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ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF XYLANASE, PROTEASE, LIPASE, LACCASE AND ALPHA-AMYLASE PRODUCED BY SOIL BACTERIA

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Ünzile GÜVEN GÜLHAN

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APPROVAL PAGE

This is to certify that I have read this thesis written by Ünzile GÜVEN GÜLHAN and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Genetics and Bioengineering.

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September 2014

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M.S. Thesis – Genetics and Bioengineering
September 2014

Thesis Supervisor: Prof. Dr. Halil Rıdvan ÖZ

ABSTRACT

Enzymes are very important for fundamental academic researches and also for industrial applications. This study aims to prepare the substructure of producing some important industrially applicable enzymes. Lipase, laccase, xylanase, protease and alpha-amylase enzymes are among the most important enzymes in the industry, and have been studied a lot since they are widely used in the industry. All these enzymes can be produced from bacteria which are found abundantly in soil. In this study, using the soil samples from different places in Turkey, bacteria colonies showing five enzyme activities (Xylanase, Protease, Lipase, Laccase, Alpha-amylase) were isolated, characterized and identified with 16s rRNA sequencing analysis. Enzyme activities of identified isolates were measured with spectrophotometric methods. Then isolates which have higher enzymatic activity were chosen and partially purified with ammonium sulfate precipitation method. After precipitation of proteins, enzyme activities were measured in different pH and temperature conditions to find out the stability and optimum pH and temperature for maximum enzyme activity. With this thesis, since soil samples special to Turkey will be used as bacterial source, bacteria species different from the literature will be able to isolated and identified, so that there will be novel resources for the production of industrially important enzymes. In the results of this research, there will be a bacterial bank capable of producing Xylanase, Protease, Lipase, Laccase, Alpha-amylase enzymes and there will be characterized partially purified enzymes which can be used in industrially important enzyme production.

Keywords: Isolation, Soil bacteria, Industrial Enzymes (xylanase, laccase, lipase, alpha-amylase, protease), Partial Purification, Characterization

İZOLE EDİLEN TOPRAK BAKTERİLERİ TARAFINDAN ÜRETİLEN KSİLANAZ, PROTEAZ, LİPAZ, LAKKAZ VE ALFA AMİLAZ ENZİMLERİNİN KISMİ SAFLAŞTIRILMALARI VE KARAKTERİZASYONLARI

Ünzile GÜVEN GÜLHAN

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ÖZ

Enzimler temel akademik araştırmalar ve endüstriyel uygulamalar için çok önemlidir. Bu çalışmada, endüstriyel önemi olan bazı enzimlerin üretiminin alt yapısı oluşturulmak hedeflenmiştir. Lipaz, lakkaz, alfa-amilaz, ksilanaz ve proteaz enzimleri endüstriyel önemi olan enzimlerdendir ve endüstride çok fazla kullanılmaktadırlar. Bütün bu enzimler toprakta bolca bulunan bakterilerden üretilebilmektedir. Bu çalışmada, Türkiye'nin farklı yerlerinden elde edilen toprak örnekleri kullanılarak, Lipaz, lakkaz, alfa-amilaz, ksilanaz ve proteaz enzimleri aktivitesi gösteren bakteriler izole edilmiş, karakterizasyonu yapılmış ve 16S rRNA sekanslama analizi ile tür tayinleri yapılmıştır. Tanımlanan izolatlardaki enzim aktivitelerine spektrofotometrik yöntemler ile bakılmıştır. Bu izolatlardan en yüksek enzimatik aktivite gösterenler seçilmiş ve amonyum sülfat ile çöktürme yöntemiyle kısmi saflaştırmaları yapılmıştır. Protein saflaştırmasından sonra, optimum pH ve sıcaklık değerlerini bulmak için farklı pH ve sıcaklıklarda enzim aktivitelerine bakılmış, maksimum enzim aktivitesinin ve stabilitesinin olduğu pH ve sıcaklık değerleri belirlenmiştir. Bakteri kaynağı olarak Türkiye'ye özgü toprak örnekleri kullanıldığı için, proje sonunda endüstriyel önemi olan enzimlerin üretimi için orijinal kaynaklar elde edilmiş olacaktır. Bu araştırma sonucunda, Lipaz, lakkaz, alfa-amilaz, ksilanaz ve proteaz enzimlerini üretebilecek olan bakteri bankası oluşturulacak, endüstriyel önemi olan enzimler kısmı saflaştırılacak ve karakterize edilecektir.

Anahtar Kelimeler: İzolasyon, Toprak Bakterisi, Endüstriyel Enzimler, Alfa amilaz, Proteaz), Kısmi Saflaştırma, Karakterizasyon.

To my parents

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I express my thanks and appreciation to my family and my husband for their understanding, motivation and patience. Lastly, I am thankful to all colleagues and friends who made my stay at the university a memorable and valuable experience.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

A	Absorbance
cm	Centimeter
^{o}C	Degree Celsius
dia.	Diameter
BC_{dia}	Diameter of bacteria colony
CZ_{dia}	Diameter of clear zone
EI	Enzymatic Index
EC	Enzyme Commission
gDNA	Genomic DNA
g	Gram
h	Hour(s)
l	Liter
LB	Luria-Bertani
ug	Microgram
ul	Microliter
mg	Miligram
mM	Milimolar
min	Minute(s)
OD	Optic density
PBS	Phosphate buffered saline
rpm	Revolutions per minute
s	Second(s)
TCA	Trichloroacetic acid
DNS	3,5-Dinitrosalicylic acid

CHAPTER 1

INTRODUCTION

Subject of this Project is to construct a small scale bacteria bank special to microbial flora in Turkey and research their enzyme producing capabilities by purifiyng and characterizing industrially important enzymes from soil bacteria isolated from different places in Turkey.

In the scope of this thesis, first thing to do is the isolation of bacteria colonies from soil, capable of producing industrial enzymes and showing enzyme activity by using microbiological procedures, then isolation and characterization of this bacteria, stocking the single bacteria cultures and so constructing the bacteria bank in small scale. In the second step, candidate isolates has been chosen according to their high enzyme activities, and partial purification of alpha-amylase, laccase, lipase, protease and xylanase enzymes has been done from these isolates. At last step of the thesis, partially purified enzymes have been characterized according to their maximum activity is observed in optimum pH and temperature conditions.

Research and development of industrial enzymes is very important in academic area. Discovering our country's national sources, developing these sources, leading to industrial production, and continuing this academic and industrial researches are strategically important for Turkey. Researches done in this thesis scope are aimed to serve these needs of our country.

CHAPTER 2

LITERATURE REVİEW

2.1 INTRODUCTION

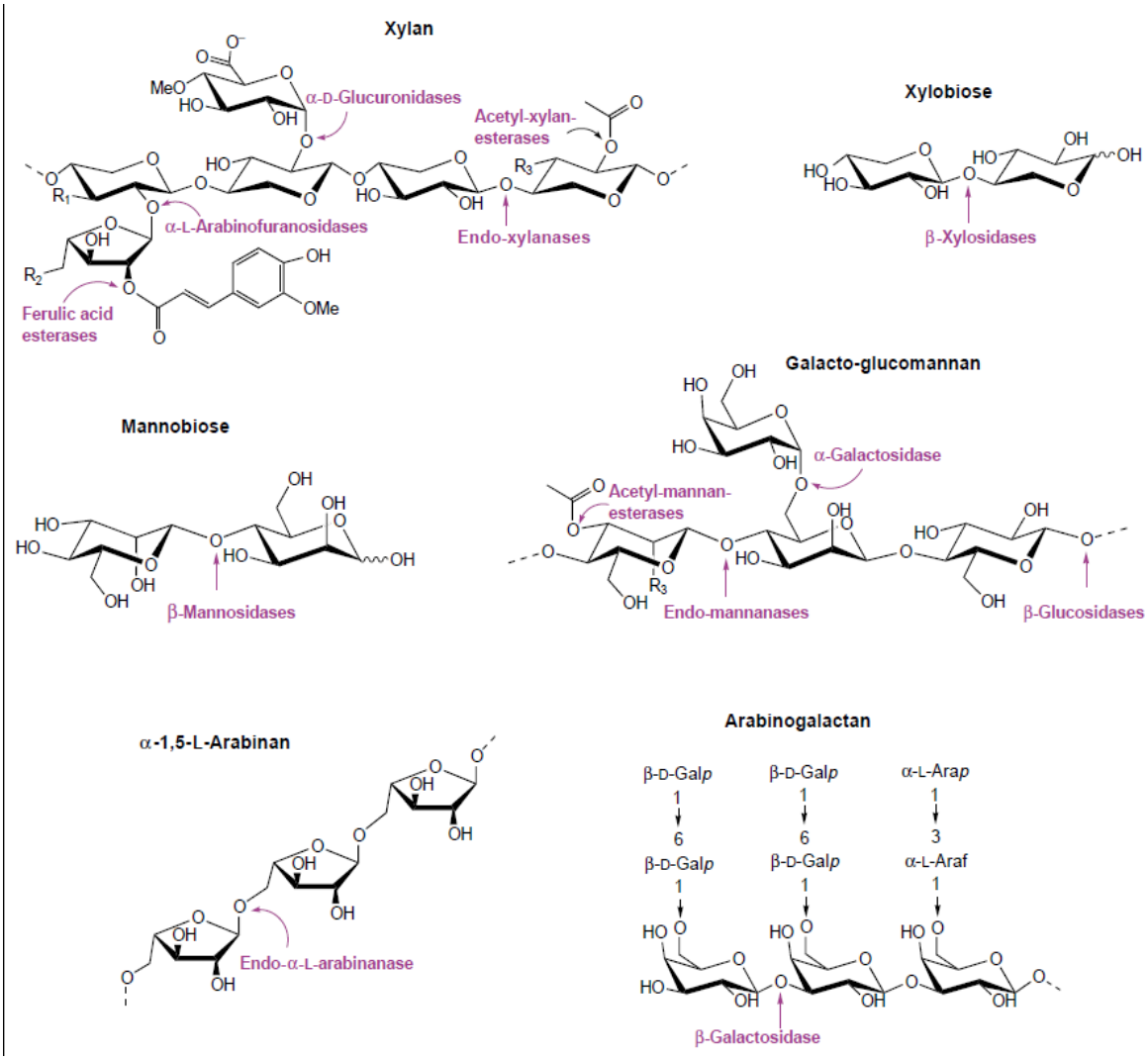
Enzymes are proteins that catalyze biochemical reactions and they have very important metabolic functions in the cells and they are a part of our daily life. Enzymes that are used abundantly in different areas in industry are generally obtained from microorganisms, since enzymes obtained from microorganisms have some advantages when compared with enzymes with plant or animal sources etc. Enzymes obtained from microorganisms have higher catalytic activity, they don't produce unwanted by-products, they are more stable and cheaper, and they can be produced in large amounts (Wiseman, 1987). Bacteria are the most abundant and predominant organisms living in the soil among the different microorganisms. Isolation of these bacteria plays a vital role in production of industrial enzymes. Protease, alpha amylase, xylanase, laccase and lipase enzymes are very important in the industry, so production of these enzymes from bacteria which can be easily isolated from soil plays very important roles in biotechnology. Below, some characteristics and industrially importance of these enzymes are given.

2.2 INDUSTRIALLY IMPORTANT ENZYMES AND SOME PROPERTIES

2.2.1 Xylanases

2.2.1.1 Properties and Industrial Importance of Xylanases

Xylanase is one of hemicellulase enzyme family which is a type of enzyme that degrades the plant cell wall polymer hemicellulose. Hemicelluloses differ from the plant cell wall structural compound cellulose, which forms a much longer chain and is comprised wall structural compound cellulose, which forms a much longer chain and is comprised entirely of glucose molecules. This polymer helps to cross-link the plant cell wall component to give the walls rigidity. Since they can be very complex molecules and be comprised of a number of different types of sugars, there are different hemicellulose polymers, so there are different types of hemicellulase enzymes degrading these different types of sugars. Various types of this enzyme have different applications in biotechnology, particularly in the food sciences. Figure 1 shows the hemicellulose types and where the degredations take place.



Current Opinion in Microbiology 2003, 6:219–228

Figure 2.1 The basic structural components found in hemicellulose and the hemicellulases responsible for their degradation.

Hemicellulose is a cross-linking agent and interconnects cellulose microfibrils. It can also link the cellulose to other cell wall components. Hemicelluloses are heterogen polymers consisting of pentose (xylose and arabinose), hexoses (mannose, glucose, galactose) and the uronic acid as building stones. Xylan is the dominant pentose sugar in hardwood hemicellulose. Xylan converts cell membrane to more robust structure with cross-ties. It hydrolysis the bonds between the various sugar groups in polysaccharide chain, allows the degradation of hemicellulose. Xylan is the fundamental component of hemicellulosic portion of higher plants' cell wall and is the

second most abundant source that is degraded into high potential useful end products (Yang, 1995; Salles, 2000).

Among hemicellulases, the two main glycosyl hydrolases depolymerising the hemicellulose backbone are endo-1,4- L-D-xylanase and endo-1,4-L-D-mannanase. Since xylan is a complex component of the hemicelluloses in wood, its complete hydrolysis requires the action of a complete enzyme system (See Figure 2.2), which is usually composed of L-xylanase, L-xylosidase, and enzymes such as K-L-arabinofuranosidase, K-glucuronidase, acetylxylan esterase, and hydroxycinnamic acid esterases that cleave side chain residues from the xylan backbone. All these enzymes act cooperatively to convert xylan to its constituents (Sunna, 1997).

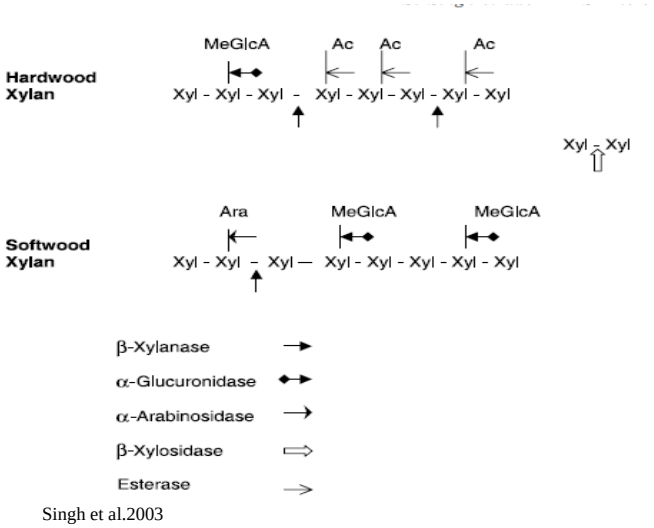


Figure 2.2 Xylanolytic enzymes involved in the degradation of hardwood and softwood xylan. Ac, acetyl group; Ara, K-arabinofuranose; MeGlcA, K-4-O-methylglucuronic acid; Xyl, xylose.

Xylanase enzyme degrades xylan and it is the most active enzyme among the studied endopolysaccharide hydrolase enzymes of aerobic fungi. It has been shown that xylanase activity is 5-7 times higher than endoglucanase activity (Borneman, 1989). Xylanases produced from thermophilic bacteria have longer half-lives, it can resist to 80°C or higher degrees.

Xylanase enzyme is one of the important industrial enzymes. It is mostly used in pulp and paper industry for bleaching of paper pulp and this is one of the promising applications to protect the environment from industrial waste. Xylanase has many usage areas in industry such as baking industry, food and feed industry and improving waste treatment. Since xylanase enzyme is effective in different industrial areas and helps to reduce industrial waste damage, it remain as an important industrial enzyme.

2.2.1.2 Some Application Areas of Xylanases

2.2.1.2.1 Pulp and Paper Industry

Usage of xylanase enzyme constitutes an important part in Pulp and Paper Industry. Today, microbial enzyme systems has gained importance in order to protect the environment from industrial waste in pulp and paper industry and xylanase is one of the enzyme used for this process. By using xylanase enzyme in the pretreatment of kraft pulp, chemicals used in bleaching process, expecially the chlorine compounds, are substantially reduced and reduces pollution (Christov, 1996). The process of bleaching of pulp using xylanase has great promising future.

2.2.1.2.2 Food Industry

Hemicellulases, expecially xylanases have a wide application area in food industry. It has been shown that xylanases has positive effects on dough and bread quality. It is used in bakery and frozen dough products in order to provide dough quality and product optimization (volume, texture, shelf life). By using xylanase enzyme in bread making and cooking, it changes water absorption, controls and increase oven spring (Jeffries, 1998).

Xylanase is also used in grape juice and fruit juice purification, obtaining fruit and vegetable juice (Wong, 2000).

2.2.1.2.3 Feed Industry

Another important usage area of xylanase is feed industry. Weight gain and feed conversion efficiency of rye fed to broiler chickens is associated with the contraction of intestinal viscosity. Addition of xylanase to rye-based diet of chicken reduces intestinal

viscosity, and increases the effectiveness in both weight gain of chickens and feed conversion (Wong, 2000).

2.2.1.2.4 Industrial Waste Treatment

Xylan is highly found in agricultural and food industry wastes. Xylanase is used to degrade this xylan present in waste water into xylose.

Development of effective enzymatic hydrolysis processes has revealed new hopes in the treatment of hemicellulosic waste and R & D efforts are needed to increase enzyme activation (Wong, 2000).

2.2.2 Proteases

2.2.2.1 Properties and Industrial Importance of Proteases

Proteases are one of the most important groups of industrial enzymes that has about 60% of the total worldwide enzyme sales (Nascimento and Martins, 2004; Beg and Gupta, 2003; Ellaiah, 2003). Proteases plays an important role in the decay of plant, animal and microbial residues in nature, therefore it ensures the food cycle and also enables plants to take their nutrients (Aoki, 1995). Proteases form a very complex group of enzymes and have different physicochemical and catalytic properties. Although cellular mechanisms responsible for the control of protease synthesis, not fully understood yet, production of alkaline proteases is suppressed by nitrogen sources which can be metabolized quickly like amino acids or ammonium. Other medium components, small sugars and minerals affect enzyme synthesis. Reducing the cost of industrial processes is aimed by use of potential protease enzyme and maximum enzyme production (Mabrouk, 1998; Kaur, 2001).

Among proteases, bacterial proteases are seem to be more effective and significant when compared to animal and fungal proteases (Banerjee, 1999). Because of the commercial interest, microbes producing industrial proteases have been studied from a wide variety of habitats (Mehrotra, 1999).

Alcalifilic proteases are produced from different sources such as bacteria, fungi and yeast (Singh, 2000), however alcalifilic Bacillus is the most used microorganism in

biotechnology since its isolation from a wide variety of environments is relatively easy. In addition to this, Bacillus can grow both in complex and synthetic medium. Alcalifilic proteases produced from thermophilic and alcalifilic Bacillus are resistant to high temperature and pH (Johnvesly, 2001; Oberoi, 2001; Aunstrup, 1981; Fogarty, 1979).

Proteases are one of the most important enzyme groups in the industry and it has been used in various areas, firstly in detergent and food industry, textile, leader, photography, silk and feed industry, in different clinical applications and pharmacy (Bulut, 2007).

2.2.2.2 Some Application Areas of Proteases

2.2.2.2.1 Food Industry

Protease enzyme used in food industry affects proteins in flour. They are used to resolve the difficulty in kneading and processing of strong flour. Proteases are especially used to improve brittleness particularly in biscuits, crackers and wafers (Suhartati, 2008).

2.2.2.2.2 Detergent Industry

Proteases are classified as acid, neutral and alkaline proteases according to the optimum pH range for protease activity. Among these, alcalifilic proteases are used as detergent additive. Alcalifilic protease maintains the stability in presence of bleaching / oxidizing agents and in high temperatures and pH range. Protease must be active and stable in high temperature and pH values. The most important parameter of proteases to be used in detergent industry is its pH range that the enzyme is active and stable (Suhartati, 2008). There is always a chance to increase this stability and activity by examining microbial diversity deeply and to explore microorganisms producing more useful commercial enzymes (Oberoi, 2001).

In 1956, the first detergent containing the bacterial enzyme was introduced to the market under the trade name Bio-40. Today, with alkaline proteases derived from Bacillus species, significant shares in the enzyme market were obtained (Mukherjee, 2008).

2.2.2.2.3 Other Applications of Proteases

Other applications of proteases are deproteinization of sea wastes (Yang, 1999; Oh, 1999), pharmaceutical, leather, laundry and waste processing industries (Pastor, 2001; Ward, 1985)

2.2.3 Lipases

2.2.3.1 Properties and Industrial Importance of Lipases

Lipases (triacylglycerol acylhydrolase; E.C.3.1.1.3) are ubiquitous enzymes produced by most biological systems including animals, plants and microorganisms. In eukaryotes, lipases are involved in various stages of lipid metabolism and are found in energy reservoir tissues. Lipases have become one of the prominent industrial enzymes they act over a wide range of pH and temperature, possess high specificity, do not require cofactors, and can catalyze a wide range of reactions. They catalyze the hydrolysis and the synthesis of esters formed from glycerol and long chain fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface (Maritinelle, 1995).

Lipases hydrolyze triglycerides to diglycerides, monoglycerides, glycerol and fatty acids. In addition, they also serve as biocatalysts for alcoholysis, acidolysis, esterification and ammonolysis. This versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries. Lipase enzymes performs catalysis of the emulsion in the oil-water transition phase and the enzyme reaction performs rate depends on the surface area formed. Lipases exhibit specificity according to chain length of the fatty acids, degree of saturation, position of fatty acids and physical condition of the substrate. Fatty acids with 4-10 carbon are hydrolysed faster than fatty acids with longer Carbon chain, seperated from oil structure and released (Abbas, 2002).

Microbial lipases constitute an important group of industrially valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications. Lipases are used in two distinct fashions. They are used as biological catalysts to

manufacture other products (such as food ingredients) and by their application as such (in making fine chemicals). Also following proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume (Eugene, 1974).

2.2.3.2 Some Application Areas of Lipases

2.2.3.2.1 Detergent Industry

Lipases are used in detergent industry to remove oil stains (Hiol, 2000). For detergent industry applications, hydrolytic lipases are added to the detergents, which are used mainly in household and industrial laundry and in household dishwashers. Also lipase can be immobilized on surfaces to facilitate oil removal from the surfaces and to alter wettability of the surfaces of fabric (Eugene, 1974).

2.2.3.2.2 Textile Industry

For textile industry applications, lipases are used to assist in the removal of size lubricants, in order to provide a fabric with greater absorbency for improved levelness in dyeing. Its use also reduces the frequency of streaks and cracks in the denim abrasion systems. Commercial preparations used for the desizing of denim and other cotton fabrics, contains both alpha amylase and lipase enzymes (Eugene, 1974).

2.2.3.2.3 Food Industry

Lipases are used in imparting flavor to butter, in chocolate industry, in cream and caramel production in food industry. Also butter products modified with lipase are used in margarines, shortenings, oven products and herbal products as flavour developer (Hiol, 2000). Lipases are used to modify flavour by synthesis of esters of short chain fatty acids and alcohols, which are known flavour and fragrance compounds. Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. Current applications include the flavour enhancement of cheeses, the acceleration of cheese ripening, the manufacturing of cheese like products, and the lipolysis of butterfat and cream (Eugene, 1974).

For fat industry applications, Lipase catalysed transesterification of cheaper oils can be used to produce cocoa butter from palm mid-fraction. Also immobilized lipase in

organic solvent catalyse the reactions of enzymatic interesterification for production of vegetable oils such as; corn oil, sunflower oil, peanut oil, olive oil and soybean oil containing omega-3 polyunsaturated fatty acids (Eugene, 1974).

2.2.3.2.4 Paper Industry

In pulp industry, lipases is used to increase the pulping rate of pulp for wastepaper deinking, increase whiteness and intensity, decrease chemical usage, prolong equipment life, reduce pollution level of waste water, save energy and time and reduce composite cost (Eugene, 1974).

