14000 rpm for 15 min. Pellet was washed with 1 ml 70 % ethanol twice and centrifuged as before. Fosmid DNA was left to dry for 15 min at RT, dissolved in sterile water. To determine the average insert size, extracted fosmids were subjected to NotI enzyme digestion. Digestion reaction components are shown in Table 2.8.

Table 2.8 : Enzyme digestion reaction of fosmids

Chemicals	**Amount**
Nuclease free water	16 µl
10X Buffer O	2 µl
DNA	1 µl
NotI	1 µl

Reaction was performed at 37 °C for 1 hour and enzyme was inactivated by incubation at 80 °C for 20 min. Isolated fosmids and products of digestion reaction were loaded onto 1 % agarose gel and electrophoresed to confirm the presence of insert DNA and to determine its average size.

2.2.6 Screening of the genomic library

After the library production was confirmed, the remaining packaged phage solution was mixed with appropriate amount of cell culture, incubated at 37 °C for 1 hour and spread out onto LB agar plates containing 12.5 µg/ml chloramphenicol, 1 % skim milk and induction solution for direct screening of fosmid library. Skim milk was used as a substrate for protease activity. Colonies were also replicated into LB + tributyrin plates for lipase screening.

3. RESULTS AND DISCUSSION

The major purpose of this research was to construct a library of a pre-enriched metagenome. Sediment sample from acid mine drainage environment were collected and used as a template for environmental genomic library. In the first part of the study, it was attempted to isolate total genomic DNA directly from environmental sample. However, some obstacles that are probably associated with low cell number, presence of nucleases and other inhibitory compounds caused failing in high-quality metagenomic DNA isolation. To overcome this problem, environmental sample was inoculated into a medium designed for sulphur oxidizing acidophilic bacteria which are mainly found in AMD.

3.1 Direct DNA isolation from sediment sample

DNA isolation from environmental sample was conducted using both Fast DNA Spin Kit and a manual isolation procedure according to Zhou et al, 1999 [57]. Isolated DNA were electrophoresed in 1 % agarose gel and visualized under UV radiation. In the isolation by using Fast DNA Spin Kit, a total amount of 4 g sample.

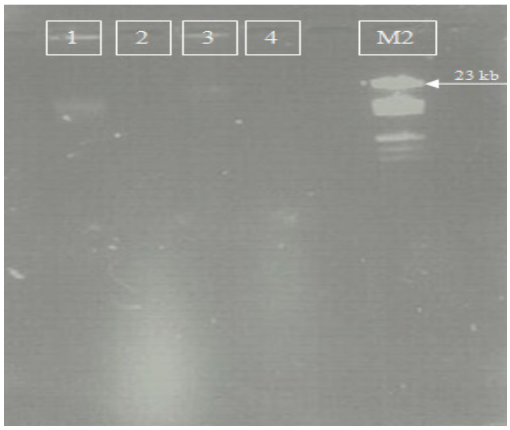

Figure 3.1 : Results of DNA isolation from environmental sample. Lane 1: DNA isolated with commercial kit, Lane 2, Lane 3 and Lane 4: DNA isolated manually from same samples collected at different time intervals; Lane M2: Molecular marker 2 (23130, 9416, 6557, 4361, 2322, 2027, 564, 125 bp).

Agarose gel electrophoresis show (Figure 3.1) that, the amount of the DNA was not high enough to construct a metagenomic library by using the commercial kit. In manual isolation method, isolated DNA was over-sheared and a reddish cloud caused by humic substances was observed on the agarose gel.

3.2 Enrichment culture

Enrichment cultures containing sulphur were used to enrich the acidophilic microbial population. Samples, obtained from acid mine drainage environments are known to be habitats for iron and sulfur oxidizing bacteria. For this reason, 35S medium containing elemental sulfur was inoculated with samples.