2.2.3.2.5 Other Applications of Lipases

In leader industry, lipases clean oil inside the leather and on leather surface, makes the leather more suitable for tanning and dyeing processes, and gives flexibility to leather (Hiol, 2000). In addition, lipasas are used in biomedical applications, production of biosensors and pesticides, environmental management, cosmetics and perfume industry. In cosmetic industry, they are used as a biocatalyst in the production of the creams and they can be used in hair waving preparation, as a component of topical antiobese creams etc. (Eugene, 1974).

Lipases have become one of the most important groups of enzymes for its applications in organic syntheses. They can be used as biocatalyst in the production of useful biodegradable compounds (Eugene, 1974).

2.2.4 Laccases

2.2.4.1 Properties and Industrial Importance of Laccases

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are copper-containing oxidase enzymes that are widely distributed in higher plants and fungi. In fungi, laccase is present in Ascomycetes, Deuteromycetes, Basidiomycetes and is particularly abundant in many white-rot fungi that degrade lignin. Laccases catalyse reduction of molecular oxygen with water and provide oxidation of phenolic and nonphenolic compounds. These compounds include industrial dyes, policyclic aromatic hydrocarbons, alkenes and agricultural pesticides (Amann, 1995). Up to now, more than

100 laccases are purified from fungi and some of them were characterized. Molecule in monomer structure is between 50-100 kDa and optimum pH is around 4 (Riva, 2006). Up to now, most of industrial laccases have been found in white fungus - basidomycetes- that are used in biotechnological processes and for lignin degradation (Xu, 2007). It has been shown that laccass can be also found in most bacteria species rather than fungi and developed plants, by protein data bank and bacteria genome researches (Claus, 2003; Sharma, 2007) . There are opportunities of bacterial laccases according to fungal laccases. Such as, laccases are produced in higher amounts and in high yield; however production of bacterial laccases is much easier than production in fungi. In addition to this, presence of introns and disulfide bridges in fungal laccase genes increases the importance of bacterial laccases (Yaver, 1999; Kiiskinen, 2002) . When compared with fungal laccases, bacterial laccases have more efficient expression levels and higher temperature stabilities. Also some of them show alkaline laccase activities (Ruijssenaars and Hartmans, 2004). Although bacterial laccases have these advantageous properties, little bacterial laccases have been purified and identified (Sharma, 2007; Singh, 2007; Koschorreck, 2008). Because of that, increase of these studies on bacterial laccase production is very important.

Laccase enzyme is very important in the industry. It has a vast amount of industrial applications including pulp and paper, textile, organic synthesis, environmental, pollutants, production of useful chemicals from lignin, pharmaceuticals and nanobiotechnology (Minussi, 2007).

2.2.4.2 Some Application Areas of Laccases

2.2.4.2.1 Pulp and Paper Industry

For pulp and paper application, the pre-treatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose. Laccases are able to delignify pulp when they are used together with mediator (Kunamneni, 2008).

2.2.4.2.2 Food Industry

For food applications, many laccase substrates, such as carbohydrates, unsaturated fatty acids, phenols, and thiol-containing proteins, are important components of various

foods and beverages. The flavor quality of food can be improved with using laccases. Laccases may be also used as O2- scavengers for better food packing. In addition, wine stabilization is one of the main applications of laccase in the food industry as alternative to physical-chemical adsorbents (Kunamneni, 2008).

2.2.4.2.3 Textile Industry

For textile applications, laccase is used to improve the whiteness in conventional bleaching of cotton and recently biostoning. Potential benefits of the application include chemicals, energy, and water saving. Laccase also can be used in situ to convert dye precursors for better, more efficient fabric dyeing (Kunamneni, 2008).

2.2.4.2.4 Other Applications of Laccases

Laccases can act in wide range of substrates and can join in a lot of catalysis reactions, so they can be used for a lot of industrial applications. Laccases can also be used in organic synthesis area, environmental technologies, pharmaceutical and nanobiotechnology areas. In addition, they can be used in decolorization of different types of stubborn paint, bioremedation of soil and water, wrapping paper recycling, dimerization of phenolic and non-phenolic acids, synthesis if natural product like pigments and antioxidans, and a lot more biotechnological applications (Morozova, 2007; Mustafa, 2005; Riva, 2006).

2.2.5 Alpha-amylases

2.2.5.1 Properties and Industrial Importance of Alpha-amylases

Alpha-amylase is and endoenzyme and hydrolysis alpha-1,4-glycosidic linkages randomly. The main products produced as a result of hydrolysis are dextrins. Alpha-amylase is important in formation of low molecular weight sugar from starch. It hydrolyses alpha-1,4 linkages in starch molecule and produces glucose, maltose, maltotriose and alpha-limit dextrins. Starch is a polysaccharide composed of numerous glucose molecule in different ways. Enzymes such as alpha-amylase, beta-amylase, glycoamylase and glycoisomerase produced by soma bacteria and fungi have the ability to degrade starch (Lee, 1996).

Milled flour naturally contains sufficient amounts of alpha amylase enzyme. However maltose produced by alpha-amylase to be used in fermentation process is not enough. Because of that, extra alpha-amylase needs to be added to flour (Mathewson, 2000).

Alpha-amylase enzyme is the first microbial enzyme used in bread making in 1950 (Puldermans and Schoppink, 1999), and still it is the most important enzyme used in bread making. Another important usage area of alpha-amylases in industry is to extend the bread staling. In order to retard the staling of bakery products, diffraction of amylopectin crystal sections or preventing recrystallisation of amorphous amylopectin after cooking is required; and alpha-amylase can make this process. Thus, staling time can be extended noticably (Başaran, 2001).

Alpha-amylases can be both produced from fungi and bacteria. Bacterial alpha-amylases are more resistant to higher temperatures (70°C and higher) and provide longer enzyme activity according to the usage area. Alpha-amylase enzyme is a carbohydrase and first used enzyme commercially (Radley, 1976; Aira, 1983). Alpha-amylases with bacteria origin can be active in higher temperatures and more stable according to fungal alpha-amylases (thermostable), thus mostly bacterial alpha amylases are preferred for studies on main enzyme source (Wiseman, 1987).

2.2.5.2 Some Application Areas of Alpha-amylase

2.2.5.2.1 Food Industry

Alpha-amylase is widely used in bread making. Wheat contains many enzymes. Among these, the most concerned enzyme because of it technological importance is alpha-amylase. Changes during fermentation of dough and at the start of cooking depend on the activity of alpha amylase enzyme. The use of this enzyme retards the staling of the bread and extends the shelf life (2-3 days) (Başaran, 2001).

In food industry, end products of degradation of starch by alpha amylase are widely used. Among these end products, dextrin is the first short molecule product before starch is hydrolysed up to glucose. Dextrins have high resolution; they are durable products and are an intense syrupy substance. These products are used in food in order to increase viscosity that is, used as thickener filling material (Keskin, 1982).

Another usage area of alpha-amylase enzyme in food sector is juice industry. It is particularly used in apple and pear juice clarification. When the fruits are collected before being fully mature, since the fruits still have starch there will be blur occuring in juice. This problem is overcomed with addition of alpha-amylase (Ekşi, 1988).

2.2.5.2.2 Textile Industry

In textile industry, during weaving, yarns are treated with a solution of starch to make yarns rigid and flat, and to prevent from rupture. This process is called sizing. After the fabric has been woven, excess starch in fabric must be removed. This process is desizing. Alpha amylase is commonly used as desizing agent in the industry (Tarakçıoğlu, 1979).

2.2.5.2.3 Other Applications of Alpha-amylases

Thermostable alpha-amylases has wide range of application field. These enzymes are used in the textile and paper industry, to liquefy the starch, production of bread, glucose and fructose syrup, glue, alcohol fermentations (Bailey and Ollis, 1987; Bajpai and Bajpai., 1989; Igarashi et al., 1998).

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Screening Media

Media used in screening of enzyme activities on agar are included in Appendix A.

3.1.2 Chemicals and Solutions

Chemicals, solutions, kits and reagents are listed in Appendix B.

3.1.3 Samples

Sampling of soil was done from different places in Turkey which are listed below:

Table 3.1 Soil Sampling places.

Type of sample	Sample no	Sampling place
Farm field soil	**1**	Karadeniz- Trabzon
Bush soil	**2**	Karadeniz- Trabzon
Soil under walnut tree	**3**	Karadeniz- Trabzon
Vineyard soil	**4**	Karadeniz- Trabzon
Ornamental plant soil	**5**	Karadeniz- Trabzon
Soil under tree behind E block	**6**	Fatih Üniversitesi
Soil near ventilation exit behind E block	**7**	Fatih Üniversitesi
Soil under tree near autopark	**8**	Fatih Üniversitesi
Soil under walnut tree	**9**	Bursa, Nilüfer Tahtalı köyü
Soil under quince tree	**10**	Bursa, Nilüfer Tahtalı köyü
Soil under oak tree	**11**	Bursa, Nilüfer Tahtalı köyü
Soil under peach tree (ploughed)	**12**	Bursa, Nilüfer Tahtalı köyü
Soil under plum tree	**13**	Bursa, Nilüfer Tahtalı köyü
Soil under nut tree	**14**	Bursa, Nilüfer Tahtalı köyü
Decomposed organic waste	**15**	Bursa, Nilüfer Tahtalı köyü
Soil under peach tree	**16**	Bursa, Nilüfer Tahtalı köyü
Soil under Taxodium ascendens brogne	**17**	İstanbul Atatürk Orman Çiftliği
Soil under Populus alba L.(silver poplar)	**18**	İstanbul Atatürk Orman Çiftliği
Soil under Liquidambar styraciflua	**19**	İstanbul Atatürk Orman Çiftliği
Soil under Skimmia Japonica Thunb	**20**	İstanbul Atatürk Orman Çiftliği
Soil under Acca sellowiana burret	**21**	İstanbul Atatürk Orman Çiftliği
Soil under Robinia pseudoaccia L.	**22**	İstanbul Atatürk Orman Çiftliği
Soil under Juniperus sabina L.	**23**	İstanbul Atatürk Orman Çiftliği
Soil around the lake	**24**	İstanbul Büyükçekmece Lake
Soil from the edge of lake	**25**	İstanbul Büyükçekmece Lake

3.2 METHODS

3.2.1 Collection of Soil Samples

Different soil and compost sample were collected for the isolation of soil bacteria capable of producing industrially important enzymes. Sampling places are listed in Table 3.1.

3.2.2 Isolation of Soil Bacteria

Isolation of soil bacteria capable of producing industrial enzymes has been carried out with screening media including selected enzymes' substrates and indicators. Emphasis was given to the selection of high enzyme-producing bacteria.

Inoculations has been carried out in four steps including soil inoculation on screening agar media, streak plate of bacteria colonies showing enzyme activity, needle inoculations of single bacteria colonies with three repeats and making serial dilutions in order to isolate single bacteria colonies when necessary.

In first step, soil samples were spread sparsely on screening media. After overnight incubation at 37 ^{o}C, bacteria colonies showing clear zones around (making color change in laccase screening media) were chosen. In second step, in order to dilute the intense colonies on agar, streak plate of selected colonies were done. In third step, after getting single colonies on streak plate, triple needle inoculations were done in order to calculate enzymatic indexes and determine enzyme activities. In second step, if no single colony on agar were observed, serial dilutions were done to get single colony on agar and calculate enzymatic indexes.

Screening of five industrial enzymes was done according to above four major steps and below procedures.

3.2.2.1 Screening for Xylanase Activity

The medium rich in xylan (Sigma, X4252) described in Appendix A was used for xylanase screening. After inoculation and incubation for 3 days at 37 ^{o}C, clear zones around the colonies on a red background were taken as the evidence for the xylanase activity.

3.2.2.2 Screening for Protease Activity

The medium rich in skim milk (Merck, 115363) described in Appendix A were used in protease screening. After inoculation of the isolates, the plates were incubated for 2 days at 37 ^{o}C. Opaque halos around the colonies were taken as the indication of protease activity.

3.2.2.3 Screening for Lipase Activity

The medium rich in tributyrin (Sigma, 91015) described in Appendix A were used in lipase screening. After inoculation of the isolates, the plates were incubated for 3 days at 37 ^{o}C. Opaque halos around the colonies were taken as the indication of lipase activity.

3.2.2.4 Screening for Laccase Activity

The medium containing guaiacol (Sigma, G5502) as and indicator, as described in Appendix A were used in laccase screening. After inoculation of the isolates, the plates were incubated for 7 days at 37 ^{o}C. Turning the color of colonies into yellowish and reddish brown was taken as the evidence of laccase activity.

3.2.2.5 Screening for Alpha-amylase Activity

Media rich in starch (Merck, 101252) used for alpha-amylase screening were listed in Appendix A. After inoculation of isolates, the plates were incubated for 2 days at 37 ^{o}C. Clear zones around the colonies indicated the presence of alpha-amylase activity.

3.2.3 Calculation of Enzymatic Indexes (EI) and Selection of Isolates

After observing clear zones around the colonies in screening media, their enzymatic indexes were calculated indicating their enzyme producing capabilities. For enzymatic index calculation, diameter of the clear zone (CZ_{dia}) and diameter of bacteria colonies (BC_{dia}) were measured. The value of CZ_{dia} over BC_{dia} gives EI value. EI value has been used for determination of enzyme activity of bacteria isolates on agar Petri.

3.2.4 Gram Reaction and Morphology

After isolation of single bacteria colonies, they were gram stained in order to examine physiological characteristics of colonies. Bacteria colonies were grouped according to their gram reaction (positive or negative) and colony shape (rod, cocci, and spiral).

3.2.5 Identification of Selected Soil Bacteria Isolates

Bacteria colonies with higher enzymatic indexes were chosen to be identified via 16S rDNA sequencing technique. 17 isolates were chosen for molecular identification.

3.2.5.1 Genomic DNA Isolation

Genomic DNA of selected isolates was isolated using Gene Jet DNA purification kit (Thermo Scientific). Concentration of gDNAs was measured with NanoDrop.

3.2.5.2 Molecular Characterization (16S rDNA sequencing)

gDNA isolations of selected isolates were sent to Macrogen Europe for 16S rDNA sequencing. Molecular identification of isolates was done according to sequence results and bioinformatics studies. 16S rDNA sequence results of isolates were analyzed through BLAST program, species with highest score (%99 identity) were chosen and isolates were identified.

3.2.6 Crude Extract Enzyme Activity Assays

After identification of 17 bacteria isolates, some of them were chosen for partial purification of proteins. In order to choose candidate isolates, supernatant and cell free extracts of 17 isolates were obtained and below enzyme activities were measured spectrophotometrically. First of all, 17 isolates were grown in 50 ml studier medium (Appendix A) which is known to have auto induction effect (Studier FW, 2005) and increase the expression of proteins resulting with higher enzyme production. After isolates were grown overnight in studier medium, they were used for enzyme extraction procedure. Cultures obtained were centrifuged at 10000 rpm, 0 ^{o}C for 30 min. Supernatant including extracellular enzymes were taken in a sterile tube and remaining pellet were used for intracellular enzyme extraction. For this purpose, pellets were

suspended with 10 ml Buffer A solution. After vortexing, 100 ul PMSF and powder lysozyme enzyme were added. The mixture were incubated firstly at 37^{o}C for 5-6 min, than on ice for 30-45 min. Blasting of bacterial cells was done with sonicator (Bandelin Electronic) with 15 cycles of 15 s sonication (%60-70 vibration) and 45 s incubation on ice. After sonication, tubes were centrifuged for 30 min at 10000 rpm. Supernatants including cell free enzymes were stocked and used for enzyme activity assays.

3.2.6.1 Xylanase Assay

300 ul enzyme extract was added to 400 ul %1 xylan substrate. The mixture was incubated at 45 ^{o}C for 10 min. Enzyme reaction stopped by adding 1000 ul DNS reagent and boiling for 5 min. After boiling, the tubes were cooled and poured in falcon tubes including 10 ml distilled water. The absorbance was measured at 530 nm with two repeats (Kishishita S, 2014). For each repeat, one tube without enzyme was used for blank measurement.

3.2.6.2 Protease Assay

50 ul from enzyme extracts of isolates were taken and mixed with 200 ul %1 casein substrate solution. The mixture was incubated at 100 rpm at 30 ^{o}C for 30 seconds. Than the reaction is stopped by adding 1,7 ml from 5% TCA to the mixture. After cooling, tubes were poured into falcon tubes including 10 ml distilled water. The absorbance was measured at 280 nm with two repeats (Asker, 2013). For each repeat, one tube without enzyme was used for blank measurement.

3.2.6.3 Laccase Assay

575 ul from 100 mM sodium acetate buffer, 125 ul from 0,1 mM CuSO4, 200 ul from 10 mM guaiacol solution was mixed and 200 ul enzyme extract was added. At 0. second after enzyme extract was added, absorbance at 470 nm was taken as blank measurement. Then after 30 seconds of incubation at 37 ^{o}C, absorbance was measured again showing enzyme activity measurement. Activities of commercial laccase enzyme from *Trametes versicolor* (Sigma, 51639) were also measured and compared with 17 isolates' laccase activities.

3.2.6.4 Lipase Assay

500 ul from 20 mM Tris-HCl (pH 7) buffer, 200 ul from %1 Tween 80 solution, 200 ul from 80 mM $CaCl_2$ solution and 200 ul from enzyme extracts were mixed and absorbance was measured at 450 nm and taken as blank measurement. Then after 30 seconds of incubation at 25 ^oC, absorbance was measured again showing enzyme activity measurement. Activities of commercial lipase enzyme from *Thermomyces lanuginosus* (Sigma, L0777) were also measured and compared with 17 isolates' laccase activities.

3.2.6.5 Alpha-amylase Assay

200 ul from enzyme extract was added to 400 ul %0,25 starch solution. The mixture was incubated at 37^oC for 10 min. After incubation, enzyme reaction was stopped by adding 1 ml DNS reagent and boiling for 5 min. After boiling, the tubes were cooled and poured in falcon tubes including 10 ml distilled water. For each sample two repeats were prepared and for each repeat one tube without enzyme extract was prepared for blank measurement. The absorbances were measured at 540 nm. Activities of commercial alpha amylase enzyme from *Bacillus licheniformis* (Sigma, A4862) were also measured and compared with 17 isolates' alpha amylase activities.

3.2.7 Partial Purification of Proteins

Partial purification of proteins was done with ammonium sulfate precipitation. For this purpose, selected isolates according to their enzymatic activities, were grown in studier medium (Appendix A) for production of enzymes. After overnight growth in 50 ml studier medium, bacteria cultures were used for ammonium sulfate precipitation experiments. In order to search the optimum ammonium sulfate concentration that purified enzyme has maximum activity, serial precipitation fractions with different ammonium sulfate concentrations were done (%40, %60, %80, %100).

3.2.7.1 Ammonium Sulfate Precipitation

3.2.7.1.1 Xylanase Precipitation

Supernatant extract from La.11.S5 isolate (Bacillus subtilis - % 100 relative xylanase activities) was chosen for partial purification of xylanase enzyme. La.11.S5 isolate grown in studier medium was centrifuged at 4000xg for 20 min to get supernatant extract. Supernatant extract was taken into a beaker on ice. Precipitations were done with %40, %60, %80 and %100 ammonium sulfate concentrations respectively. Amounts of solid ammonium sulfate to be added were calculated according to Table 3.2.

Table 3.2 Ammonium sulfate saturation table using solid ammonium sulfate.

Initial concentration of ammonium sulphate, % saturation	Final concentration of ammonium sulphate—% saturation at 0 °C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	g solid ammonium sulphate to add to 100 ml of solution																
0	10·6	13·4	16·4	19·4	22·6	25·8	29·1	32·6	36·1	39·8	43·6	47·6	51·6	55·9	60·3	65·0	69·7
5	7·9	10·8	13·7	16·6	19·7	22·9	26·2	29·6	33·1	36·8	40·5	44·4	48·4	52·6	57·0	61·5	66·2
10	5·3	8·1	10·9	13·9	16·9	20·0	23·3	26·6	30·1	33·7	37·4	41·2	45·2	49·3	53·6	58·1	62·7
15	2·6	5·4	8·2	11·1	14·1	17·2	20·4	23·7	27·1	30·6	34·3	38·1	42·0	46·0	50·3	54·7	59·2
20	0	2·7	5·5	8·3	11·3	14·3	17·5	20·7	24·1	27·6	31·2	34·9	38·7	42·7	46·9	51·2	55·7
25		0	2·7	5·6	8·4	11·5	14·6	17·9	21·1	24·5	28·0	31·7	35·5	39·5	43·6	47·8	52·2
30			0	2·8	5·6	8·6	11·7	14·8	18·1	21·4	24·9	28·5	32·3	36·2	40·2	44·5	48·8
35				0	2·8	5·7	8·7	11·8	15·1	18·4	21·8	25·4	29·1	32·9	36·9	41·0	45·3
40					0	2·9	5·8	8·9	12·0	15·3	18·7	22·2	25·8	29·6	33·5	37·6	41·8
45						0	2·9	5·9	9·0	12·3	15·6	19·0	22·6	26·3	30·2	34·2	38·3
50							0	3·0	6·0	9·2	12·5	15·9	19·4	23·0	26·8	30·8	34·8
55								0	3·0	6·1	9·3	12·7	16·1	19·7	23·5	27·3	31·3
60									0	3·1	6·2	9·5	12·9	16·4	20·1	23·9	27·9
65										0	3·1	6·3	9·7	13·2	16·8	20·5	24·4
70											0	3·2	6·5	9·9	13·4	17·1	20·9
75												0	3·2	6·6	10·1	13·7	17·4
80													0	3·3	6·7	10·3	13·9
85														0	3·4	6·8	10·5
90															0	3·4	7·0
95																0	3·5
100																	0

 Adapted from Dawson et al. (1986).
 Values given are the amount of solid $(NH_4)_2SO_4$ required to bring a solution of known initial saturation to a desired final saturation. For the data listed, the saturation is relative to full saturation at 0°C.

Fractions after precipitation by slowly mixing on ice with magnetic stirrer, was taken in a falcon tube and centrifuged at 0^oC, 10000 rpm for 10 min. Pellet of each fraction was kept on ice and supernatants after centrifugation were used for other

fractionations. When last fraction was collected, last supernatant was also kept on ice for enzyme activity measurements. Later, pellets obtained and last supernatant fraction was used for dialysis experiments to remove salts after ammonium sulfate precipitation. Before dialysis process, spectrophotometric xylanase activity measurements were done for all fractions (%40, %60, %80, %100) to determine the fraction that did not lose its enzyme activity at all. According to these activity results, best fraction with highest enzyme activity was determined. Pellet of best fraction was suspended in 5 ml 0,1 M NaPi buffer and put into 33 mm dialysis bag (Biobasic, TX0111). Dialysis bag was placed into a beaker containing 500 ml 50 mM NaPi buffer. The beaker was incubated for 3 hours at +4 degrees, than buffer inside the beaker was renewed and dialysis bag inside the buffer was left for overnight incubation at +4 degrees. After overnight incubation, dialysed solute inside the bag was taken in a sterile tube; enzyme activity of this solute, including dissolved proteins was measured spectrophotometrically.

3.2.7.1.2 Protease Precipitation

Cell free extract from M.5.S4.1 isolate (Bacillus sp. - % 100 relative protease activities) was chosen for partial purification of protease enzyme. M.5.S4.1 isolate grown in studier medium was centrifuged at 4000xg for 20 min. Pellet was taken and its cell free extract was obtained with sonication. Cell free extract was taken into a beaker on ice. Precipitations were done as described in 3.2.6.1.1.

3.2.7.1.3 Laccase Precipitation

Cell free extract from L.13.S2.2 isolate (Bacterium BS72 - %100 relative laccase activity) was chosen for partial purification of laccase enzyme. Its precipitation was done as described in 3.2.6.1.2.

3.2.7.1.4 Lipase Precipitation

Cell free extract from L.13.S2.2 isolate (Bacterium BS72 - %100 relative lipase activity) was chosen for partial purification of lipase enzyme. Its precipitation was done as described in 3.2.6.1.2.

3.2.7.1.5 Alpha-amylase Precipitation

Supernatant extract from M.22.S2.3 isolate (Bacillus pumilus - %6 relative alpha-amylase activity) was chosen for partial purification of lipase enzyme. M.22.S2.3 isolate grown in studier medium was centrifuged at 4000xg for 20 min. to get supernatant extract. Supernatant extract was taken into a beaker on ice. Precipitations were done as described in 3.2.6.1.1.

3.2.7.2 Dialysis

Dialysis of pellets of desired fractions in 5 ml 0,1 M PBS buffer was done using 33 mm dialysis bags (Biobasic). Suspensions were poured into dialysis bags and bags were placed in beaker containing 500 ml PBS buffer with lower concentration than the suspension inside the bag. The beaker was incubated at 4^{o}C bu gently mixing. After 3 hours PBS buffer inside the beaker was changed with fresh buffer, then overnight incubation was done. After overnight incubation, solutions inside the dialysis bag were taken into sterile tubes. Partially purified proteins' enzymatic activities were measured with spectrophotometry and they were characterized.

3.2.8 Characterization of Partially Purified Proteins

For characterization of partially purified enzymes, the effect of pH and temperature on enzyme activities was measured and optimum pH and temperature values showing highest enzyme activities were found.

3.2.8.1 Effect of pH on Enzyme Activity

Buffers with different pH values (between 2-10) were prepared in order to measure the effect of pH values on enzyme activities. Enzyme-substrate solutions were mixed with these buffers having different pH values and enzymatic activities were measured spectrophotometrically. According to the activity results, pH graph was created and optimum pH was found where the activity is shown to be maximum.

3.2.8.1.1 Effect of pH on Xylanase Activity

20 mM sodium acetate buffer was prepared for pH 2, pH 3, pH 5, pH 8, pH 9 solutions. 20 mM glycin-NaOH buffer was prepared for pH 10 and pH 12 solutions.

Enzyme activity measurements were done as described in 3.2.6.1. %1 xylan substrates were prepared with these buffers and used in activity measurements. According to activity results, optimum pH value was determined.

3.2.8.1.2 Effect of pH on Protease Activity

20 mM glycin-HCl buffer was prepared for pH 2 and pH 3 solutions. 20 mM sodium acetate buffer was prepared for pH 4and pH 5 solutions. 20 mM sodium phosphate buffer was prepared for pH 6 and pH 7 solutions; 20mM Tris-HCl buffer was prepared for pH 8, pH 9 and pH 10 solutions (Asker, 2013). Enzyme activity measurements were done as described in 3.2.6.2. %1 casein substrates were prepared with these buffers and used in activity measurements. According to activity results, optimum pH value was determined.

3.2.8.1.3 Effect of pH on Laccase Activity

20 mM glycin-HCl buffer was prepared for pH 2 and pH 3 solutions. 20 mM sodium acetate buffer was prepared for pH 4and pH 5 solutions. 20mM Tris-HCl buffer was prepared for pH 8 and pH 9 solutions. 20 mM Na_2HPO_4-NaOH was prepared for pH 10 solution (Lu, 2013). Enzyme activity measurements were done as described in 3.2.6.3 with using these buffers having different pH values. According to activity results, optimum pH value was determined.

3.2.8.1.4 Effect of pH on Lipase Activity

20 mM sodium acetate buffer was prepared for pH 3, pH 4 and pH 5 solutions. 20 mM sodium phosphate buffer was prepared for pH 6 and pH 7 solutions. 20mM Tris-HCl buffer was prepared for pH 8, pH 9 and pH 10 solutions (Emtenani, 2013). Enzyme activity measurements were done as described in 3.2.6.4 with using these buffers having different pH values. According to activity results, optimum pH value was determined.

3.2.8.1.5 Effect of pH on Alpha-amylase Activity

20 mM sodium acetate buffer was prepared for pH 3, pH 4 and pH 5 solutions. 20 mM sodium phosphate buffer was prepared for pH 6 and pH 7 solutions; 20mM Tris-HCl buffer was prepared for pH 8 and pH 9 solutions (Xie, 2014). Enzyme activity

measurements were done as described in 3.2.6.5. %0,25 starch substrates were prepared with these buffers and used in activity measurements. According to activity results, optimum pH value was determined.

3.2.8.3 Effect of temperature on Enzyme Activity

Optimum pH buffers that showed highest enzyme activity were used to measure the effect of different temperatures on enzyme activities. Spectrophotometric activity measurements were done by incubating enzyme-substrate solutions at different temperatures. According to the activity results, temperature graph was created and optimum temperatures were found where the activity is shown to be maximum.

3.2.8.3.1 Effect of temperature on Xylanase Activity

20 mM sodium acetate buffer at pH 8 was used and enzymatic activities were measured as described in 3.2.6.1 with incubations at different temperatures (30^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C, 70 ^{o}C, 80 ^{o}C). According to the activity results, temperature graph was created and optimum temperature was found where the activity is shown to be maximum.

3.2.8.3.2 Effect of temperature on Protease Activity

20 mM sodium phosphate buffer at pH 6 was used and enzymatic activities were measured as described in 3.2.6.2 with incubations at different temperatures (0^{o}C, 10 ^{o}C, 15 ^{o}C, 20 ^{o}C, 25 ^{o}C, 30 ^{o}C, 35 ^{o}C). According to the activity results, temperature graph was created and optimum temperature was found where the activity is shown to be maximum.

3.2.8.3.3 Effect of temperature on Laccase Activity

20 mM Tris-HCl buffer at pH 8 was used and enzymatic activities were measured as described in 3.2.6.3 with incubations at different temperatures (30^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C, 70 ^{o}C, 80 ^{o}C, 90^{o}C). According to the activity results, temperature graph was created and optimum temperature was found where the activity is shown to be maximum.

3.2.8.3.4 Effect of temperature on Lipase Activity

20 mM sodium phosphate buffer at pH 7 was used and enzymatic activities were measured as described in 3.2.6.4 with incubations at different temperatures (30^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C, 70 ^{o}C, 80 ^{o}C, 90^{o}C). According to the activity results, temperature graph was created and optimum temperature was found where the activity is shown to be maximum.

3.2.8.3.5 Effect of temperature on Alpha-amylase Activity

20 mM sodium phosphate buffer at pH 6 was used and enzymatic activities were measured as described in 3.2.6.4 with incubations at different temperatures (30^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C, 70 ^{o}C, 80 ^{o}C, 90^{o}C). According to the activity results, temperature graph was created and optimum temperature was found where the activity is shown to be maximum.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 ISOLATION OF SOIL BACTERIA

Bacteria isolation was carried out with samples listed in Table 3.1. Isolations were done in screening media for xylanase, protease, laccase, lipase and alpha-amylase enzyme activity.

4.1.1 Isolated Bacteria with Xylanase Activity

After soil inoculations on xylanase screening media, 2 single bacteria colonies showed zone of clearance in xylan agar that indicates xylanase activity. These single colonies called XB.24.S1.3 and XB.26.S2.2 were isolated (Figure 4.1) and stocked in both -80 ^{o}C in %50 glycerol and at +4 ^{o}C as slant agars.

Figure 4.1 Needle inoculation of Xylanase (+) isolates.

4.1.2 Isolated Bacteria with Protease Activity

After soil inoculations on protease screening media, 22 bacteria colonies capable of producing protease showed zone of clearance in skim milk agar. These single colonies were isolated and stocked in both -80 ^{o}C in 50% glycerol and at +4 ^{o}C as slant agars. Bacteria isolated are listed in Table 4.1 (Figure 4.2).

Table 4.1 Bacteria isolates on protease screening media.

No	Isolate	No	Isolate
1	M.1.S1.3	12	M.5.S3.1
2	M.1.S2.2	13	M.5.S4.1
3	M.1.S3.1	14	M.9.S1.1
4	M.2.S1.1	15	M.12.S1.2
5	M.3.S1.2	16	M.13.S1.1
6	M.4.S1.1	17	M.14.S1.3
7	M.4.S2.3	18	M.15.S1.3
8	M.4.S3.3	19	M.16.S1.1
9	M.4-2.S1.1	20	M.23.S2.2
10	M.5.S1.1	21	M.23.S1.2
11	M.5.S2.1	22	M.22.S2.3

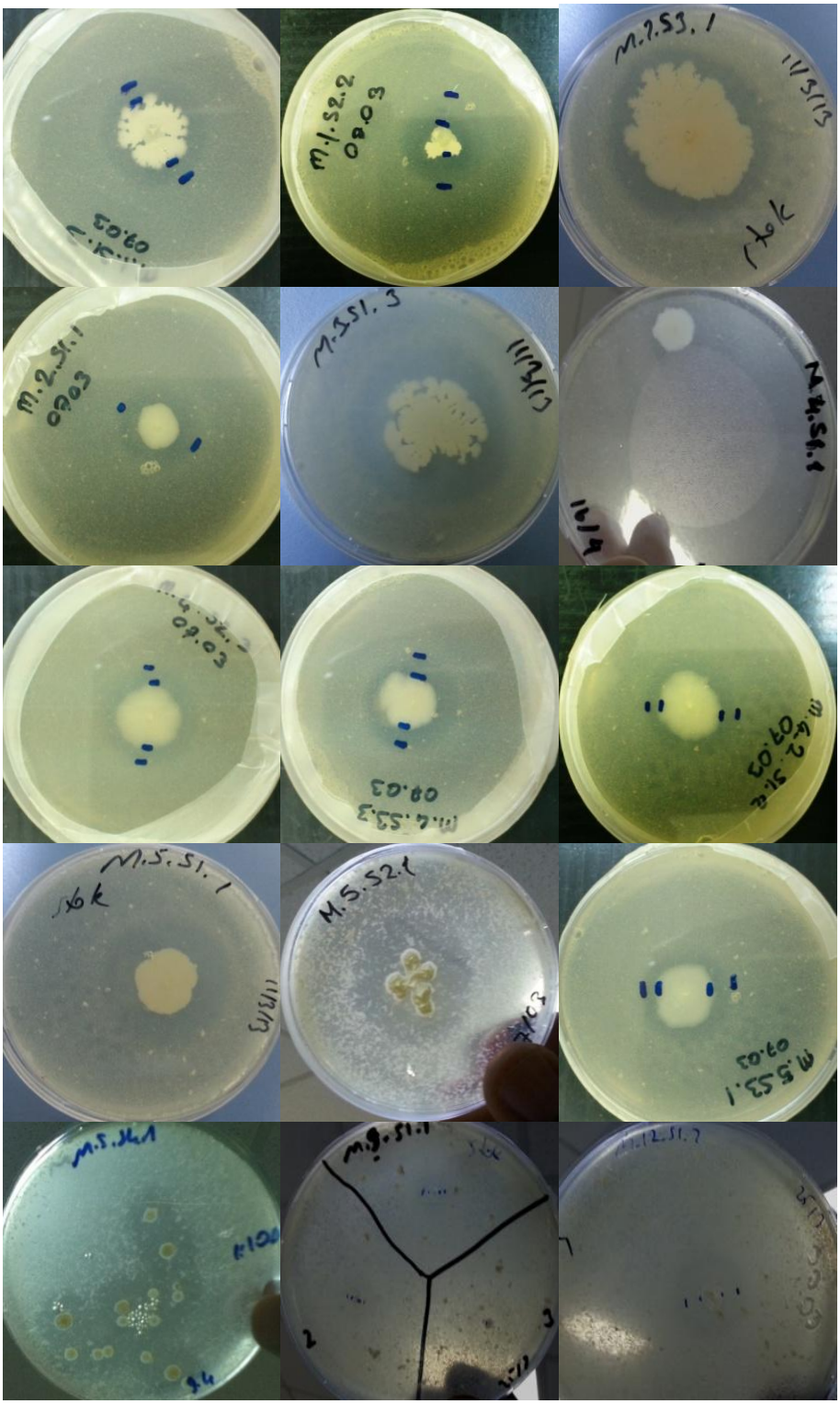

Figure 4.2 Petri plate photos of protease (+) isolates (left to right: M.1.S1.3, M.1.S2.2, M.1.S3.1, M.2.S1.1, M.3.S1.2, M.4.S1.1, M.4.S2.3, M.4.S3.3, M.4-2.S1.1, M.5.S1.1, M.5.S2.1, M.5.S3.1, M.5.S4.1, M.9.S1.1, M.12.S1.2, M.13.S1.1, M.14.S1.3, M.15.S1.3, M.16.S1.1, M.23.S2.2, M.23.S1.2, M.22.S2.3).

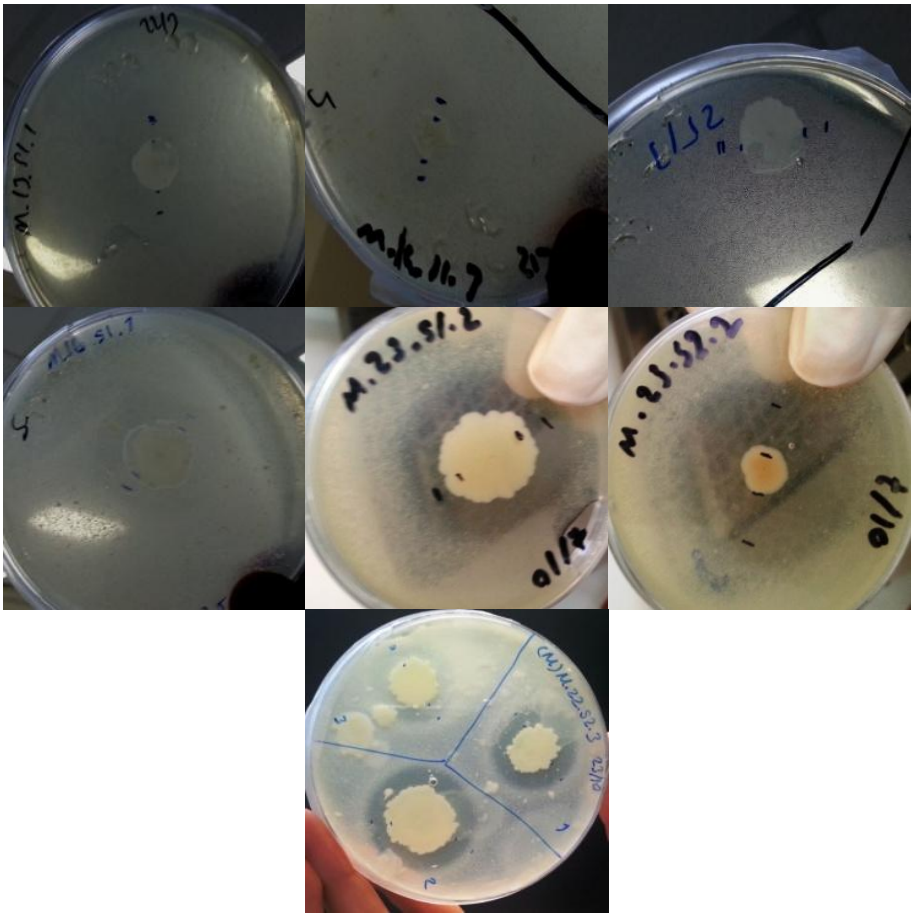

Figure 4.2 (cont.)

4.1.3 Isolated Bacteria with Laccase Activity

After soil inoculations on laccase screening media, 33 bacteria colonies capable of producing laccase showed zone of clearance in nutrient agar containing guaiacol as an indicator. These single colonies were isolated and stocked in both -80 ^{o}C in 50 % glycerol and at +4 ^{o}C as slant agars. Bacteria isolated are listed in Table 4.2 (Figure 4.3).

Table 4.2 Bacteria isolates on laccase screening media.

No	Isolate	No	Isolate	No	Isolate
1	La.9.S1	**12**	La.12.S1	**23**	La.13.S6
2	La.9.S2	**13**	La.12.S2	**24**	La.14.S1
3	La.9.S3	**14**	La.12.S3	**25**	La.14.S2
4	La.9.S4	**15**	La.12.S4	**26**	La.14.S3
5	La.10.S1	**16**	La.12.S5	**27**	La.14.S4
6	La.10.S2	**17**	La.12.S6	**28**	La.14.S5
7	La.10.S3	**18**	La.13.S1	**29**	La.15.S1
8	La.11.S1	**19**	La.13.S2	**30**	La.15.S2
9	La.11.S3	**20**	La.13.S3	**31**	La.15.S5
10	La.11.S4	**21**	La.13.S4	**32**	La.16.S1
11	La.11.S5	**22**	La.13.S5	**33**	La.16.S2

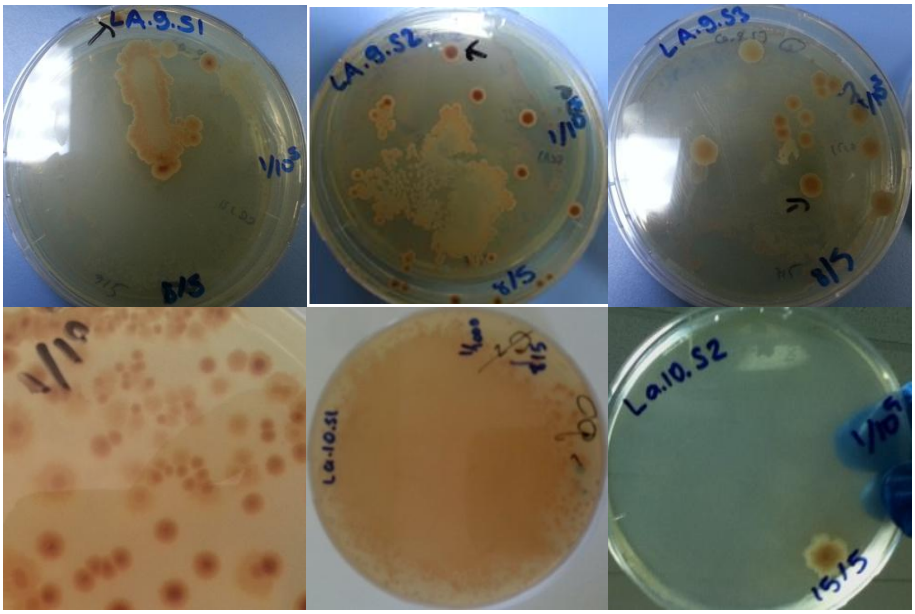

Figure 4.3 Petri plate photos of laccase (+) isolates (left to right: La.9.S1, La.9.S2, La.9.S3, La.9.S4, La.10.S1, La.10.S2, La.10.S3, La.11.S1, La.11.S3, La.11.S4, La.11.S5, La.12.S1, La.12.S2, La.12.S3, La.12.S4, La.12.S5, La.12.S6, La.13.S1, La.13.S2, La.13.S3, La.13.S4, La.13.S5, La.13.S6, La.14.S1, La.14.S2, La.14.S3, La.14.S4, La.14.S5, La.15.S1, La.15.S2, La.15.S5, La.16.S1, La.16.S2).

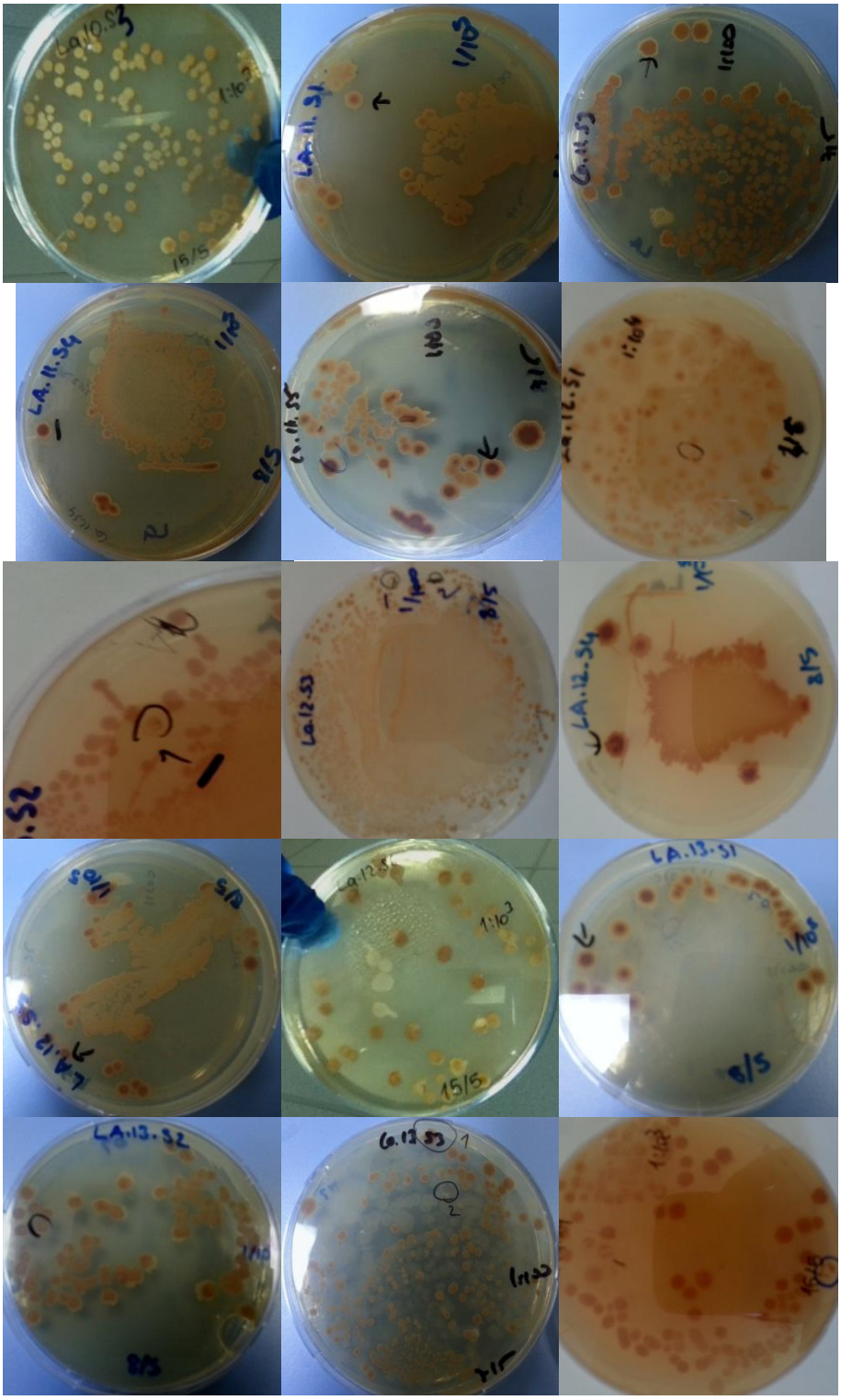

Figure 4.3 (cont.)