During incubation period, 35S enrichment culture was sampled and pH was measured regularly. The changes in acidity are shown in Figure 3.2. pH of the medium was decreased due to the oxidation of sulfur to yield sulfuric acid according to the equation given below:

$$2S + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4$$

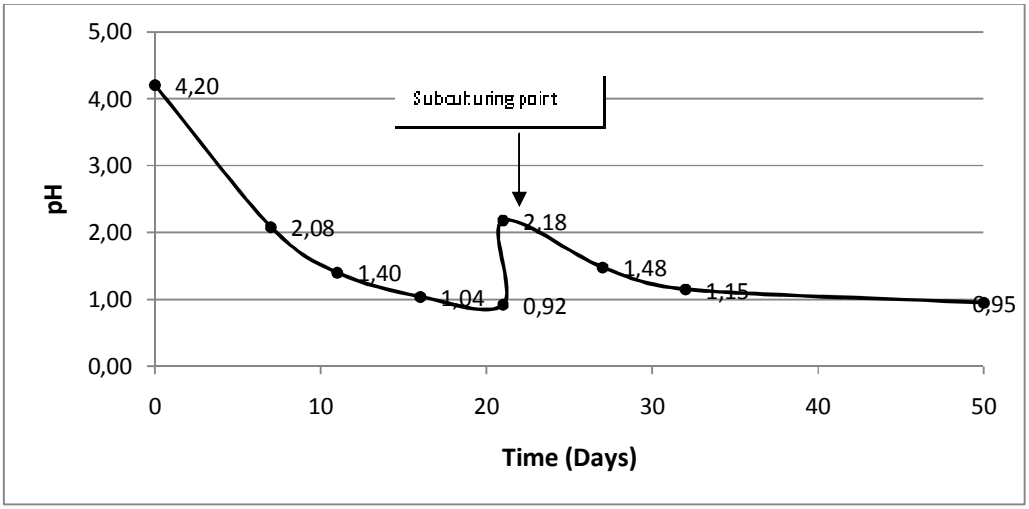

Figure 3.2 : pH changes in 35S enrichment culture during incubation.

The subculturing point shown in the figure indicates the time that 20 ml of the first enrichment culture was taken and inoculated into 200 ml fresh media with a pH of 4.2. As shown in the figure, pH was decreased during the incubation period and this indicates the oxidation of sulfur.

3.3 Determination of Enriched Microbial Population

16S rDNA method was used to determine the microbial population present in 35S medium, For this purpose, genomic DNA from culture was isolated with MoBio UltraClean™ Microbial DNA Isolation Kit according to manufacturer's instructions. Isolated DNA was analyzed on agarose gel and shown in Figure 3.4.

Targeted 16S rDNA region of about 1500 bp was amplified by PCR using pA-F and pH-R primers. After confirmation of the presence of PCR products, they were purified by gel extraction. Purified PCR products were electrophoresed on agarose gel and amplification of the region with expected size was confirmed as shown in Figure 3.3.

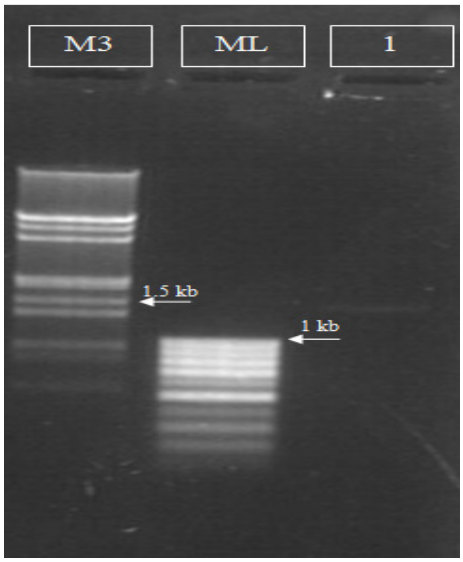

Figure 3.3 : Purified PCR product. Lane M3: Molecular marker 3 (21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 bp), Lane ML: DNA Ladder, Lane 1: Purified PCR product.