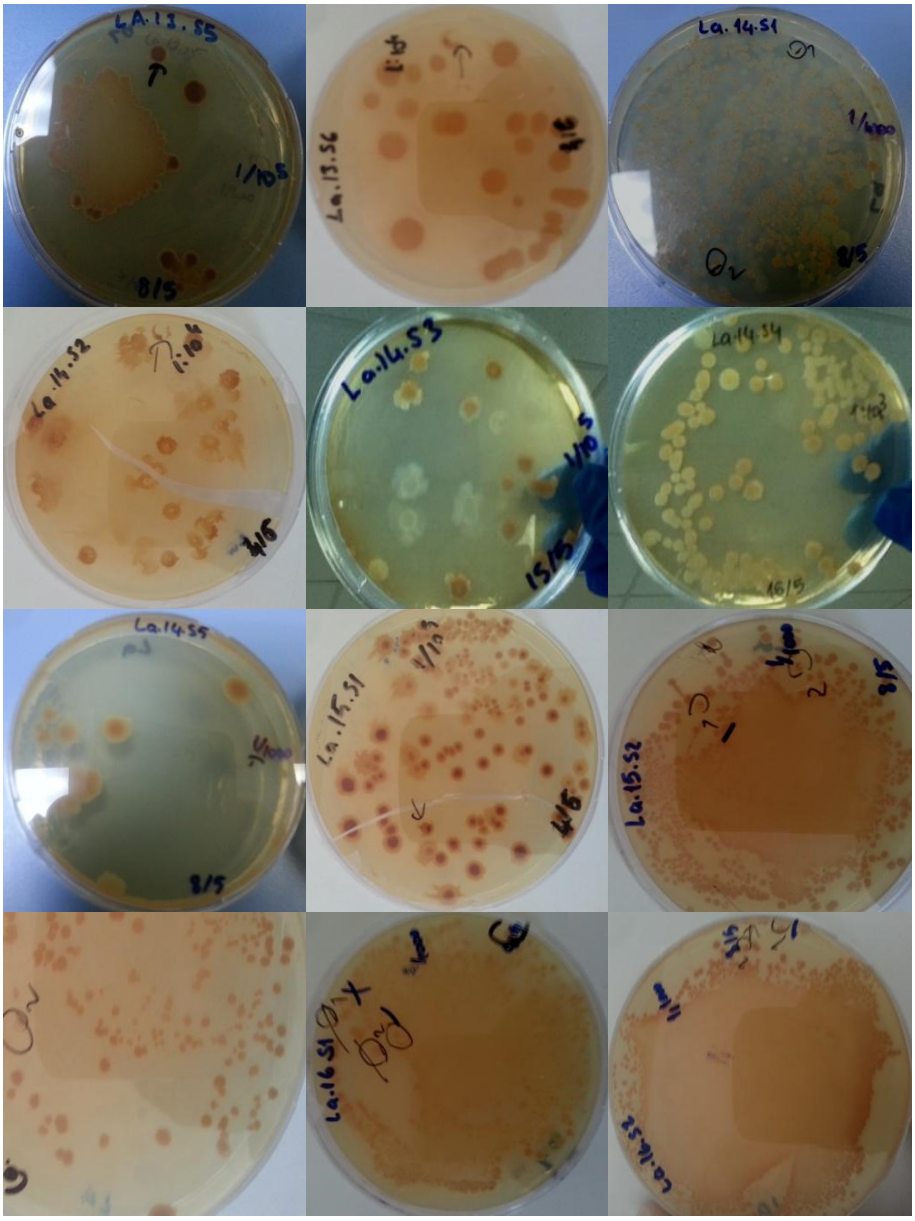

Figure 4.3 (cont.)

4.1.4 Isolated Bacteria with Lipase Activity

After soil inoculations on lipase screening media, 28 single bacteria colonies were isolated and listed in Table 4.3 (Figure 4.4).

Table 4.3 Bacteria isolates on lipase screening media.

No	Isolate	No	Isolate	No	Isolate
1	L.7.S1.1	**11**	L.16.S4.2	**20**	L.23.S3.1
2	L.13.S2.2	**12**	L.17.S2	**21**	L.23.S5.3
3	L.14.S1.2	**13**	L.18.S2.1	**22**	L.24.S2.2
4	L.14.S2.2	**14**	L.18.S4.3	**23**	L.24.S3.2
5	L.14.S3.3	**15**	L.18.S5.3	**24**	L.24.S5.1
6	L.15.S3.1	**16**	L.18.S6.3	**25**	L.26.S2.2
7	L.15.S3.2.3	**17**	L.20.S1.3	**26**	L.26.S3.2
8	L.16.S1.1	**18**	L.20.S2.1	**27**	L.26.S5.2
9	L.16.S2.1	**19**	L.21.S1.1	**28**	L.X.2.3
10	L.16.S3				

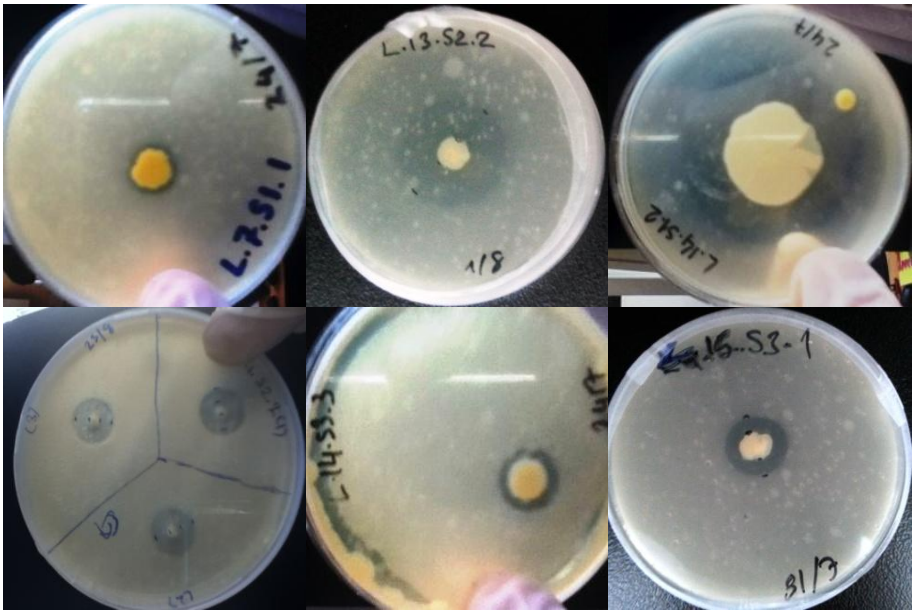

Figure 4.4 Petri plate photos of lipase (+) isolates (left to right: L.7.S1.1, L.13.S2.2, L.14.S1.2, L.14.S2.2, L.14.S3.3, L.15.S3.1, L.15.S3.2.3, L.16.S1.1, L.16.S2.1, L.16.S3, L.16.S4.2, L.17.S2, L.18.S2.1, L.18.S4.3, L.18.S5.3, L.18.S6.3, L.20.S1.3, L.20.S2.1, L.21.S1.1, L.23.S3.1, L.23.S5.3, L.24.S2.2, L.24.S3.2, L.24.S5.1, L.26.S2.2, L.26.S3.2, L.26.S5.2, L.X.2.3).

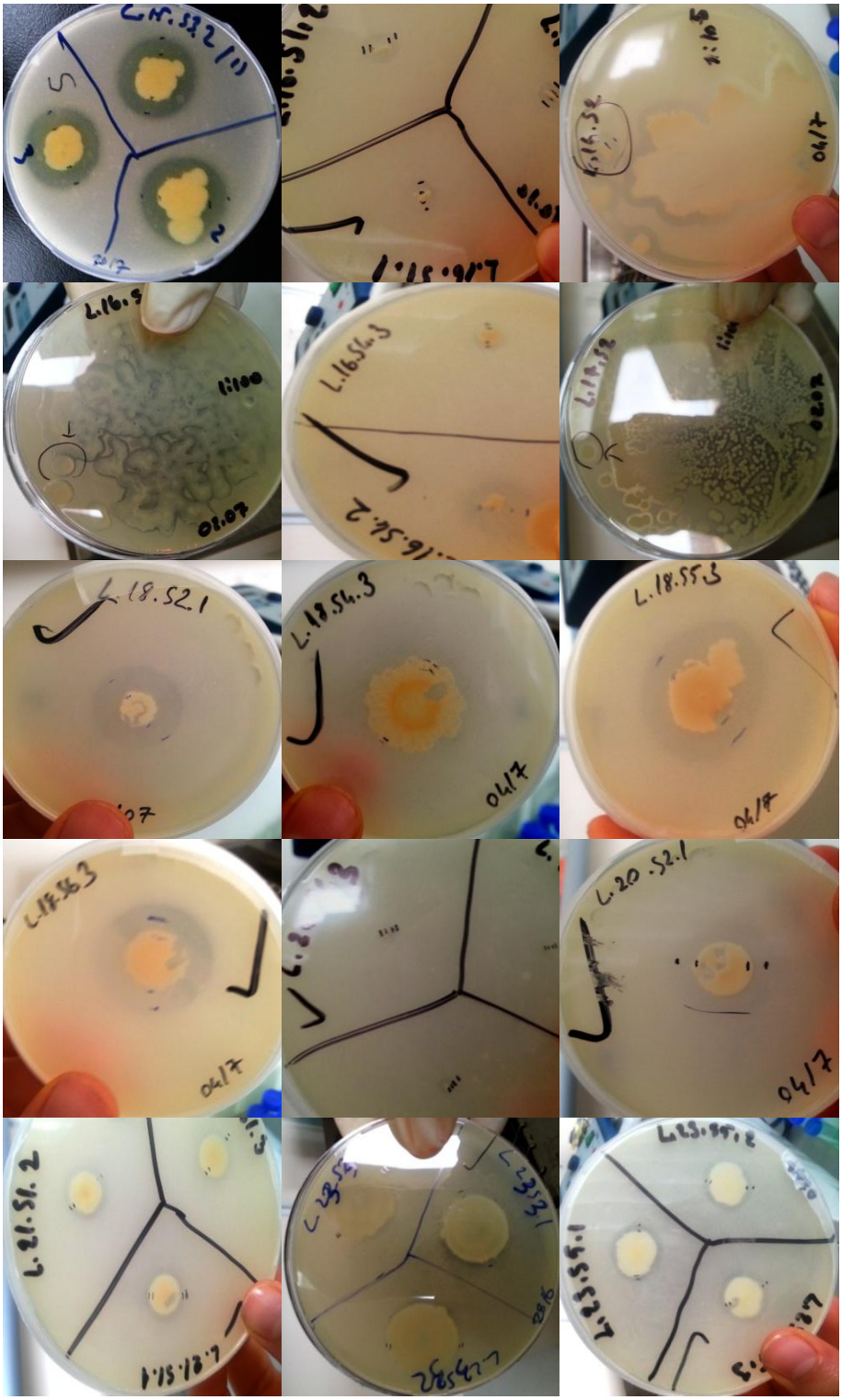

Figure 4.4 (cont.)

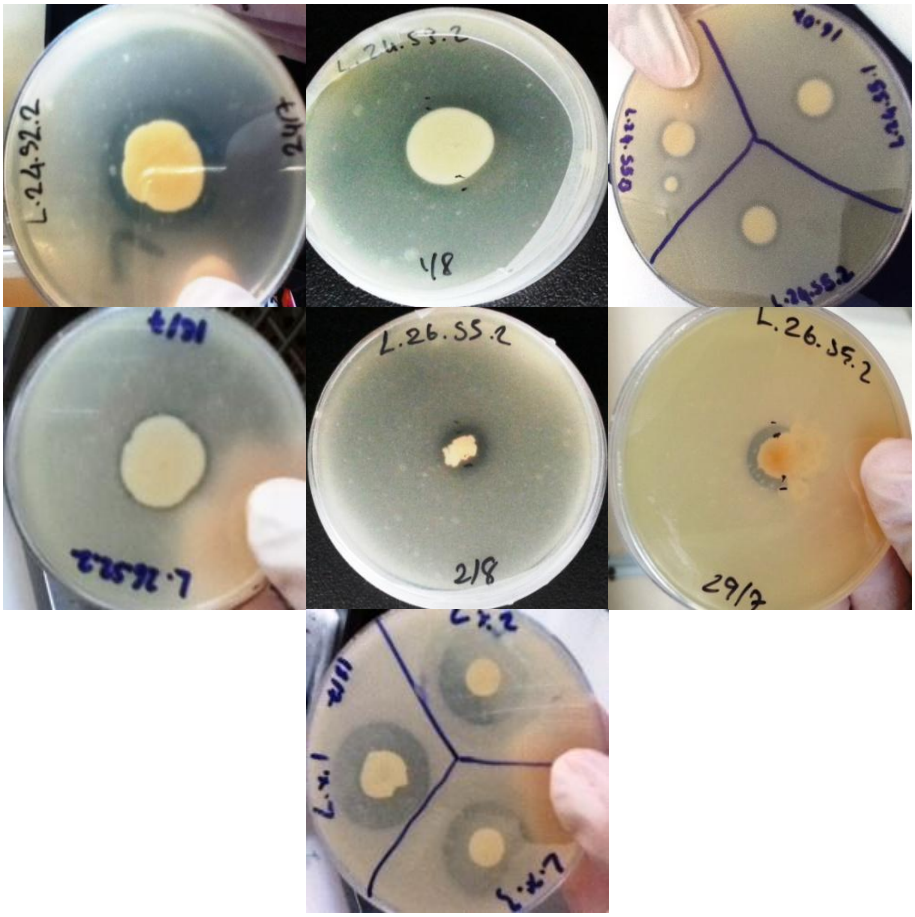

Figure 4.4 (cont.)

4.1.5 Isolated Bacteria with Alpha-amylase Activity

After soil inoculations on lipase screening media, 27 single bacteria colonies were isolated and listed in Table 4.4 (Figure 4.5).

Table 4.4 Bacteria isolates on alpha-amylase screening media.

No	Isolate	No	Isolate	No	Isolate
1	A1.1.S1.1	**10**	A1.21.S3.1	19	A2.11.S1.3
2	A1.3.S1	**11**	A1.21.S5.3	20	A2.11.S2.2
3	A1.3.S2.1	**12**	A1.22.S2.2	21	A2.11.S3.2
4	A1.5.S3.1	**13**	A2.1.S1.2	22	A2.11.S4.2
5	A1.5-2.S2.2	**14**	A2.1.S1.3	23	A2.12.S2.1
6	A1.13.S2.3	**15**	A2.5.S2.1	24	A2.22.S1.3
7	A1.18.S2.1	**16**	A2.9.S1.3	25	A2.23.S2.1
8	A1.19.S1.3	**17**	A2.9.S2.2	26	A2.23.S3.1
9	A1.20.S2.2	**18**	A2.9.S3.1	27	A2.23.S4.2

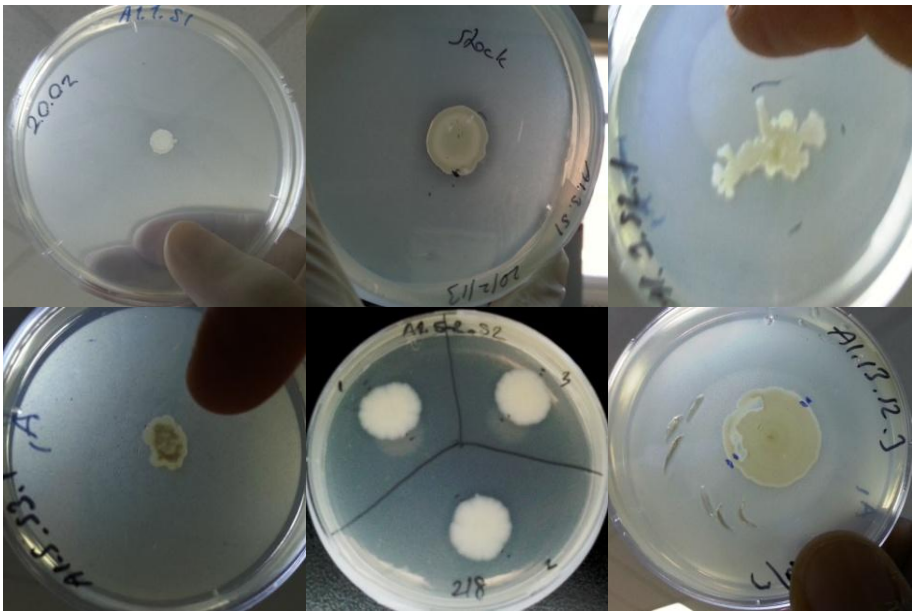

Figure 4.5 Petri plate photos of alpha amylase (+) isolates (left to right: A1.1.S1.1, A1.3.S1, A1.3.S2.1, A1.5.S3.1, A1.5-2.S2.2, A1.13.S2.3, A1.18.S2.1, A1.19.S1.3, A1.19.S2.2, A1.20.S2.2, A1.21.S3.1, A1.21.S5.3, A1.22.S2.2, A2.1.S1.3, A2.5.S2.1, A2.9.S1.3, A2.9.S2.2, A2.9.S3.1, A2.11.S1.3, A2.11.S2.2, A2.11.S3.2, A2.11.S4.2, A2.12.S2.1, A2.22.S1.3, A2.23.S2.1, A2.23.S3.1, A2.23.S4.2).

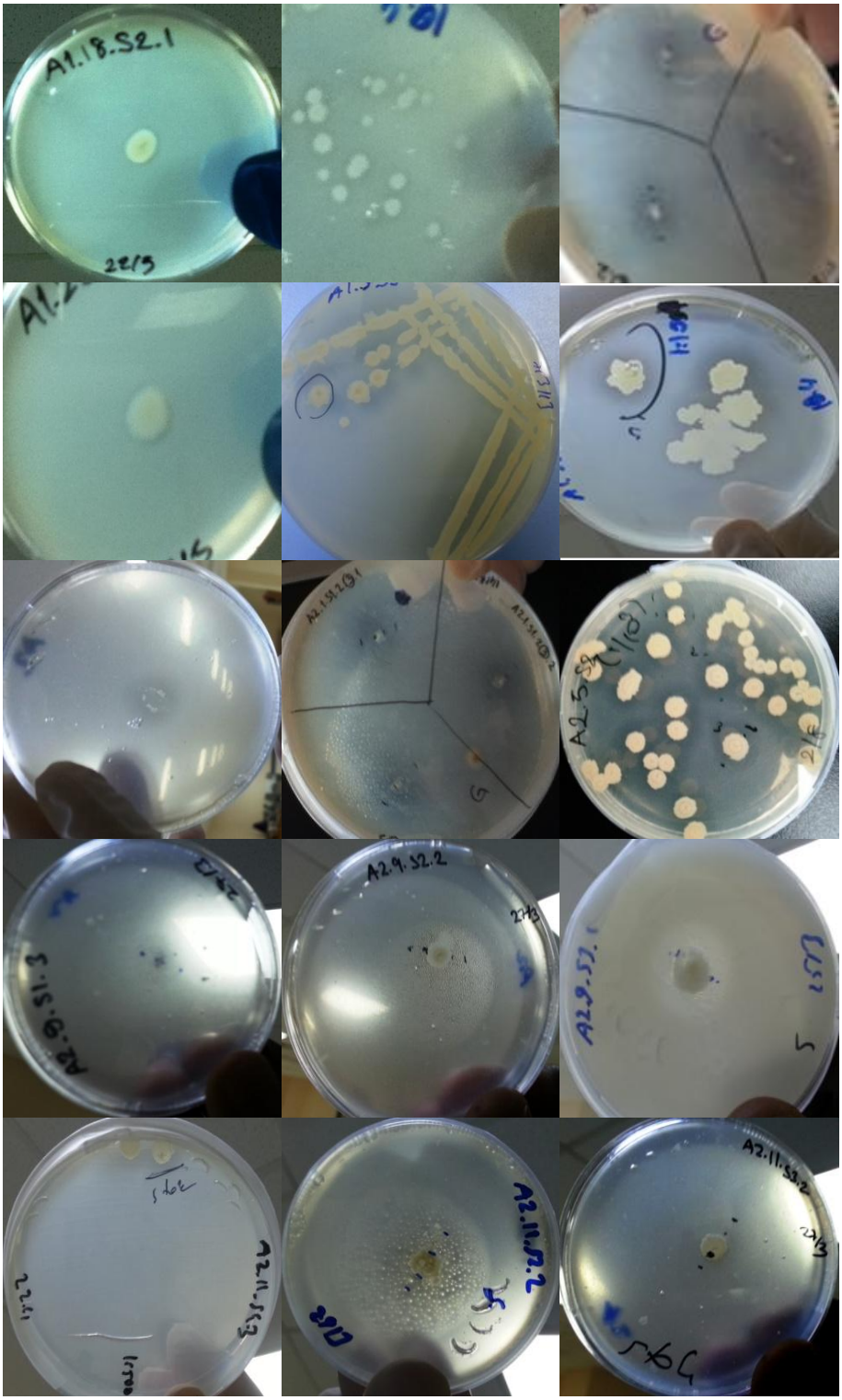

Figure 4.5 (cont.)

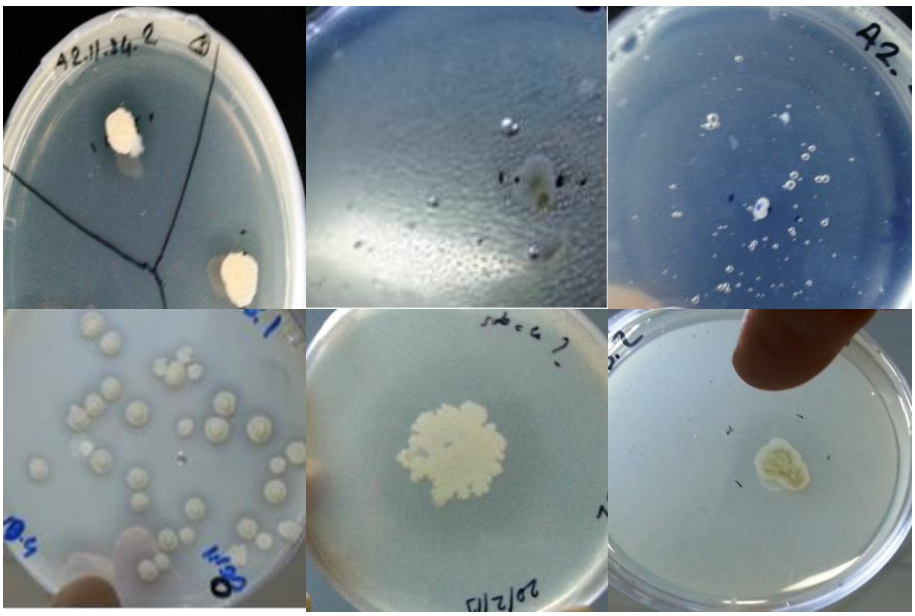

Figure 4.5 (cont.)

4.2 ENZYMATIC INDEXES OF ISOLATES

After observing enzyme activities in Petri plates, enzymatic indexes (EI) of isolates were calculated using diameter of zone of clearance and colony diameter. These enzymatic indexes for all enzymes are given in Table 4.5. According to the EI results, isolates having higher EI values have more enzyme activity and isolates with high EI values can be a good candidate for purification of enzymes. For laccase enzyme activity measurement, the color of bacteria colonies was examined. After one week incubation on nutrient agar containing guaiacol which is naturally occuring yellowish aromatic oil with the formula $C_6H_4(OH)(OCH_3)$, laccase producing bacteria turns into reddish brown colonies (Nishida, 1998). If the color of the colony is more intense, bacteria isolate has higher laccase activity.

Table 4.5 Enzymatic indexes of isolates.

Isolate No	Zone dia. (CZ_{dia})	Colony dia. (BC_{dia})	Enzymatic Index (CZ_{dia}/ BC_{dia})	Isolate No	Zone dia. (CZ_{dia})	Colony dia. (BC_{dia})	Enzymatic Index (CZ_{dia}/ BC_{dia})
XB.24.S1.3	16	4,6	3,50	XB.25.S2.2	13,5	6	2,33
M.1.S1.3	32	27	1,18	M.5.S3.1	17	9	1,89
M.1.S2.2	18	6	3,00	M.5.S4.1	27	11	2,45
M.1.S3.1	29	21	1,38	M.9.S1.1	6	3	2,00
M.2.S1.1	16	8	2,00	M.12.S1.2	19	6	3,16
M.3.S1.2	9,5	4	2,38	M.13.S1.1	22	11	2,00
M.4.S1.1	23	14	1,64	M.14.S1.3	11,5	6,5	1,76
M.4.S2.3	17	8	2,13	M.15.S1.3	10	18	1,80
M.4.S3.3	27	20	1,35	M.16.S1.1	17	24	1,41
M.4-2.S1.1	18	12	1,50	M.23.S2.2	25	8	3,13
M.5.S1.1	7	3	2,33	M.23.S1.2	25	15	1,67
M.5.S2.1	8	3	2,67	M.22.S2.3	18	6	3,00
L.7.S1.1	7	9,5	1,36	L.18.S5.3	23	14	1,64
L.13.S2.2	22	6	3,67	L.18.S6.3	18	11	1,63
L.14.S1.2	20	35	1,75	L.20.S1.3	4	1	4,00
L.14.S2.2	12	4	3,00	L.20.S2.1	16	9	1,78
L.14.S3.3	6	10	1,67	L.21.S1.1	12	9	1,33
L.15.S3.1	6	12	2,00	L.23.S3.1	25	21	1,19
L.15.S3.2.3	23	12	1,92	L.23.S5.3	15	12	1,25
L.16.S1.1	7	3	2.33	L.24.S2.2	15	22	1,47
L.16.S2.1	10	6	1,67	L.24.S3.2	20	16	1,29
L.16.S3	10	7	1,43	L.24.S5.1	15	10	1,50
L.16.S4.2	7	4	1,75	L.25.S2.2	2	23	1,05
L.17.S2	6,5	4	1,63	L.25.S3.2	3	7	2,33
L.18.S2.1	10	3	3,33	L.26.S5.2	11	4	2,75
L.18.S4.3	18	15,5	1,16	L.X.2.3	5	18,5	3,70

Table 4.5 (cont.)