Purified PCR products were ligated to TOPO TA vector. This is a plasmid DNA which includes ampicilline resistance gene that enables selection of transformed hosts. In addition, when poly(A) added insert is ligated to the vector, β-galactosidase coding gene called *lac*Z which is present within the original vector is inactivated. Because of this property, colonies having no insert can produce β-galactosidase and gain a blue colour with the hydrolysis of X-gal present in the medium. As a result, white and ampicilline resistant colonies which are formed after transformation constitute the cloning library. Plasmids were isolated from sixteen colonies and targeted region was amplified by PCR using M13-F and M13-R primers. Pure DNA

samples were sequenced using the ABI Prism 3100-Avant automated sequencer at the Molecular Biology and Genetics Dept., ITU. Sequence results were compared in NCBI database using Basic Local Alignment Search Tool (BLAST). Results of sequence analysis are listed in Table 3.1.

Table 3.1 : Microorganisms found in enrichment medium.

Microorganism	Query Recoverage	Sequence Similarity	Frequency*
Acidithiobacillus thiooxidans strain OGCS3	100 %	100 %	33.3 %
Acidithiobacillus thiooxidans strain LYS	100 %	100 %	
Acidithiobacillus sp. SH	99 %	100 %	
Acidithiobacillus sp. lsh-01	94 %	98 %	
Acidithiobacillus ferrooxidans	99 %	98 %	
Sulfobacillus sp. RIV14	100 %	100 %	40 %
Sulfobacillus sp. L15	94 %	99 %	
Uncultured *Sulfobacillus sp*. clone D47	100 %	97 %	26.7 %
Uncultured bacterium clone M19bMb7	95 %	100 %	
Uncultured bacterium clone BS-C11	91 %	99 %	

*Frequency is the percentage of genus found in total analysis.

As shown in the table, major species found in enrichment culture belongs to two different genuses, *Acidithiobacillus* and *Sulfobacillus,* while uncultured clones also constitute a significant proportion of the flora. *Acidithiobacillus* species are gram negative and belong to γ-proteobacteria. *A. caldus*, *A. ferrooxidans* and *A. thiooxidans*, which are mesophilic chemolithoautrophic bacteria, are the most studied microorganisms due to their industrial importance [60]. Those bacteria are used in bioleaching or biomining processes in which they oxidize insoluble metal sulfides

and produce soluble metal sulphates [61]. *Sulfobacillus* species are gram positive bacteria belonging to Clostridiales order and are known to assist metal extraction during bioleaching of sulfide ores [62]. Considering the acid mine drainage environment where sediment samples were collected from, it is reasonable for those species to grow in the enrichment culture. According to the records accessed through NCBI database, uncultured clones found in enrichment culture have been defined in various environments. Those are listed in Table 3.2.

Table 3.2 : Sources of 16S rRNA sequences of uncultured bacteria found in enrichment culture

Uncultured clone	Source	Reference
Uncultured *Sulfobacillus sp*. clone D47	low-grade copper bioleaching heap	63
Uncultured bacterium clone M19bMb7	andesitic hydrothermal environments and acidic, hydrothermally modified volcanic soil	64
Uncultured bacterium clone BS-C11	thermophilic microflora enriched by different energy sources	65

Presence of uncultured bacterium in enrichment culture indicates that this culture may serve as a source for genetic material which have not been discovered or defined yet. Considering this, the enrichment culture was used to construct a genomic library to screen for targeted enzymes such as lipase and protease.

3.4 Genomic Library Production

To construct the genomic library of enrichment culture, high quality genomic DNA was isolated manually according to Zeng, et al, 2007 [58]. In this high temperature and SDS based procedure, genomic DNA was sheared minimally and amount of the DNA was satisfactory for library production as shown in Figure 3.4. As indicated in the figure, manual isolation method produced much high quality DNA compared to the DNA isolated with commercial kit.

Because of the methods used in genomic DNA isolation, DNA molecules are often sheared. Repairing the ends of DNA molecules is required for ligation reaction. End-repair is the reaction in which 5'-phophorylated and blunt ended DNA molecules was generated for this purpose. After that, DNA was electrophoresed on LMP

agarose gel for 8 hours to ensure resolution of large DNA fragments. DNA molecules with average size of 40 kb were recovered from agarose gel and small aliquot of the solution was electrophoresed to confirm the presence of the DNA in the correct size.