A1.1.S1.1	5	14	2,80	A2.5.S2.1	11,5	5	2,30
A1.3.S1	16	14	1,14	A2.9.S1.3	9	2	4,50
A1.3.S2.1	27	13	2,08	A2.9.S2.2	13	6	2,17
A1.5.S3.1	12	7	1,72	A2.9.S3.1	10	8	1,25
A1.5-2.S2.2	21	17	1,23	A2.11.S1.3	3	9	3,00
A1.13.S2.3	17	14	1,21	A2.11.S2.2	14	6	2,33
A1.18.S2.1	11	9	1,22	A2.11.S3.2	12	5	2,40
A1.19.S1.3	6	4,5	1,33	A2.11.S4.2	11	8	1,38
A1.19.S2.2	3	13	4,33	A2.12.S2.1	16	9	1,78
A1.20.S2.2	8	6	1,33	A2.22.S1.3	11	2,5	4,40
A1.21.S3.1	8	5	1,60	A2.23.S2.1	10	8	1,12
A1.21.S5.3	6	2	3,00	A2.23.S3.1	13	3	4,34
A1.22.S2.2	8	2	4,00	A2.23.S4.2	8	4	2,00
A2.1.S1.3	3,5	10,5	3,00				

In total, 112 bacteria colonies were isolated. A distribution of these isolations according to enzymatic indexes and laccase (+) isolations was done with isolation sources (Table 4.6).

Table 4.6 Isolation sources and distribution of isolates according to enzymatic index values and laccase (+) isolate numbers.

Isolation Sources	Isolate numbers according to their enzymatic indexes				Laccase (+) isolates
	1-2	**2-3**	**3-4**	**4-5**	**#**
Karadeniz Farm field soil 1	2	1	2	1	-
Soil Under walnut tree 3 9	2	4	-	1	4
Vineyard soil 4	3	1	-	-	-
Ornamental plant soil 5	3	4	-	-	-

Table 4.6 (cont.)

Fatih University Campus soil 6 7 8	1	-	-	-	-
Soil under quince tree 10	-	-	-	-	3
Soil under oak tree 11	1	2	1	-	4
Soil under peach tree 12 16	5	1	1	-	8
Soil under plum tree 13	1	1	-	1	6
Soil under nut tree 14	3	-	1	-	5
Decomposed organic waste 15	2	1	-	-	3
Soil under Taxodium ascendens brogne	1	-	-	-	-
Soil under Populus alba L.(silver poplar)	4	-	3	-	-
Soil under Liquidambar styraciflua	1	-	-	-	-
Soil under Skimmia Japonica Thunb	2	-	-	1	-
Soil under Acca sellowiana burret (sweetsop)	2	-	1	-	-
Soil under Robinia pseudoaccia L. (top acacia)	-	-	1	2	-
Soil under Juniperus sabina L.	4	1	1	1	-
Soil from Büyükçekmece Lake	4	3	1	-	-
TOTAL	41	19	12	7	**33**

According to these results, %37 of 112 isolates form hydrolysis zones (clear zones) with enzymatic indexes between 1-2, %17 of isolates have enzymatic indexes between 2-3, %11 of isolates have enzymatic indexes between 3-4, and only %6 of isolates have enzymatic indexes between 4-5. The rest %29 of isolates showed laccase (+) activity on agar with color change to reddish brown.

According to the EI results, isolates showing alpha amylase, protease, laccase and lipase activities were chosen for molecular characterization with 16S rDNA sequencing method. For laccase, isolates were chosen according to their color indications. Isolates showing higher enzyme activities on Petri assay were picked for gDNA isolation to be used in 16S rDNA sequencing (Table 4.7).

Table 4.7 Enzymatic indexes of bacteria isolates chosen for molecular identification.

Isolate name	Enzymatic Index	Isolate name	Enzymatic Index
A2.22.S1.3	4,40	L.16.S1.1	2,33
XB.26.S2.2	3,5	L.26.S5.2	2,75
XB.24.S1.3	2,33	L.13.S2.2	3,67
M.22.S2.3	3,00	L.18.S2.1	3,33
M.3.S1.2	2,38	La.9.S2	-
M.5.S4.1	2,45	La.11.S4	-
M.12.S1.2	3,16	La.11.S3	-
M.1.S2.2	3,00	La.13.S5	-
		La.11.S5	-

4.3 GENOMIC DNA ISOLATION

According to EI values, isolates that have higher activity for each enzyme were chosen for gDNA isolation and were sent for 16S rDNA sequencing. gDNA isolations of 17 isolates chosen are listed in Table 4.8.

Table 4.8 gDNA isolations of selected isolated.

Isolate name	gDNA concentration (ng/ul)	Isolate name	gDNA concentration (ng/ul)
A2.22.S1.3	15	L.16.S1.1	19.97
XB.26.S2.2	10	L.26.S5.2	25.53
XB.24.S1.3	15.5	L.13.S2.2	52.9
M.22.S2.3	51.77	L.18.S2.1	20.5
M.3.S1.3	28.03	La.9.S2	108.57
M.5.S4.1	51.1	La.11.S4	32.33
M.12.S1.2	73.23	La.11.S3	11.2
M.1.S2.1	10.8	La.13.S5	18.9
		La.11.S5	90

4.4 IDENTIFICATION OF SOIL BACTERIA

4.4.1 Phenotypic Characterization

Phenotypic characterization of 112 bacteria isolates were done with gram staining according to their bacterial cell wall properties. Gram reactions (positive or negative) and colony shape (morphology) were observed under microscope. The results are given in Table 4.9.

Table 4.9 Gram reaction results of bacteria isolates.

Isolate no	Morphology	Gram reaction	Isolate no	Morphology	Gram reaction
XB.24.S1.3	rod	positive	XB.26.S2.2	rod	positive
M.1.S1.3	rod	positive	M.5.S3.1	rod	positive
M.1.S2.2	rod	positive	M.5.S4.1	rod	positive
M.1.S3.1	rod	positive	M.9.S1.1	cocci	positive
M.2.S1.1	rod	positive	M.12.S1.2	rod	positive
M.3.S1.2	rod	positive	M.13.S1.1	rod	positive
M.4.S1.1	rod	positive	M.14.S1.3	rod	positive
M.4.S2.3	rod	positive	M.15.S1.3	rod	positive
M.4.S3.3	rod	positive	M.16.S1.1	rod	positive
M.4-2.S1.1	rod	positive	M.23.S2.2	rod	positive
M.5.S1.1	rod	positive	M.23.S1.2	rod	positive
M.5.S2.1	rod	positive	M.22.S2.3	rod	positive
La.9.S1	rod	positive	La.13.S1	rod	positive
La.9.S2	rod	positive	La.13.S2	rod	positive
La.9.S3	rod	positive	La.13.S3	rod	positive
La.9.S4	rod	positive	La.13.S4	rod	positive
La.10.S1	rod	positive	La.13.S5	rod	positive
La.10.S2	rod	positive	La.13.S6	rod	positive
La.10.S3	rod	positive	La.14.S1	rod	positive
La.11.S1	rod	positive	La.14.S2	rod	positive

Table 4.9 (cont.)

La.11.S3	rod	positive	La.14.S3	rod	positive
La.11.S4	rod	positive	La.14.S4	rod	positive
La.11.S5	rod	positive	La.14.S5	rod	positive
La.12.S1	rod	positive	La.15.S1	rod	positive
La.12.S2	rod	positive	La.15.S2	rod	positive
La.12.S3	rod	positive	La.15.S5	rod	positive
La.12.S4	rod	negative	La.16.S1	rod	positive
La.12.S5	rod	positive	La.16.S2	rod	positive
La.12.S6	rod	positive			
L.7.S1.1	rod	negative	L.18.S5.3	rod	negative
L.13.S2.2	rod	positive	L.18.S6.3	rod	positive
L.14.S1.2	long rod	positive	L.20.S1.3	rod	positive
L.14.S2.2	cocci	positive	L.20.S2.1	rod	negative
L.14.S3.3	rod	positive	L.21.S1.1	rod	positive
L.15.S3.1	rod	positive	L.23.S3.1	rod	negative
L.15.S3.2.3	rod	negative	L.23.S5.3	rod	positive
L.16.S1.1	rod	negative	L.24.S2.2	rod	negative
L.16.S2.1	rod	positive	L.24.S3.2	rod	positive
L.16.S3	rod	positive	L.24.S5.1	rod	positive
L.16.S4.2	rod	negative	L.26.S2.2	rod	positive
L.17.S2	rod	negative	L.26.S3.2	rod	negative
L.18.S2.1	cocci	positive	L.26.S5.2	rod	positive
L.18.S4.3	rod	positive	L.X.2.3	cocci	positive
A1.1.S1.1	rod	positive	A2.5.S2.1	rod	positive
A1.3.S1	rod	positive	A2.9.S1.3	rod	negative
A1.3.S2.1	rod	positive	A2.9.S2.2	rod	negative
A1.5.S3.1	rod	positive	A2.9.S3.1	rod	negative
A1.5-2.S2.2	rod	positive	A2.11.S1.3	rod	positive
A1.13.S2.3	rod	positive	A2.11.S2.2	rod	positive
A1.18.S2.1	rod	positive	A2.11.S3.2	rod	positive
A1.19.S1.3	rod	positive	A2.11.S4.2	rod	positive
A1.19.S2.2	rod	positive	A2.12.S2.1	rod	positive

Table 4.9 (cont.)

A1.20.S2.2	rod	positive	A2.22.S1.3	rod	positive
A1.21.S3.1	rod	positive	A2.23.S2.1	rod	positive
A1.21.S5.3	rod	negative	A2.23.S3.1	rod	negative
A1.22.S2.2	branched rod	positive	A2.23.S4.2	rod	positive
A2.1.S1.3	rod	negative			

Microscope images of xylanase (+), protease (+), laccase (+), lipase (+) and alpha amylase (+) isolates after gram staining are given in Figure 4.6, 4.7, 4.8, 4.9 and 4.10.

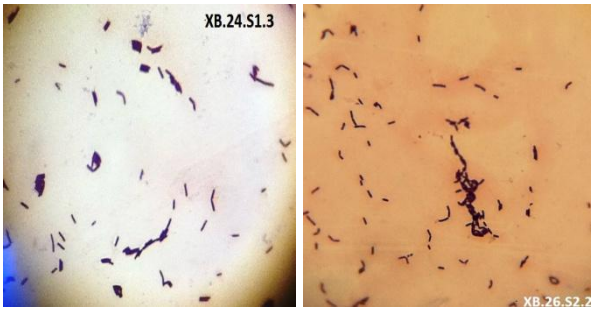

Figure 4.6 Microscope images of xylanase (+) isolates after gram staining (XB.24.S1.3, XB.25.S2.2).

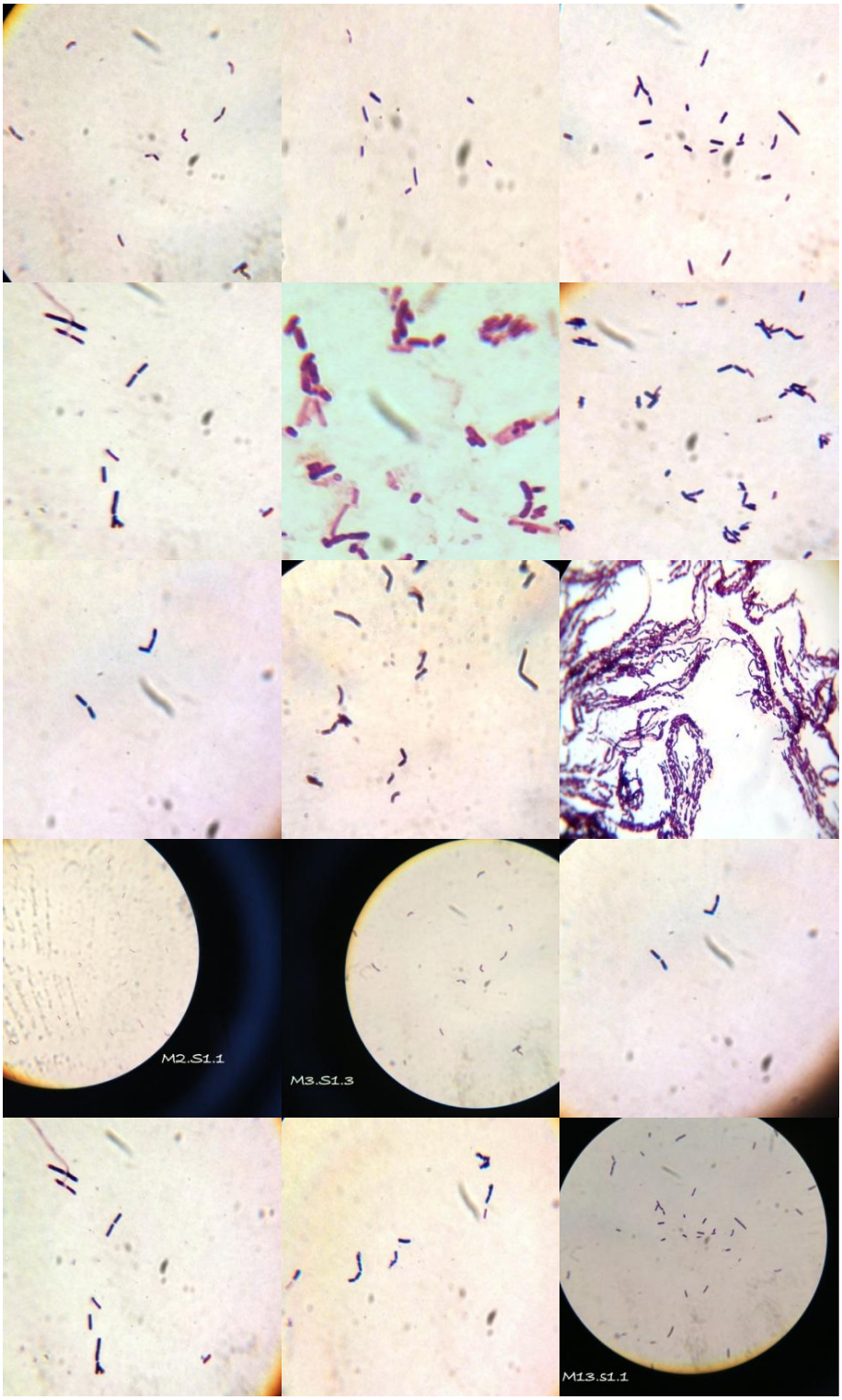

Figure 4.7 Microscope image of protease (+) isolates after gram staining (left to right: M.3.S1.2, M.22.S2.3, M.13.S1.1, M.5.S3.1, M.14.S1.3, M.16.S1.1, M.5.S1.1, M.5.S4.1, M.1.S2.2, M.2.S1.1, M.5.S2.1, M.15.S1.3, M.23.S2.2, M.23.S1.2, M.12.S1.2, M.1.S1.3, M.1.S3.1, M.4.S1.1, M.4.S2.3, M.4.S3.3, M.4-2.S1.1, M.9.S1.1).

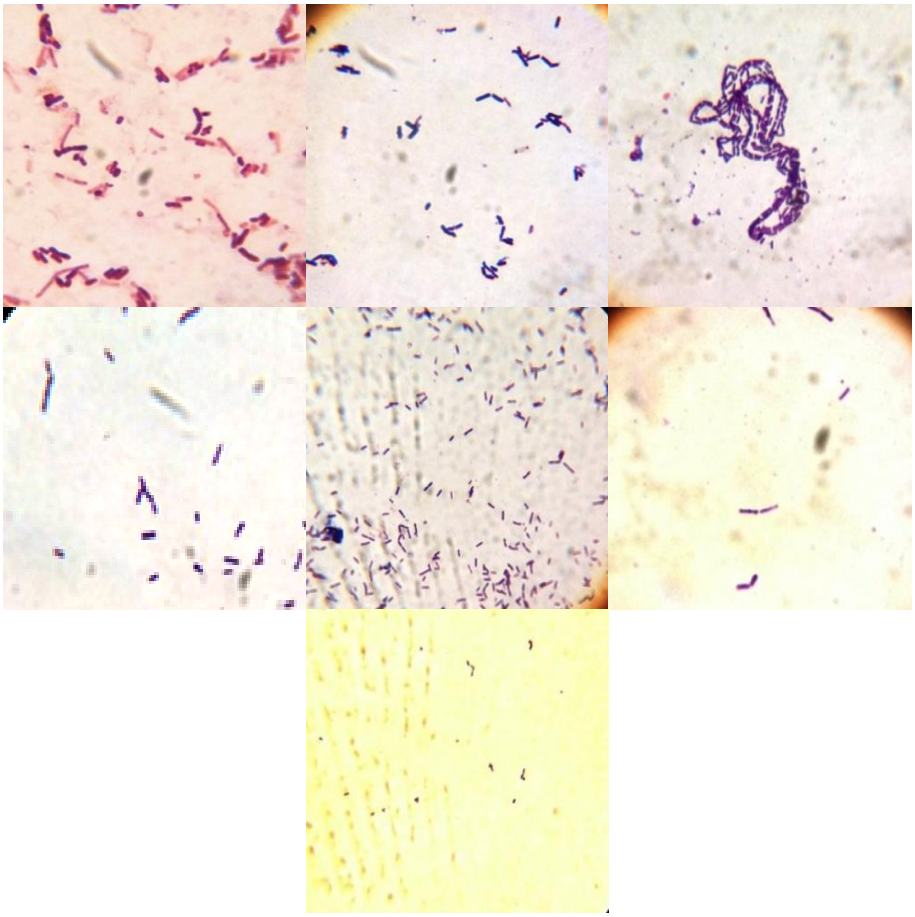

Figure 4.7 (cont.)

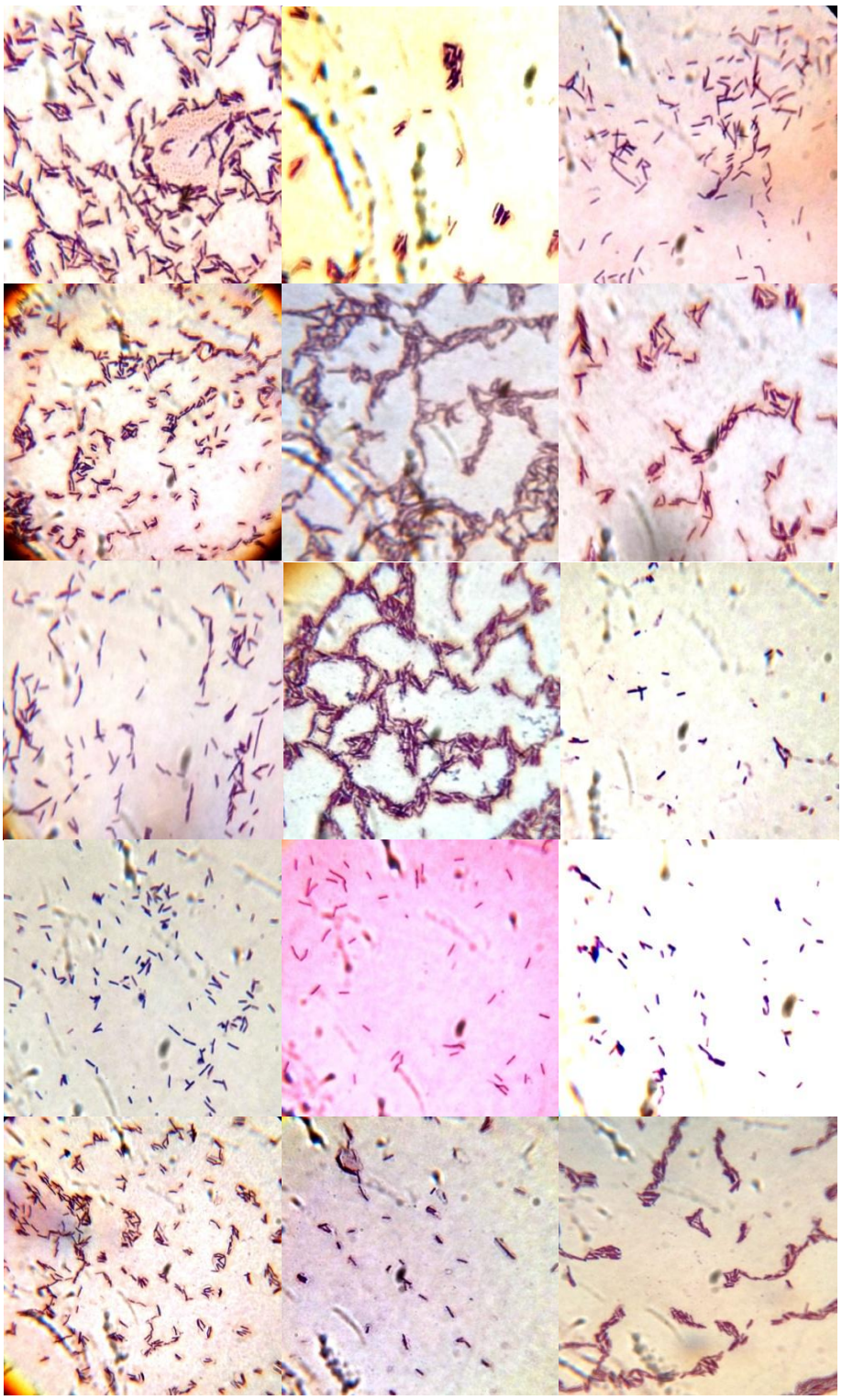

Figure 4.8 Microscope image of laccase (+) isolates after gram staining (left to right: La.9.S1, La.9.S2, La.9.S3, La.9.S4, La.10.S1, La.10.S2, La.10.S3, La.11.S1, La.11.S2, La.11.S3, La.11.S4, La.11.S5, La.12.S2, La.12.S3, La.12.S5, La.12.S6, La.13.S1, La.13.S2, La.13.S3, La.13.S5, La.14.S1, La.14.S3, La.14.S4, La.14.S5, La.15.S2, La.15.S5,La.16.S1, La.16.S2, La.12.S1, La.13.S6, La.14.S2, La.15.S1, La.15.S2, La.12.S4).

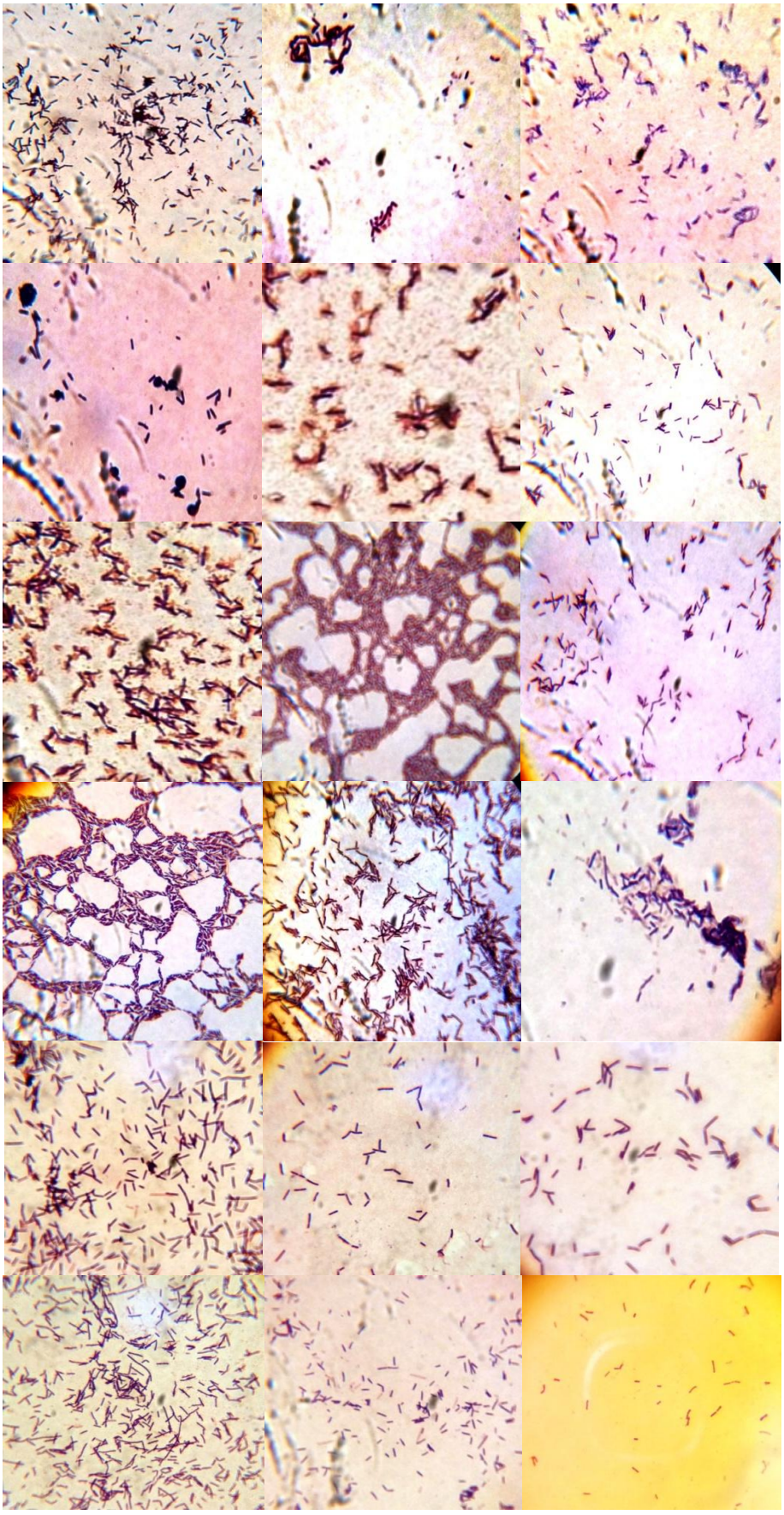

Figure 4.8 (cont.)

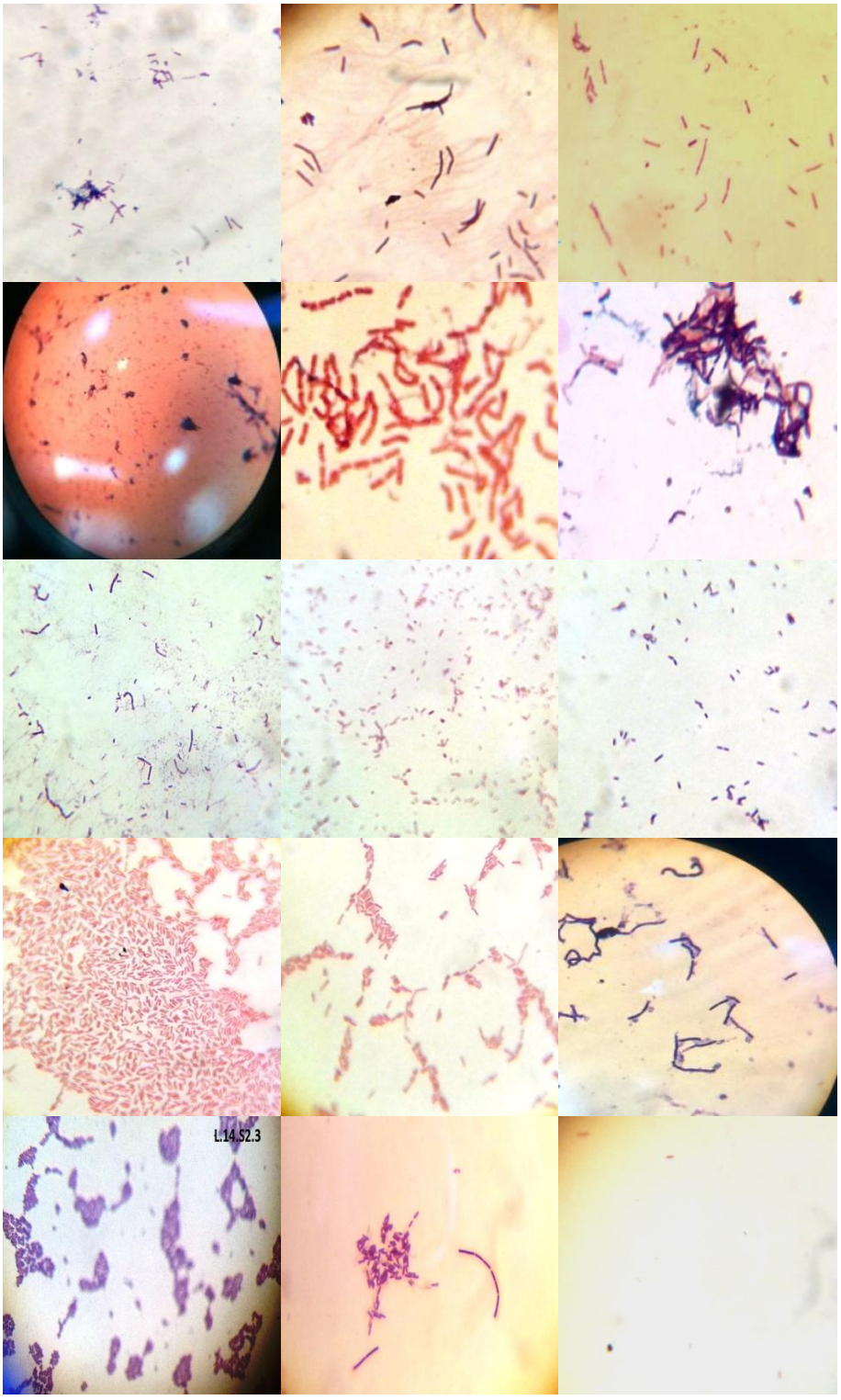

Figure 4.9 Microscope image of lipase (+) isolates after gram staining (left to right: L.21.S1.1, L.18.S2.1, L.16.S1.1, L.18.S4.3, L.20.S2.1, L.16.S2.1, L.20.S1.3, L.16.S4.2, L.18.S6.3, L.7.S1.1, L.18.S5.3, L.24.S5.1; L.14.S2.2, L.15.S3.1, L.15.S3.2.3, L.24.S3.2, L.26.S5.2, L.X.2.3, L.14.S1.2, L.23.S3.1, L.26.S2.2, L.14.S3.3, L.16.S3, L.17.S2, L.26.S3.2, L.23.S5.3, L.13.S2.2, L.24.S2.2).

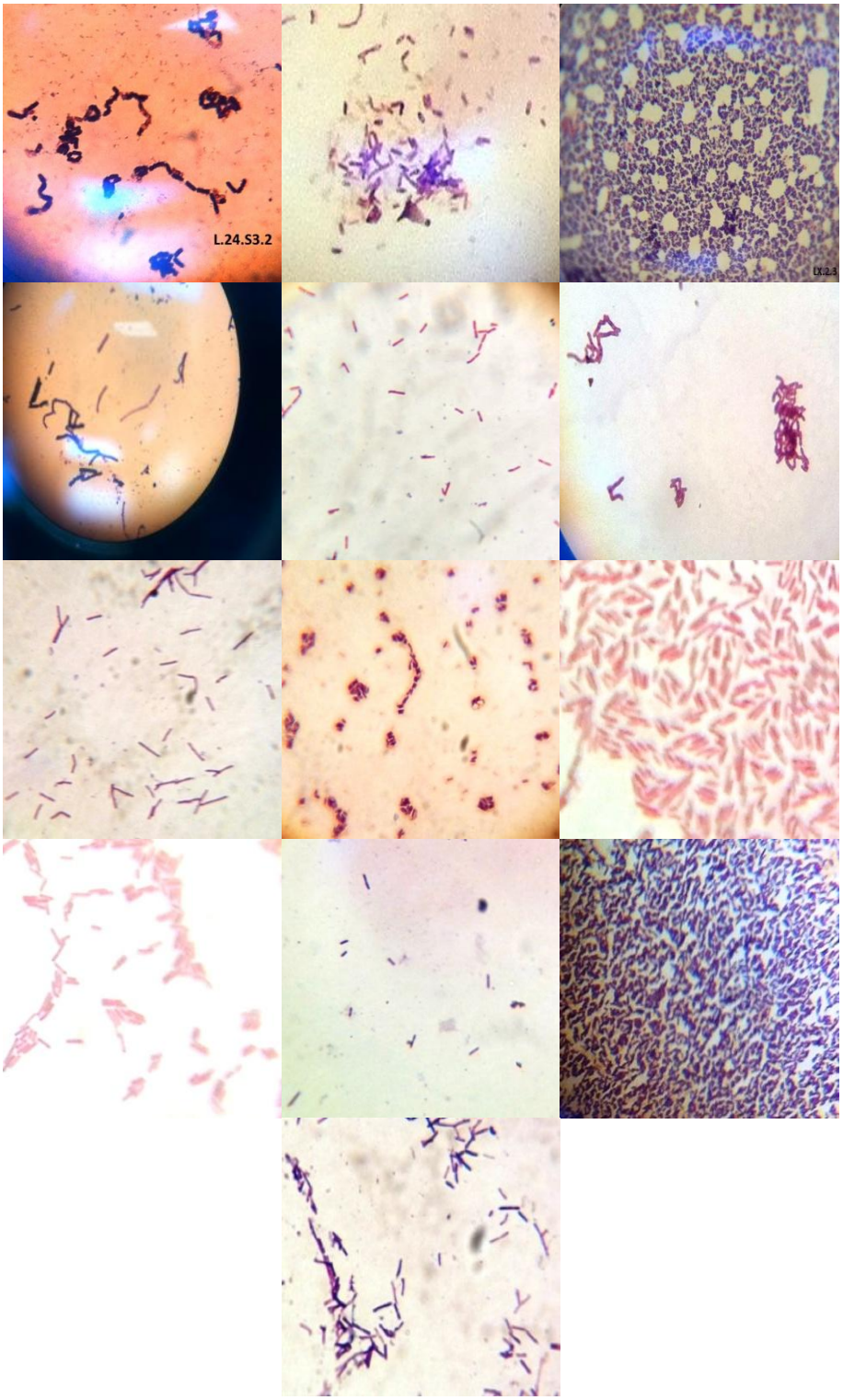

Figure 4.9 (cont.)

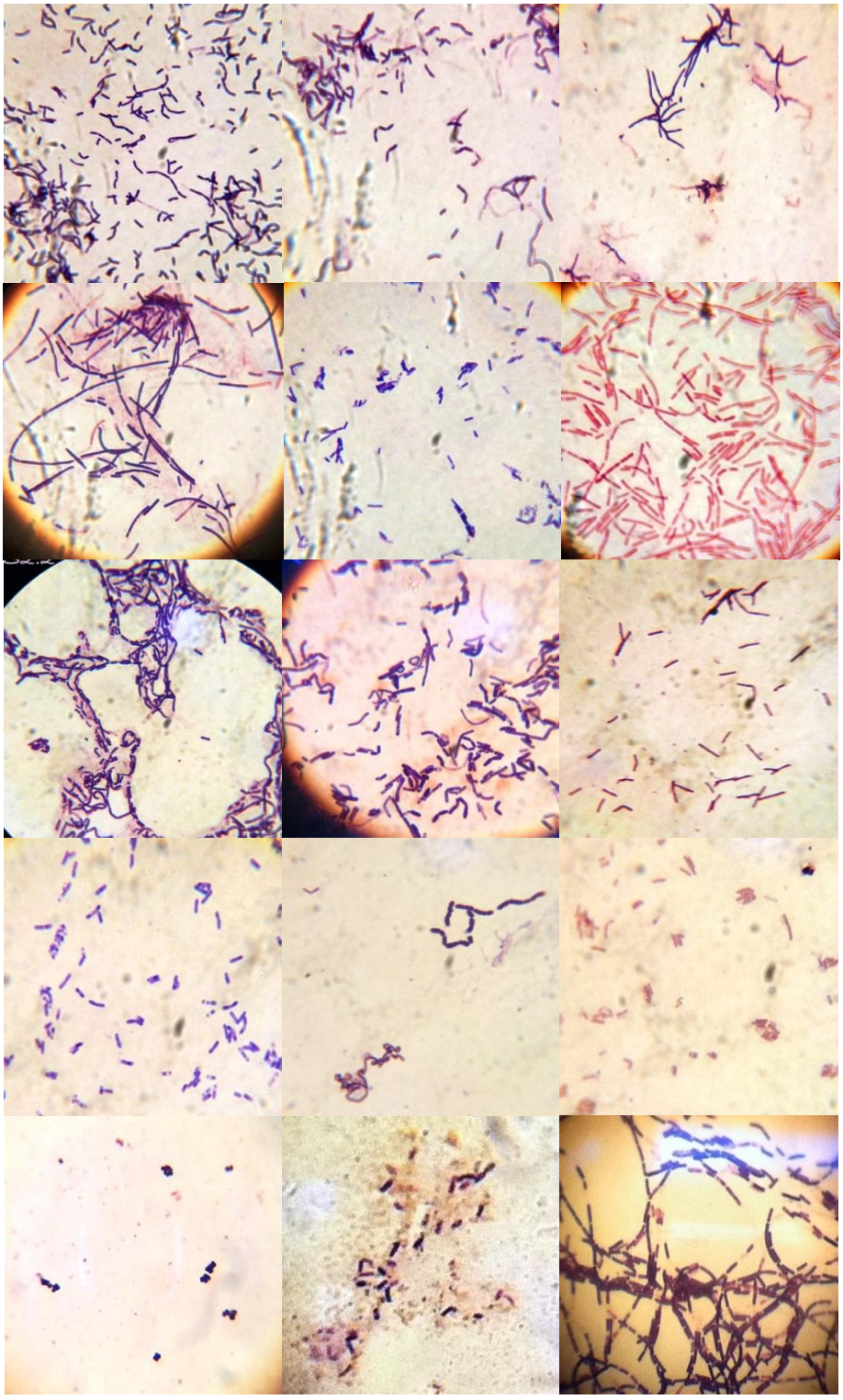

Figure 4.10 Microscope image of alpha amylase (+) isolates after gram staining (left to right: A1.1.S1.1, A1.3.S2.1, A1.22.S2.2, A2.22.S1.3, A1.13.S2.3, A2.1.S1.3, A1.5.S3.1, A1.18.S2.1, A2.11.S2.2, A2.23.S4.2, A1.21.S3.1, A2.9.S3.1, A2.9.S2.2, A1.3.S1, A2.11.S1.3, A2.5.S2.1, A1.21.S5.3, A2.11.S3.2, A1.5-2.S2.2, A1.19.S1.3, A1.19.S2.2, A1.20.S2.2, A2.9.S1.3, A2.11.S4.2, A2.12.S2.1, A2.23.S2.1, A2.23.S3.1).

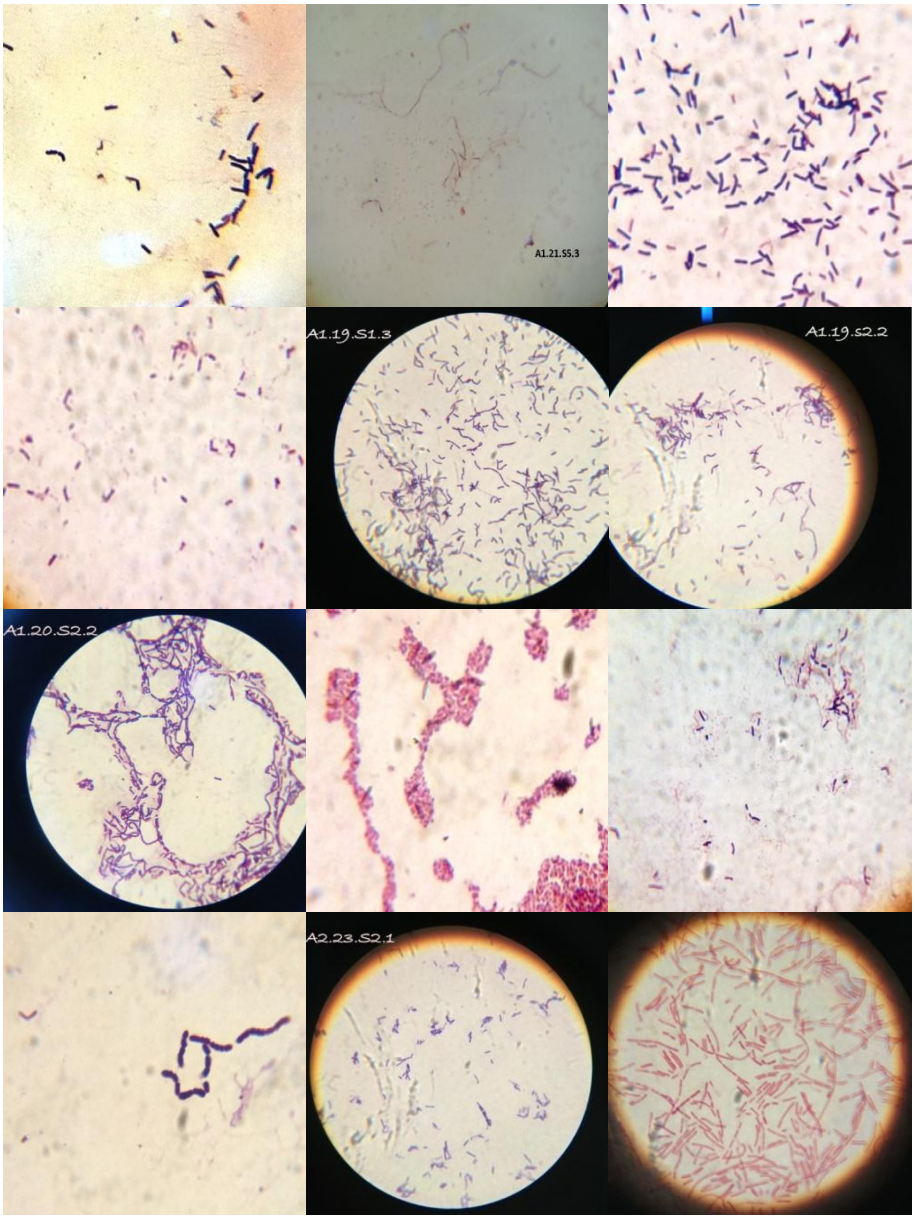

Figure 4.10 (cont.)

As soil bacteria are known to be mostly in bacilli form which is rod shaped and in gram positive character, most of the isolates were in rod shaped and showed Gram positive reaction after staining and observation under microscope. From the distribution of gram reactions of 112 isolates given in Table 4.10, it is seen that %85 of the isolates were in gram positive character and %97 of isolates were in rod shape.

Table 4.10 Distribution of gram reactions of 112 isolates.

		Number of isolates	
Sample name	**Colony morphology**	**Gram (+)**	**Gram (-)**
Protease (+)	Rod (21), cocci (1)	22	-
Alpha amylase (+)	Rod (26), branched rod (1)	21	6
Laccase (+)	Rod (33)	32	1
Lipase (+)	Rod (26), cocci (2)	18	10
Xylanase (+)	Rod (2)	2	-

4.4.2 Genotypic Characterization

Genotypic characterization was done with 16S rDNA sequencing of bacteria isolates and according to sequencing results molecular identification of isolates were done with bioinformatics analysis. After identification, it was seen most of the isolated bacteria belongs to *Bacillus* genus. Bacillus strains are commonly found in soil environment and *Bacillus* strains have been widely used in enzyme production for decades since these bacteria are able to overproduce many enzymes including industrially important enzymes (Soenshein, 1993). So, *Bacillus* isolates are used in this thesis for purification of industrially important enzymes. Identified isolates are listed in Table 4.11.

Table 4.11 Identified bacteria isolates.

Isolate	Bacteria species
A2.22.S1.3	Bacillus sp. MN3-10
L.18.S2.1	Lysinibacillus sp.
L.26.S5.2	Bacillus licheniformis
L.16.S1.1	Paenibacillus sp. B17a
L.13.S2.2	Bacterium BS72
La.11.S4	Bacillus pumilus
La.12.S5	Pseudomonas aeruginosa
La.13.S5	Bacillus pumilus
La.11.S5	Bacillus subtilis
M.22.S2.3	Bacillus pumilus
M.3.S1.3	Bacillus sp. CNE 8
M.5.S4.1	Bacillus sp. SAP02_1
M.12.S1.2	Pseudomonas aeruginosa
XB.26.S2.2	Bacillus sp. LS05
XB.24.S1.3	Bacillus subtilis
M.1.S2.2	Bacillus mojavensis
La.9.S2	Bacillus pumilus

4.5 CRUDE ENZYME ACTIVITY ASSAYS

4.5.1 Xylanase Assay

Xylanase supernatant and cell free crude enzyme activities of 17 isolates are given in Table 4.12 and Table 4.13.

Table 4.12 Xylanase activities in supernatant crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	99 ± 1,47
L.16.S1.1	Paenibacillus sp. B17a	0
L.26.S5.2	Bacillus licheniformis	0
L.13.S2.2	Bacterium BS72	0
L.18.S2.1	Lysinibacillus sp.	0
La.11.S4	Bacillus pumilus	0
La.11.S3	Pseudomonas aeruginosa	25± 4,12
La.13.S5	Bacillus pumilus	0
La.11.S5	Bacillus subtilis	100 ± 1,18
M.22.S2.3	Bacillus pumilus	0
M.3.S1.3	Bacillus sp. CNE 8	0
M.5.S4.1	Bacillus sp. SAP02_1	0
M.12.S1.2	Pseudomonas aeruginosa	0
XB.26.S2.2	Bacillus sp. LS05	13 ± 0,59
XB.24.S1.3	Bacillus subtilis	99 ± 1,47
M.1.S2.2	Bacillus mojavensis	13 ± 7,68
La.9.S2	Bacillus pumilus	17 ± 5,60

Table 4.13 Xylanase activities in cell free crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	17 ± 2
L.16.S1.1	Paenibacillus sp. B17a	31 ± 20
L.26.S5.2	Bacillus licheniformis	69 ± 6
L.13.S2.2	Bacterium BS72	100 ± 16
L.18.S2.1	Lysinibacillus sp.	99 ± 2
La.11.S4	Bacillus pumilus	36 ± 4
La.11.S3	Pseudomonas aeruginosa	76 ± 2
La.13.S5	Bacillus pumilus	75 ± 18
La.11.S5	Bacillus subtilis	24 ± 10
M.22.S2.3	Bacillus pumilus	10 ± 14
M.3.S1.3	Bacillus sp. CNE 8	75 ± 16
M.5.S4.1	Bacillus sp. SAP02_1	0
M.12.S1.2	Pseudomonas aeruginosa	99 ± 24
XB.26.S2.2	Bacillus sp. LS05	85 ± 12
XB.24.S1.3	Bacillus subtilis	36 ± 10
M.1.S2.2	Bacillus mojavensis	18 ± 4
La.9.S2	Bacillus pumilus	60 ± 27

Highest supernatant xylanase activity was measured at La.11.S5 (Bacillus subtilis - %100 relative activity), A2.22.S1.3 (Bacillus sp. MN3-10 - %99 relative activity) and XB.24.S1.3 (Bacillus subtilis - %99 relative activity) isolates.