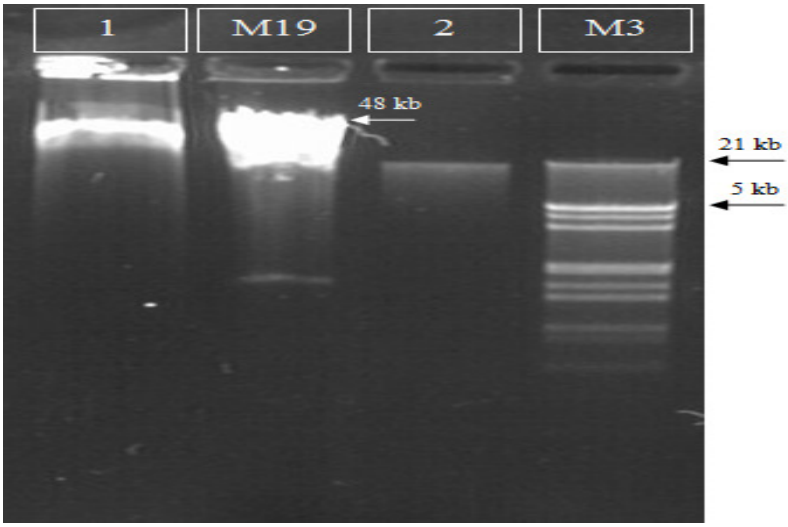

Figure 3.4 : Genomic DNA isolated from enrichment culture. Lane 1: DNA isolated by manual method, Lane M19: Molecular marker 19, Lane 2: DNA isolated using commercial kit, Lane M3: Molecular marker 3 (21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564).

Figure 3.5 show the size of DNA after recovery from LMP agarose gel. The concentration of end-repaired DNA was calculated as 0.05 µg/µl. Required amount of DNA solution was used in ligation reaction and products were packaged by phage extracts. After treating the *E. coli* cells with phage extracts, they were spread on LB agar plates containing 12.5µg/µl chloramphenicol.

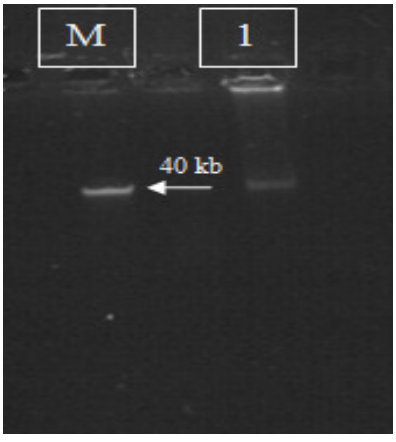

Figure 3.5 : Genomic DNA after size selection and gel purification. Lane M: Fosmid control DNA (40 kb), Lane 1: Size selected and gel purified genomic DNA.

To confirm the ligation of insert, fosmids were extracted from selected clones and digested with NotI which has two restriction sites within the fosmid vector including insert ligation region. As shown in Figure 3.6, insert ligation was successful and average insert size is higher than 21 kb. All of the infected cell solution was spread on relevant agar plates and about 12,000 of clones were obtained. 12,000 individuals, each carrying a genome fragment of bigger than 21 kb; generate a library containing microbial genetic information more than 252 Mbp.