Highest cell free xylanase activity was measured at L.13.S2.2 (Bacterium BS72 - %100 relative activity), L.18.S2.1 (Lysinibacillus sp. - %99 relative activity) and M.12.S1.2 (Pseudomonas aeruginosa - %99 relative activity) isolates.

Supernatant extract of La.11.S5 isolate (Bacillus subtilis) that has %100 relative enzyme activity when compared with other 16 isolates was chosen for xylanase purification. Industrial xylanase enzyme from Bacillus subtilis were obtained in previous studies (Jalal, 2009) that can be used in pulp and paper industry. Purified xylanase enzyme from our Bacillus subtilis (La.11.S5) isolate can be a canditate for usage in pulp and paper industry as well. In further studies, the relative activity of this La.11.S5 isolate can be compared with commercial xylanase enzymes to see the differences between our protease's activities with industrially important xylanases.

4.5.2 Protease Assay

Protease supernatant and cell free crude enzyme activities of 17 isolates are given in Table 4.14 and Table 4.15.

Table 4.14 Protease activities in supernatant crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	16 ± 3
L.16.S1.1	Paenibacillus sp. B17a	0
L.26.S5.2	Bacillus licheniformis	0
L.13.S2.2	Bacterium BS72	6 ± 2,9
L.18.S2.1	Lysinibacillus sp.	0
La.11.S4	Bacillus pumilus	11 ± 5,1
La.11.S3	Pseudomonas aeruginosa	0
La.13.S5	Bacillus pumilus	0
La.11.S5	Bacillus subtilis	4 ± 0,3
M.22.S2.3	Bacillus pumilus	100 ± 3,8
M.3.S1.3	Bacillus sp. CNE 8	0
M.5.S4.1	Bacillus sp. SAP02_1	1 ± 0,8
M.12.S1.2	Pseudomonas aeruginosa	0
XB.26.S2.2	Bacillus sp. LS05	12 ± 7
XB.24.S1.3	Bacillus subtilis	3 ± 4,7
M.1.S2.2	Bacillus mojavensis	0
La.9.S2	Bacillus pumilus	4 ± 5,2

Table 4.15 Protease activities in cell free crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	0
L.16.S1.1	Paenibacillus sp. B17a	8 ± 8,9
L.26.S5.2	Bacillus licheniformis	0
L.13.S2.2	Bacterium BS72	0
L.18.S2.1	Lysinibacillus sp.	0
La.11.S4	Bacillus pumilus	50 ± 0,1
La.11.S3	Pseudomonas aeruginosa	16 ± 0,6
La.13.S5	Bacillus pumilus	1 ± 1,6
La.11.S5	Bacillus subtilis	83 ± 2,1
M.22.S2.3	Bacillus pumilus	87 ± 3,4
M.3.S1.3	Bacillus sp. CNE 8	89 ± 6,7
M.5.S4.1	Bacillus sp. SAP02_1	100 ± 3,3
M.12.S1.2	Pseudomonas aeruginosa	0
XB.26.S2.2	Bacillus sp. LS05	6 ± 0,3
XB.24.S1.3	Bacillus subtilis	0
M.1.S2.2	Bacillus mojavensis	0
La.9.S2	Bacillus pumilus	0

Highest supernatant protease activity was measured at M.22.S2.3 (Bacillus pumilus - %100 relative activity) isolate. Highest cell free protease activity was measured at M.5.S4.1 (Bacillus sp. - %100 relative activity) isolate. In previous studies, protease from Bacillus sp. isolates was obtained (Kim, 2000). In this thesis, cell free extract of Bacillus sp. SAP02_1 (M.5.S4.1) isolate was chosen for protease purification

since it has % 100 relative enzyme activities when compared with other 16 identified isolates. In further studies, the relative activity of this M.5.S4.1 isolate can be compared with commercial protease enzymes to see the differences between our protease's activities with industrially important proteases.

4.5.3 Laccase Assay

Laccase supernatant and cell free crude enzyme activities of 17 isolates are given in Table 4.16 and Table 4.17.

Table 4.16 Laccase activities in supernatant crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	0
L.16.S1.1	Paenibacillus sp. B17a	0
L.26.S5.2	Bacillus licheniformis	0
L.13.S2.2	Bacterium BS72	0
L.18.S2.1	Lysinibacillus sp.	0
La.11.S4	Bacillus pumilus	0
La.11.S3	Pseudomonas aeruginosa	0
La.13.S5	Bacillus pumilus	0
La.11.S5	Bacillus subtilis	0
M.22.S2.3	Bacillus pumilus	0
M.3.S1.3	Bacillus sp. CNE 8	0
M.5.S4.1	Bacillus sp. SAP02_1	0
M.12.S1.2	Pseudomonas aeruginosa	0
XB.26.S2.2	Bacillus sp. LS05	0
XB.24.S1.3	Bacillus subtilis	0
M.1.S2.2	Bacillus mojavensis	0
La.9.S2	Bacillus pumilus	0
Laccase (commercial)	Trametes versicolor	100 ± 0

Table 4.17: Laccase activities in cell free crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	21 ± 3
L.16.S1.1	Paenibacillus sp. B17a	7 ± 3
L.26.S5.2	Bacillus licheniformis	40 ± 3
L.13.S2.2	Bacterium BS72	100 ± 0
L.18.S2.1	Lysinibacillus sp.	0
La.11.S4	Bacillus pumilus	14 ± 0
La.11.S3	Pseudomonas aeruginosa	14 ± 0
La.13.S5	Bacillus pumilus	10 ± 0
La.11.S5	Bacillus subtilis	21 ± 3
M.22.S2.3	Bacillus pumilus	31 ± 3
M.3.S1.3	Bacillus sp. CNE 8	21 ± 3
M.5.S4.1	Bacillus sp. SAP02_1	19 ± 7
M.12.S1.2	Pseudomonas aeruginosa	52 ± 0
XB.26.S2.2	Bacillus sp. LS05	21 ± 3
XB.24.S1.3	Bacillus subtilis	31 ± 3
M.1.S2.2	Bacillus mojavensis	19 ± 0
La.9.S2	Bacillus pumilus	50 ± 10
Laccase (commercial)	Trametes versicolor	100 ± 7

No laccase activity was found in supernatant enzyme extracts, so it was concluded that our laccase enzyme is not an extracellular enzyme.

Highest cell free laccase activity was measured at L.13.S2.2 (*Bacterium BS72* - %100 relative activity) isolate. As a control, activity of commercial laccase enzyme from *Trametes versicolor* was measured and 17 isolates' % activities were calculated according to commercial laccase relatively. According to these results, L.13.S2.2 cell free extract's relative enzyme activity was same as 1 unit of commercial laccase's relative activity. So, it can be concluded that our laccase enzyme can be as valuable as commercial laccase enzymes that are used in the industry. With further studies and improvements on characterization and purification of this laccase enzyme from cell free extract of L.13.S2.2 isolate, a novel enzyme with high enzyme activity can be produced and used in industry for different purposes.

4.5.4 Lipase Assay

Lipase supernatant and cell free crude enzyme activities of 17 isolates are given in Table 4.18 and Table 4.19.

Table 4.18 Lipase activities in supernatant crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	0
L.16.S1.1	Paenibacillus sp. B17a	74 ± 1,5
L.26.S5.2	Bacillus licheniformis	0
L.13.S2.2	Bacterium BS72	0
L.18.S2.1	Lysinibacillus sp.	25± 1,0
La.11.S4	Bacillus pumilus	0
La.11.S3	Pseudomonas aeruginosa	0
La.13.S5	Bacillus pumilus	0
La.11.S5	Bacillus subtilis	4± 1,9
M.22.S2.3	Bacillus pumilus	49± 5,8
M.3.S1.3	Bacillus sp. CNE 8	0
M.5.S4.1	Bacillus sp. SAP02_1	0
M.12.S1.2	Pseudomonas aeruginosa	0
XB.26.S2.2	Bacillus sp. LS05	75± 9,7
XB.24.S1.3	Bacillus subtilis	100± 15,5
M.1.S2.2	Bacillus mojavensis	0
La.9.S2	Bacillus pumilus	0
Lipase (commercial)	Thermomyces lanuginosus	42± 4,8

Table 4.19 Lipase activities in cell free crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	0
L.16.S1.1	Paenibacillus sp. B17a	0
L.26.S5.2	Bacillus licheniformis	0
L.13.S2.2	Bacterium BS72	100 ± 35
L.18.S2.1	Lysinibacillus sp.	0
La.11.S4	Bacillus pumilus	0
La.11.S3	Pseudomonas aeruginosa	0
La.13.S5	Bacillus pumilus	0
La.11.S5	Bacillus subtilis	0
M.22.S2.3	Bacillus pumilus	0
M.3.S1.3	Bacillus sp. CNE 8	0
M.5.S4.1	Bacillus sp. SAP02_1	0
M.12.S1.2	Pseudomonas aeruginosa	0
XB.26.S2.2	Bacillus sp. LS05	0
XB.24.S1.3	Bacillus subtilis	0
M.1.S2.2	Bacillus mojavensis	0
La.9.S2	Bacillus pumilus	38 ± 18
Lipase (commercial)	Trametes versicolor	113 ± 18

In supernatant enzyme extracts, highest lipase relative activity was found at XB.24.S1.3 (*Bacillus subtilis* - %100 relative activity) isolate. Relative activity of

commercial *Thermomyces lanuginosus* lipase (Sigma- L0777) was found lower than our XB.24.S1.3 supernatant enzyme extract, which is 42%.

In cell free enzyme extracts, highest lipase relative activity was measured at L.13.S2.2 (*Bacterium BS72* - %100 relative activity) isolate. Relative activity of commercial *Thermomyces lanuginosus* lipase (Sigma- L0777) was measured as 113%, which is closer to our L.13.S2.2 cell free enzyme extract. There was no laccase gene purified from Bacterium BS72 in the literature, so cell free extract of L.13.S2.2 (Bacterium BS72) isolate that has closer enzymatic activity relatively to commercial lipase was chosen for purification of laccase enzyme.

4.5.5 Alpha-amylase Assay

Alpha-amylase supernatant and cell free crude enzyme activities of 17 isolates are given in Table 4.20 and Table 4.21.

Table 4.20 Alpha-amylase activities in supernatant crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	7 ± 1,2
L.16.S1.1	Paenibacillus sp. B17a	0
L.26.S5.2	Bacillus licheniformis	4 ± 2,4
L.13.S2.2	Bacterium BS72	5 ± 3,6
L.18.S2.1	Lysinibacillus sp.	0
La.11.S4	Bacillus pumilus	0
La.11.S3	Pseudomonas aeruginosa	0
La.13.S5	Bacillus pumilus	25 ± 4,8
La.11.S5	Bacillus subtilis	6 ± 1,2
M.22.S2.3	Bacillus pumilus	6 ± 1,2
M.3.S1.3	Bacillus sp. CNE 8	0
M.5.S4.1	Bacillus sp. SAP02_1	19 ± 3,6
M.12.S1.2	Pseudomonas aeruginosa	3 ± 3,6
XB.26.S2.2	Bacillus sp. LS05	5 ± 2,4
XB.24.S1.3	Bacillus subtilis	0
M.1.S2.2	Bacillus mojavensis	3 ± 2,4
La.9.S2	Bacillus pumilus	0
Alpha amylase (commercial)	Bacillus licheniformis	100 ± 4,8

Table 4.21 Alpha amylase activities in cell free crude enzyme extracts.

Isolate name	Bacteria species	%Rel. Activity
A2.22.S1.3	Bacillus sp. MN3-10	0
L.16.S1.1	Paenibacillus sp. B17a	0
L.26.S5.2	Bacillus licheniformis	0
L.13.S2.2	Bacterium BS72	0
L.18.S2.1	Lysinibacillus sp.	0
La.11.S4	Bacillus pumilus	2 ± 3,1
La.11.S3	Pseudomonas aeruginosa	0
La.13.S5	Bacillus pumilus	0
La.11.S5	Bacillus subtilis	0
M.22.S2.3	Bacillus pumilus	9 ± 0
M.3.S1.3	Bacillus sp. CNE 8	0
M.5.S4.1	Bacillus sp. SAP02_1	0
M.12.S1.2	Pseudomonas aeruginosa	10 ± 0
XB.26.S2.2	Bacillus sp. LS05	0
XB.24.S1.3	Bacillus subtilis	0
M.1.S2.2	Bacillus mojavensis	0
La.9.S2	Bacillus pumilus	0
Alpha amylase (commercial)	Trametes versicolor	100 ± 4,8

In supernatant enzyme extracts, highest alpha amylase relative activities was measured at La.13.S5 (*Bacillus pumilus* - % 25 relative activity), M.5.S4.1 (Bacillus sp. SAP02_1 - % 19 relative activity), A2.22.S1.3 (*Bacillus sp. MN3-10* - % 7 relative

activity) and M.22.S2.3 (*Bacillus pumilus* - % 6 relative activity) isolates relative to commercial *Bacillus licheniformis* alpha amylase (Sigma- A4862) activity (100 % relative activity).

In cell free enzyme extracts, highest alpha amylase relative activity was measured at M.12.S1.2 (*Pseudomonas aeruginosa* - 10 % relative activity) and M.22.S2.3 (*Bacillus pumilus* - 9 % relative activity) isolates relative to commercial *Bacillus licheniformis* alpha amylase (Sigma- A4862) activity (100 % relative activity).

Supernatant extract of M.22.S2.3 (*Bacillus pumilus*) isolate was chosen for alpha amylase purification that has %6 relative activity when compared with commercial alpha amylase. Other isolates that have shown higher enzyme activities lost their activity after purification steps, so another isolate that has high enzyme activity as well was chosen for purification of alpha amylase enzyme. Chosen isolate is Bacillus pumilus bacteria and there are not much studies on production of alpha amylase from Bacillus pumilus. There is just one putative alpha amylase gene obtained from Bacillus pumilus (Gioai, 2007) in NCBI database. So our alpha amylase enzyme that will be purified from Bacillus pumilus, characterized and improved with further studies can be a novel enzyme.

4.6 PARTIAL PURIFICATION AND CHARACTERIZATION OF PROTEINS

4.6.1 Enzyme Activity Assays After Partial Purification

Ammonium sulfate precipitations (%40, %60, %80, %100) were done for partial purification of xylanase, protease, laccase, lipase and alpha amylase enzymes. After each fraction, enzyme activities were measured and relatively compared to starter supernatant activity including cell free or supernatant extract which is used for enzyme precipitation. After enzymatic activity measurements of precipitation fractions, the fraction that preserves its enzyme activity with higher % relative activity value was chosen for dialysis experiments. After dialysis, partially purified enzymes were used for characterization experiments.

For xylanase enzyme from La.11.S5 supernatant extract, highest xylanase activity was measured at %100 ammonium sulfate precipitation fraction (Table 4.22).

Table 4.22 Xylanase activity after ammonium sulfate precipitation.

Isolate name	Species	% Rel. Activity
La.11.S5	Bacillus subtilis (starter süpernatant)	100
La.11.S5	Bacillus subtilis (%80 precipitation)	7
La.11.S5	Bacillus subtilis (%100 precipitation)	28

For protease enzyme from M.5.S4.1 cell free extract, highest protease activity was measured at %60 ammonium sulfate precipitation fraction (Table 4.23).

Table 4.23 Protease activity after %60 ammonium sulfate precipitation.

Isolate name	Species	% Rel. Activity
M.5.S4.1	Bacillus subtilis (starter süpernatant)	100
M.5.S4.1	Bacillus subtilis (%60 precipitation)	4
M.5.S4.1	Bacillus subtilis (%80 precipitation)	1

For laccase and lipase enzyme from L.13.S2.2 cell free extract, highest enzyme activity was measured at %60 ammonium sulfate precipitation fraction (Table 4.24).

Table 4.24 Laccase and lipase activity after ammonium sulfate precipitation.

		Lipase	Laccase
		% Rel. activity	% Rel. activity
L.13.S2.2	Starter supernatant	100 ± 0	100 ± 0
L.13.S2.2	%40 precipitation	25 ± 0	25 ± 12
L.13.S2.2	%60 precipitation	94 ± 27	33 ± 0
L.13.S2.2	%80 precipitation	13 ± 0	0
L.13.S2.2	%100 precipitation	19 ± 9	0
L.13.S2.2	Last supernatant	0	0

For alpha amylase enzyme from M.22.S2.3 cell free extract, highest alpha amylase activity was measured at %80 ammonium sulfate precipitation fraction (Table 4.24).

For alpha amylase enzyme from M.22.S2.3 supernatant extract, highest enzyme activity was measured at %80 ammonium sulfate precipitation fraction (Table 4.25).

Table 4.25 Alpha amylase activity after %80 ammonium sulfate precipitation.

Isolate name	Species	% Rel. Activity
M.22.S2.3	Bacillus pumilus (starter supernatant)	100
M.22.S2.3	Bacillus pumilus (%80 precipitation)	96,4

After partial purification of enzymes with ammonium sulfate precipitation, purified enzymes were used for determination of pH and temperature effects on enzyme activities.

4.6.2 Effect of pH on Enzyme Activity

4.6.2.1. Effect of pH on Xylanase Activity

Effect of pH on xylanase activity was measured according to xylanase activity measurements using buffers with different pH values (2, 3, 4, 5, 6, 7, 8, 9, 10). pH activity graph was created based on the activity results and optimum pH value was determined (Figure 4.11).

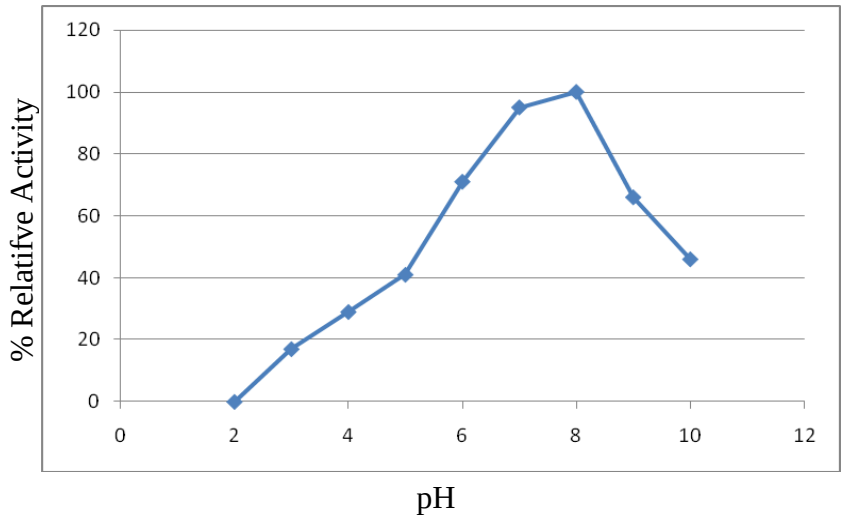

Figure 4.11 pH profile of xylanase activity.

According to pH activity results, our xylanase enzyme partially purified from La.11.S5 (*Bacillus subtilis)* isolate has shown activity in pH 3-10 range. At pH 8, maximum enzyme activity was calculated when compared with other pH values. After pH 8, the relative xylanase activity decreased up to % 46 relative activities at pH 10. So we can conclude that our xylanase enzyme can resist high pH values, further experiments can be done to see the effect of higher pH values on xylanase activity.

4.6.2.2. Effect of pH on Protease Activity

Effect of pH on protease activity was measured according to protease activity results using buffers with different pH values (2, 3, 4, 5, 6, 7, 8, 9, 10). pH activity

graph was created based on the activity results and optimum pH value was determined (Figure 4.12).

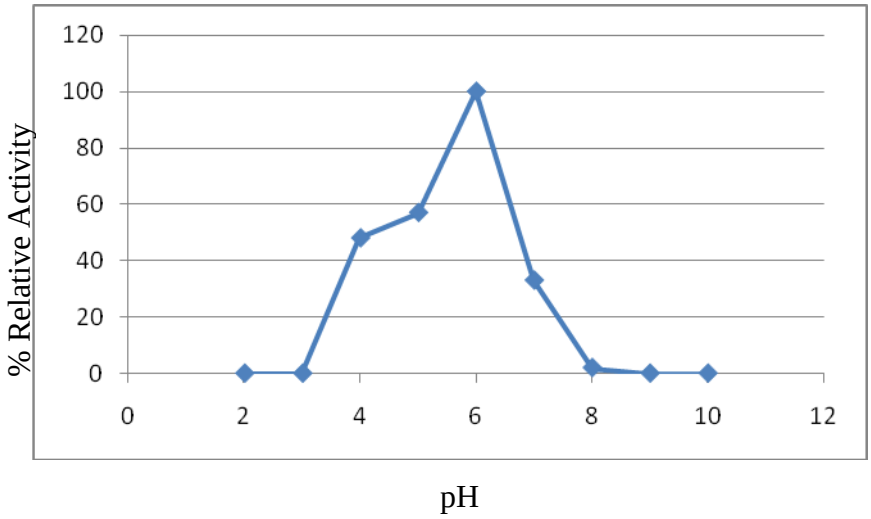

Figure 4.12 pH profile of protease activity.

Protease enzyme partially purified from M.5.S4.1 (Bacillus sp.) isolate has shown activity between pH 4-8. The enzyme lost its activity at pH 8. Optimum enzyme activity was at pH 6.

4.6.2.3. Effect of pH on Laccase Activity

Effect of pH on laccase activity was measured according to laccase activity results using buffers with different pH values (2, 3, 4, 5, 8, 9, 10). pH activity graph was created based on the activity results and optimum pH value was determined (Figure 4.13). Highest laccase activity was found at pH 8.

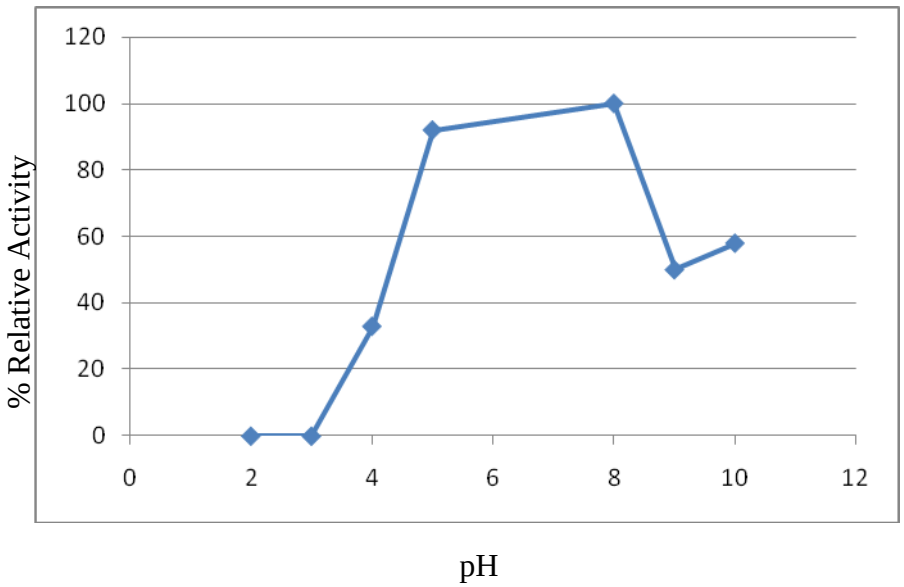

Figure 4.13 pH profile of laccase activity.