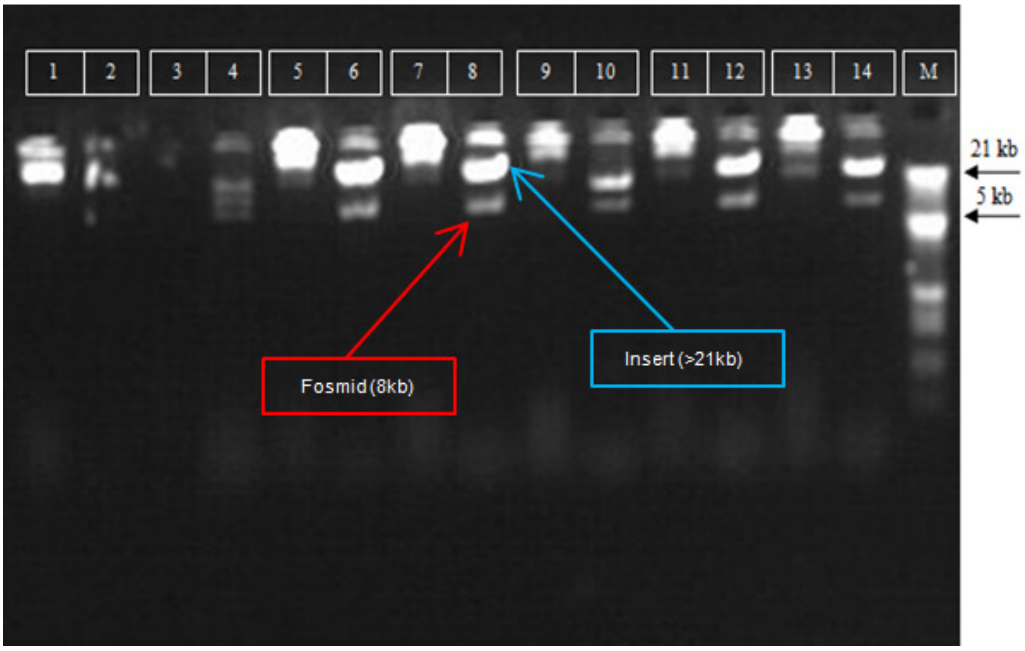

Figure 3.6 : Control of insert presence and size by fosmid digestion. Odd Numbered Lanes: Undigested fosmids; Even Numbered Lanes: Digested Fosmids; Lane M: Molecular marker 3 (21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 bp).

3.5 Screening the genomic library

After construction of the library, clones were screened separately for protease and lipase activity. LB agar plates containing 1% skim milk as protease substrate were used to screen the protease activity,. Induction solution was also added into the agar medium to induce the fosmids to high copy numbers.. All of the clones were screened but unfortunately, none of the clones showed protease activity. LB agar plates containing 1% tributyrin was emulsified by sonication. In addition, medium was supplemented with 1% gum arabic to stabilize the emulsion. In Figure 3.7, the difference in agar medium before and after sonication is shown. After sonication, it was observed that medium gains a milky appearance.

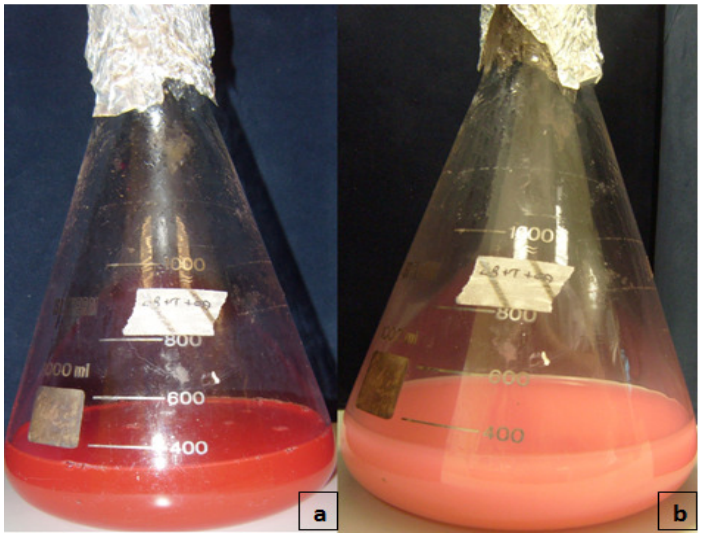

Figure 3.7 : Appearance of LB agar medium containing tributyrin before **(a)** and after **(b)** sonication treatment.

Clones were manually arrayed on agar plates by sterile toothpicks and incubated at 37°C for about 7 days. Among screened clones, 3 of them formed a clear halo around colony and these clones were streaked out again using same agar plates for control. Lipase activity on agar plate was confirmed and shown in Figure 3.8.

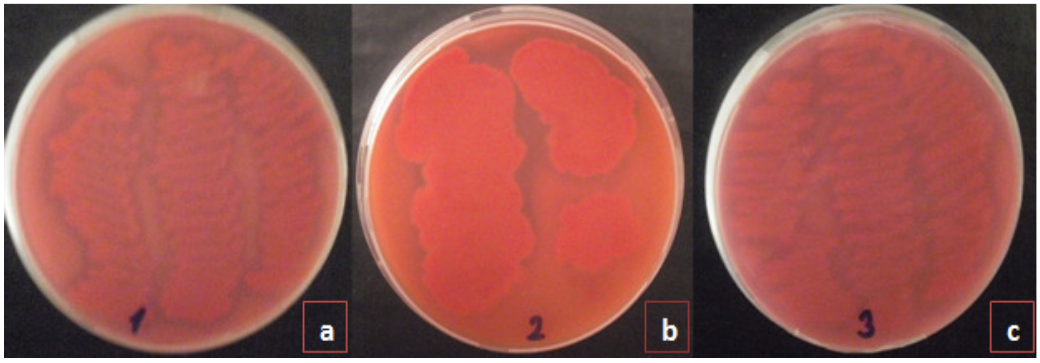

Figure 3.8 : Clones showing lypolytic activity on agar plate.

4. CONCLUSION

Today it is known that a great majority of the microorganisms on earth have not been cultivated in standard laboratory conditions. Despite this fact, which eventually seems to be preventing scientists to reach unculturable microorganisms, a new field called metagenomics or environmental genomics directly get access to genomic material present in any environmental sample. This approach overcomes the obligations such as culturing and allows direct isolation of environmental DNA. By this technique, many novel biopharmaceuticals, drug precursors, enzymes and so other industrially important products have been isolated so far. Environmental samples, especially extreme environments such as hot springs and acidic mine drainages constitute the major sources of metagenomic studies. Enzymes isolated from extremophilic microorganisms provide advantages in industrial operations in terms of process conditions such as temperature, substance solubility, pressure, water content and pH. Enzymes from halophilic microorganisms are adapted to low water activity due to high salt content and this feature makes them exploitable in organic or non-aqueous solvents. Acidophilic microorganisms are alternative sources for acidic enzymes which can catalyze reactions under high H^+ ion concentration.

Here, we aimed producing genomic library to isolate novel hydrolase enzymes from acidophilic samples. Those hydrolase enzymes are good candidates tobe used in $LCO_2/SCCO_2$ system for cleaning/disinfection and sterilization purposes. Because of the nature of $LCO_2/SCCO_2$, enzymes to be used in such a system should be active at low pH and low salt concentration. For this purpose, environmental samples from acid mine drainage environment were collected as a first step. Those samples were enriched in elemental sulfur containing medium to increase the microbial population. Microorganisms found in enrichment culture were determined using 16S rDNA method. *Acidithiobacillus* and *Sulfobacillus*, are the dominant genuses together with uncultured bacteria which represents the whole community present in enrichment culture. To produce a library, high quality of genomic DNA was isolated and genomic library which containing 12000 clones with an average insert size of >21

kb, was constructed using fosmids as vectors. The library was screened for protease and lipase activities. While none of the clones showed proteloytic activity, 3 of the clones showed clear halos around colonies in agar plates which contain tributyrin as a lipase substrate. Those zones produced by colonies were confirmed.

Following the subcloning of these 3 clones, , lipase enzymes will be characterized and evaluated in terms of activity in acidic conditions. In addition, the genomic library of the acidophilic community can be used in screening for other important enzymes. Beside acidophilic samples, studies on library production from halophilic samples are on progress. When lipase and protease enzymes from these two extreme environments will have been isolated, their potential application in $LCO_2/SCCO_2$ system will be evaluated.

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APPENDICES

APPENDIX A : pCR®2.1.-TOPO® Vector Map

APPENDIX B : CopyControl PCC1FOS™ Fosmid Vector Map

APPENDIX C : Laboratory Equipment List

APPENDIX A.

pCR®2.1.-TOPO® Vector Map

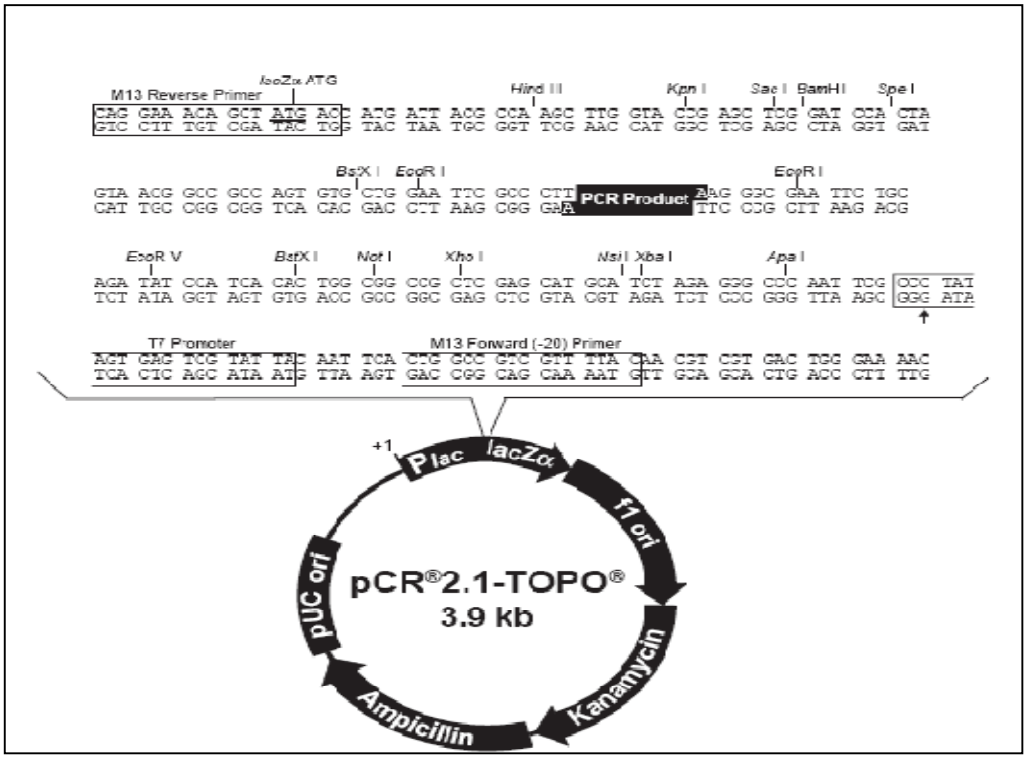

Figure A.1 : Vector map of pCR®2.1-TOPO plasmid.

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

APPENDIX B.

CopyControl PCC1FOS™ Fosmid Vector Map

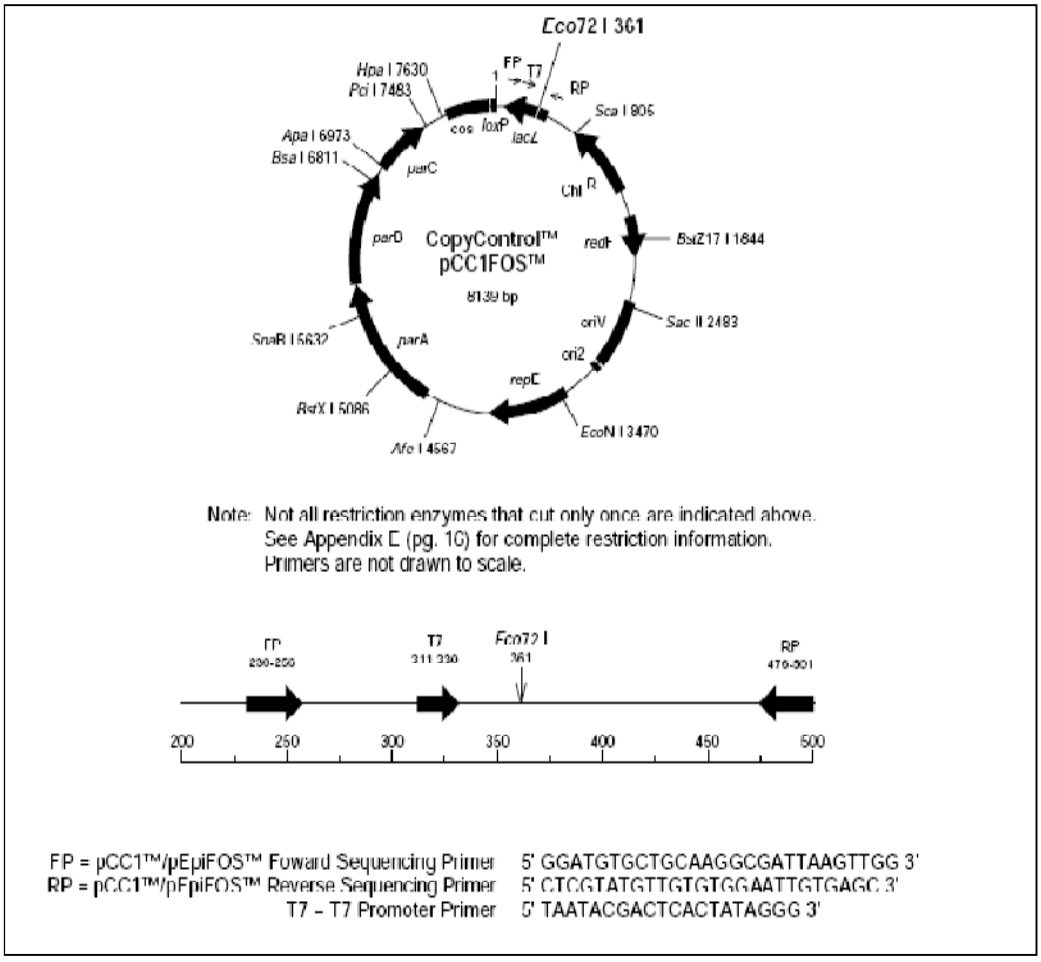

Figure A.2 : Vector map of pCCFOS™ fosmid.

APPENDIX C.

Laboratory Equipment List

Analytical Balances:	Precisa BT 610C
Autoclave:	2540 ML benchtop autoclave, Systec GmbH Labor-Systemtechnik.
Centrifuges:	Microfuge 18, Beckman Coulter; AvantiJ30I, Beckman Coulter.
Deep freezes and refrigerators:	Ultra low freezer MDF-U4086S, Sanyo; Ultra low freezer MDF-U333, Sanyo; 1061 M refrigerator, Arcelik.
Electrophoresis equipment:	Horizon 11.14, Whatman, Biometra Gel Casting System, Horizon 20-25, Whatman, Biometra Gel Casting System.
Electroporator:	Electroporator 2510, Eppendorf
Gel Documentation System:	UVIpro GAS7000, UVItec Limited.
Ice machine:	AF 10, Scotsman.
Laminar flow cabinet:	Faster Laminar Flow BH-EN 2003.
Magnetic stirrer:	Heidolph Standard
Orbital shaker:	Forma orbital shaker, Thermo Electron Corporation.
pH meter:	Inolab pH level 1, Wissenschaftlich-Technische Werkstätten GmbH & Co KG
Pipettes:	epResearch Pipettes, Eppendorf.
Sonicator:	Sonoplus, Bandelin
Thermal Cycler:	Biometra Thermal Cycler
Thermomixer:	Thermoshaker Ts1, Biometra.
Vortex:	SI-D256 Daigger
Water Bath:	Memert

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- Bıyık, H. E., Koseoglu, V. K., and Aran, N., 2009. Investigation of the antagonistic effect of *Pediococcus acidilactici* on *Listeria monocytogenes* 1/2a in a meat simulation medium and sausage formula. 2009 IFT Annual Meeting, Book of Abstracts, Abstract No: 123-53. 6-9 June Anaheim, USA.