Laccase enzyme partially purified from L.13.S2.2 (Bacterium BS72) isolate has shown activity between pH 4-10. The enzyme was not active at pH 3 and at even pH 10, enzyme did not lost all the activity. Although optimum pH for enzyme activity is 8, our enzyme can resist higher pH environments. Further experiments can be done to search more basic pH effects on enzyme activity.

4.6.2.4. Effect of pH on Lipase Activity

Effect of pH on lipase activity was measured according to lipase activity results using buffers with different pH values (3, 4, 5, 6, 7, 8, 9, 10). pH activity graph was created based on the activity results and optimum pH value was determined (Figure 4.14).

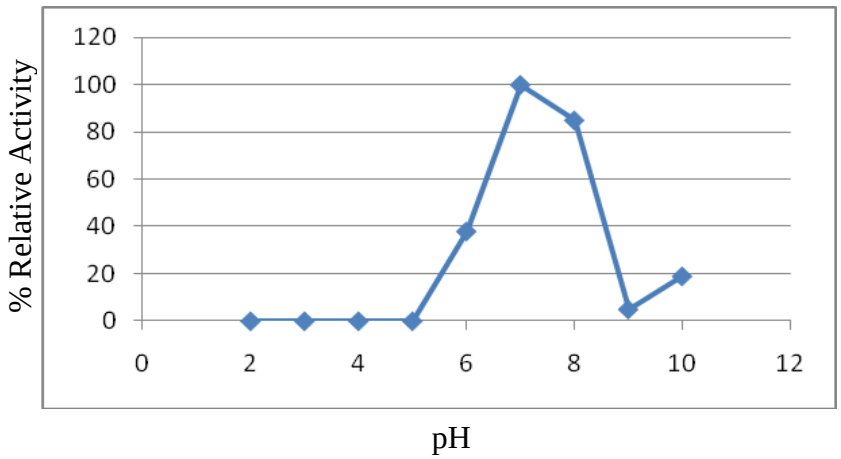

Figure 4.14 pH profile of lipase activity.

Lipase enzyme partially purified from L.13.S2.2 (Bacterium BS72) isolate did not show activity at pH2, pH 3, pH 4 and pH 5. Maximum enzyme relative activity was found at pH 7. While the enzyme activity is nearly lost at pH 9, there was very little relative enzyme activity at pH 10.

4.6.2.5. Effect of pH on Alpha-amylase Activity

Effect of pH on alpha amylase activity was measured according to alpha amylase activity results using buffers with different pH values (3, 4, 5, 6, 7, 8, 9). pH activity graph was created based on the activity results and optimum pH value was determined (Figure 4.15).

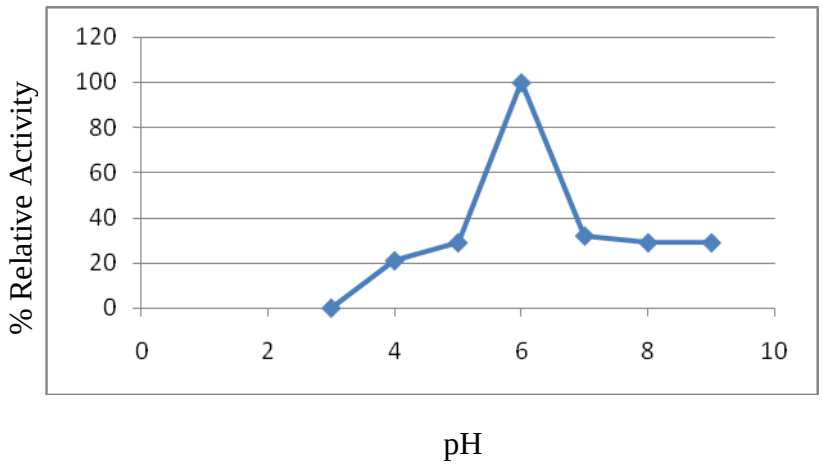

Figure 4.15 pH profile of alpha amylase activity.

Alpha amylase enzyme partially purified from M.22.S2.3 (*Bacillus pumilus*) isolate showed activity between pH 3-pH 9. Highest alpha amylase activity was found at pH 6. Enzyme activity was not lost at pH 9, there was very little enzymatic activity at pH 9.

4.6.3 Effect of Temperature on Enzyme Activity

4.6.3.1. Effect of temperature on Xylanase Activity

Effect of temperature on xylanase activity was measured according to xylanase activity results at different temperatures (25^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C, 80 ^{o}C). Temperature activity graph was created based on the activity results and optimum temperature was determined (Figure 4.16). Highest xylanase activity was found at 50 ^{o}C. Xylanase enzyme activity was lost after 60 ^{o}C.

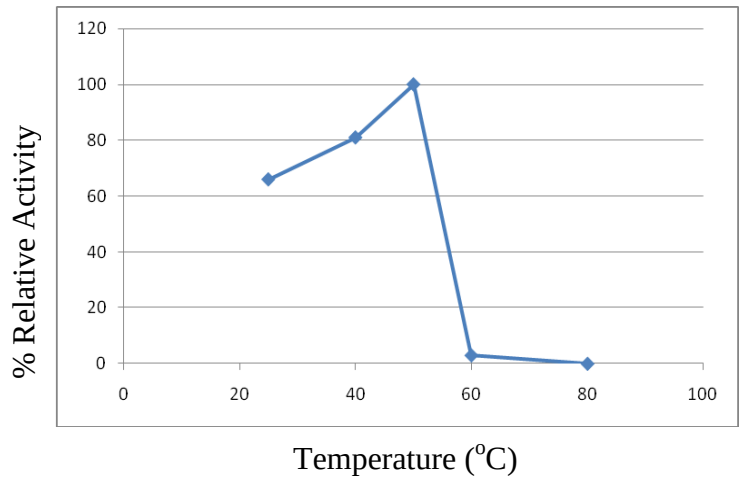

Figure 4.16 Temperature profile of xylanase activity.

4.6.3.2. Effect of temperature on Protease Activity

Effect of temperature on protease activity was measured according to protease activity results at different temperatures (0°C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C). Temperature activity graph was created based on the activity results and optimum temperature was determined (Figure 4.17). Highest protease activity was found at 25 °C. Protease enzyme activity started after 15 °C and lost after 35 °C.

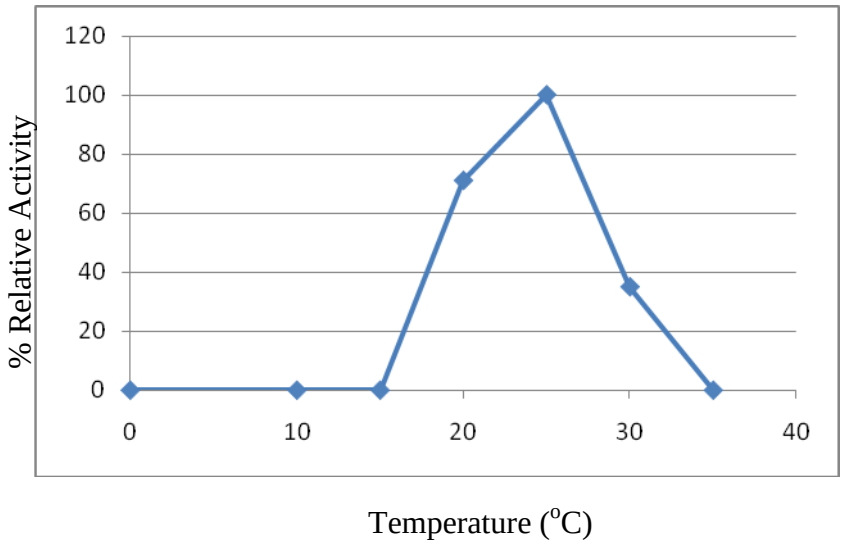

Figure 4.17 Temperature profile of protease activity.

4.6.3.3. Effect of temperature on Laccase Activity

Effect of temperature on laccase activity was measured according to laccase activity results at different temperatures (30°C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90°C). Temperature activity graph was created based on the activity results and optimum temperature was determined (Figure 4.18). Highest laccase activity was found at 50 °C. Laccase enzyme activity was lost at 90 °C.

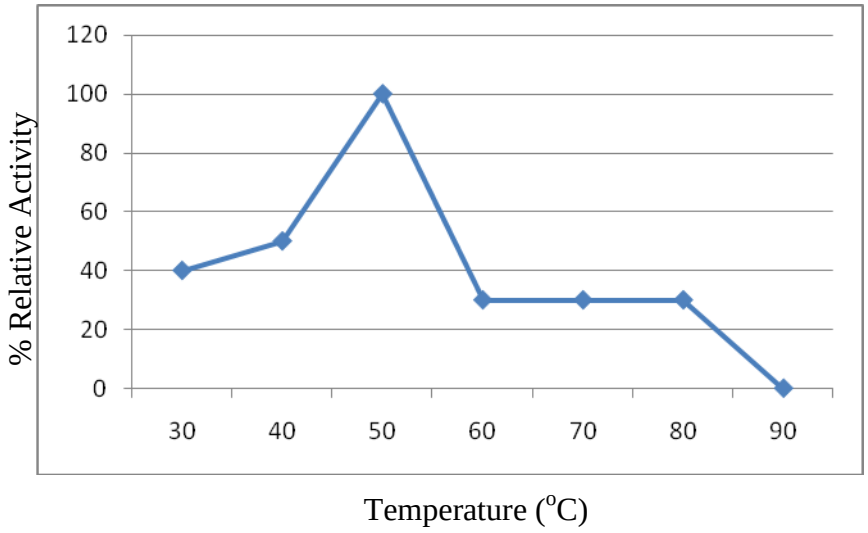

Figure 4.18 Temperature profile of laccase activity.

4.6.3.4. Effect of temperature on Lipase Activity

Effect of temperature on lipase activity was measured according to lipase activity results at different temperatures (30°C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90°C). Temperature activity graph was created based on the activity results and optimum temperature was determined (Figure 4.19). Highest lipase activity was found at 80 °C. Lipase enzyme activity was lost at 90 °C.

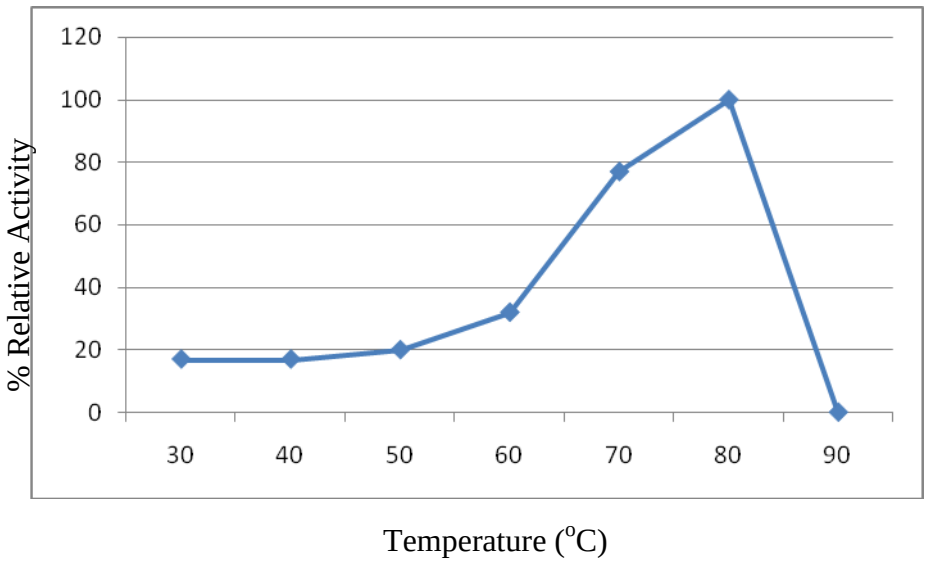

Figure 4.19 Temperature profile of lipase activity.

4.6.3.5. Effect of temperature on Alpha-amylase Activity

Effect of temperature on alpha amylase activity was measured according to alpha amylase activity results at different temperatures (30°C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90°C). Temperature activity graph was created based on the activity results and optimum temperature was determined (Figure 4.20). Highest alpha amylase activity was found at 50 °C. Alpha amylase enzyme activity started after 40 °C and lost at 90 °C.

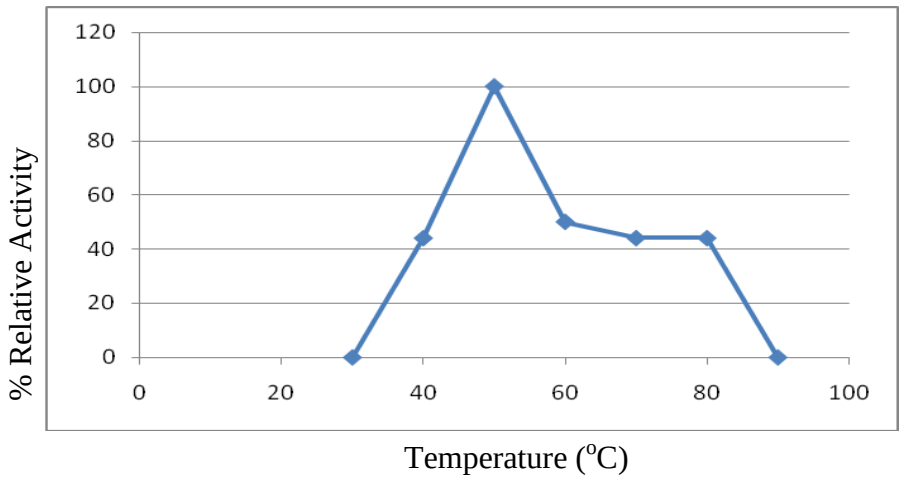

Figure 4.20 Temperature profile of alpha amylase activity.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVE

Soil has a wide range of microorganisms such as fungi and bacteria. Among these different microorganisms, bacteria are the most abundant and predominant organisms inhabiting in the soil. These soil bacteria can be capable of producing enzymes which are important for the industry. In this study, 112 soil bacteria isolates that are capable of producing industrially important enzymes were isolated from different soil environments of Turkey.

Among these isolated bacteria colonies, ones showing higher enzymatic activity on agar were chosen and 17 bacteria isolates were identified by 16S rDNA sequencing. The majority of industrial enzymes with bacterial origin are produced from *Bacillus* species and bacteria species belong to the genus *Bacillus* can be isolated from soil diversely. So in this study, after identification of 17 isolates it was seen that majority of our soil bacteria isolates belonged to *Bacillus* genus, and there were also 2 soil bacteria isolates belonged to *Pseudomonas aeruginosa* species. *Bacillus* genus belongs to *Bacillaceae* family which is Gram-positive, rod shaped (bacilli), spore forming aerobes or facultative anaerobes. Morphologically, soil bacteria are divided into three groups in which Bacilli with rod shape are most numerous following Cocci with round shape and Spirilla with spiral shape. In this study, majority of 112 bacteria isolates were shown to be gram positive and rod shaped. Many researches are made about the isolation of bacteria from different soil environments and production of industrial enzymes (Banerjee, 1999; Mehrotra, 1999; Singh, 2000; Johnvesly, 2001). After identifying bacteria isolates, supernatant and cell free extracts were taken and enzyme activity of these extracts were measured. According to these activities, candidate

isolates were chosen for partial purification of enzymes (Table 5.1). After purification of these enzymes, pH and temperature effects on enzyme activities were investigated. pH and temperature properties are very important for survival and growth of bacteria in different environments. Enzyme activity is also affected by these parameters. On the basis of pH requirements, bacteria is classified into three groups named as acidophilic, alkaliphilic and neutrophilic in which bacteria can live in acidic pH, basic pH and neutral pH environments. On the basis of temperature requirements, bacteria are classified into three major groups including psychrophilic, mesophilic and thermophilic bacteria which can live in psychrophil (-15 to 10 ^{o}C), mesophil (20-45 ^{o}C) and thermophil (45 ^{o}C and more) environments. In this study, acidophilic, alkaliphilic, neutrophilic, mesophilic and thermophilic bacteria were characterized since soil bacteria from different environments can have all these types of bacteria inside. In addition, extremophile enzymes can be isolated from soil with moderate conditions. For example, lipase enzyme produced from *Bacterium BS72* bacteria isolated from farm soil in a village of Bursa is found to be highly thermophilic enzyme that can resist high temperatures up to 80 ^{o}C.

Table 5.1 Bacteria isolates used for partial purification of enzymes.

Enzyme	Bacteria isolate
Xylanase	La.11.S5 *(Bacillus subtilis)*
Protease	M.5.S4.1 *(Bacillus sp.)*
Laccase	L.13.S2.2 *(Bacterium BS72)*
Lipase	L.13.S2.2 *(Bacterium BS72)*
Alpha amylase	M.22.S2.3 *(Bacillus pumilus)*

Enzymatic activity varies with respect to the bacterial species since xylanase, protease, laccase, lipase and alpha amylase enzymes with different activities in different pH and temperature conditions were purified from different bacteria species.

As a result of this study, after characterization of partially purified enzymes, thermo tolerant enzymes were discovered like lipase enzyme from L.13.S2.2

(Bacterium BS72) isolate showed maximum activity at 80^{o}C. Thermostable enzymes are very important in enzyme industry since most of the enzymes used in different industry sectors are resistant to high temperatures (Demirijan, 2001). So lipase characterized in this thesis has an important role in enzyme industry and can be used in industrial enzyme production with further studies. Also with further studies, thermostable property of this lipase can be improved and pH, temperature stabilities can be tested and improved as well. In addition, this thermostable lipase gene can be isolated from the bacteria and used for cloning studies, so that lipase enzyme can be produced more quickly and with higher amounts.

When we compare lipase characterized in this thesis with other lipases that are used in industry, it is seen that our lipase can resist higher temperature (Table 5.2). This feature makes our lipase enzyme more valuable. On the other hand, our lipase enzyme has lower optimum pH when compared with other lipases, but our lipase enzyme has also activity at pH 8 and pH 10. So according to this information, we can conclude that our lipase can be a good candidate for industrial lipase production after making further researches. Another important property of lipase characterized in this thesis is that there is no lipase gene discovered from *Bacterium BS72* species in the literature. So lipase partially purified and characterized from *Bacterium BS72* can be a novel lipase enzyme.

Table 5.2 Comparison of lipase enzyme of *Bacterium BS72* with other lipases.

Enzyme	Organism	Optimum pH	Optimum temperature (^{o}C)	Reference	Application area
Lipase	*Bacterium BS72*	7	80		
EC 3.1.1.3 - triacylglycerol lipase	*Bacillus sp.*	8	50	Sharma, 2002	Detergent industry
EC 3.1.1.3 - triacylglycerol lipase	*Lysinibacillus sphaericus*	8	15	Joseph, 2008	Biofuel production, Food industry
EC 3.1.1.3 - triacylglycerol lipase	*Bacillus sp.*	9,5	60	Sharma, 2001	Enantiomeric components synthesis

For protease enzyme partially purified from *Bacillus sp.* in this thesis, comparison with other proteases was done (Table 5.3). It was seen form the comparison that our protease enzyme has lower optimum pH and temperature values. In contrary to our protease enzyme, some researchers were able to purify thermo tolerant proteases (Srimathi, 2006). Since our protease loses its activity at pH 8 and 35 ^{o}C, further studies need to be done to increase its activity at higher pH and temperature values.

Table 5.3 Comparison of protease enzyme of *Bacillus sp.* with other proteases.

Enzyme	Organism	Optimum pH	Optimum temperature (^{o}C)	Reference	Application
Protease	*Bacillus sp.*	6	25		
EC 3.4.21.105 rhomboid protease	*Bacillus subtilis*	7.5	25 37	Urban, 2005; Strisovsky, 2009	Intramembrane proteolysis
EC 3.4.21.62 subtilisin	*Bacillus subtilis*	10	45	Rai, 2010	Detergent industry
EC 3.4.21.62 subtilisin	*Bacillus licheniformis*	10.5	67	Srimathi, 2006	Industry, covalent coupling to oxidized sucrose polymers
EC 3.4.21.62 subtilisin	*Bacillus sp.*	10	40	Kim, 2000	Medicine, as thrombolytic agent

For xylanase partially purified from La.11.S5 (*Bacillus subtilis*), comparison with other xylanases used in the industry was done (Table 5.4). When we compare our xylanase with other xylanase obtained from *Bacillus subtilis* (Jalal et al. 2009), our xylanase enzyme has higher optimum pH value. There are also xylanase enzymes that have optimum activity at pH 8 and can be used in pulp and paper industry. Among these studies, xylanase enzymes that have optimum activity at 70 ^{o}C (Shrinivas, 2010) and 40 ^{o}C (Wang, 2010). Our xylanase enzyme that acts at optimum 50 ^{o}C can also be a good candidate to use in pulp and paper industry.

Table 5.4 Comparison of xylanase enzyme of *Bacillus subtilis* with other xylanases.

Enzyme	Organism	Optimum pH	Optimum temperature (^{o}C)	Reference	Application
Xylanase	*Bacillus subtilis*	8	50		
EC 3.2.1.8 - endo-1,4-beta-xylanase	*Bacillus subtilis*	6	50	Jalal, 2009	Pulp and paper industry
EC 3.2.1.8 - endo-1,4-beta-xylanase	*Bacillus sp.*	8	40	Wang, 2010	Pulp and paper industry
EC 3.2.1.8 - endo-1,4-beta-xylanase	*Bacillus sp.*	8	70	Shrinivas, 2010	Pulp and paper industry
EC 3.2.1.8 - endo-1,4-beta-xylanase	*Bacillus licheniformis*	5-7	40-50	Lee, 2008	Industry (Biomass hydrolisation etc.)

For alpha amylase of *Bacillus pumilus*, comparison with other alpha amylases was done (Table 5.5). When we compare our alpha amylase with other alpha amylases obtained from *Bacillus subtilis* and *Bacillus licheniformis*, we can see that our alpha amylase enzyme have lower optimum temperature, but it has also enzyme activity at 80 ^{o}C with %44 relative activity. So out alpha amylase can be a candidate for industrial applications with further studies on improving temperature resistance. In addition, there is no known alpha amylase gene obtained from *Bacillus pumilus* bacteria. There is one study that a putative alpha amylase gene found (Gioia, 2007) and there is no much literature about production of alpha amylase from Bacillus pumilus. So, our alpha amylase obtained from our *Bacillus pumilus* isolate can be a good candidate for production of novel alpha amylase with further studies.

Table 5.5 Comparison of alpha amylase enzyme of *Bacillus pumilus* with other alpha amylases.

Enzyme	Organism	Optimum pH	Optimum temperature (^{o}C)	Reference	Application
Alpha amylase	*Bacillus pumilus*	6	50		
EC 3.2.1.1 - alpha-amylase	*Bacillus subtilis*	5-7	65	Nagarajan, 2006	Food industry (bread making)
EC 3.2.1.1 - alpha-amylase	*Bacillus subtilis*	6	65	Cho, 2007	bread making as antistaling agent
EC 3.2.1.1 - alpha-amylase	*Bacillus licheniformis*	5,5-6	95	Tee, 2009	Industry (starch liquefaction)

For laccase of *Bacterium BS72*, comparison with other laccases was done (Table 5.6). When we compare our laccase with other laccases obtained from *Bacillus pumilus*, *Bacillus sp.* and *Bacillus licheniformis*, we can see that our laccase enzyme can resist higher temperatures from a laccase obtained from Bacillus sp. (Mohammadian, 2010) although there are also laccases that can resist up to 85 ^{o}C (Koschorreck, 2008). To increase the usage area of our laccase, further studies can be done to improve its thermotolerance.

Laccases are mostly produced from fungi. Not much bacterial laccases have been characterized since there is not enough knowledge about the diversity and distribution of laccases from bacteria species. For laccase obtained from *Bacterium BS72* in this study, there is no known gene in the database. So our laccase can be a candidate for production of a novel laccase enzyme from bacterial origin. Future studies on this enzyme will make a major contribution to the literature as well.

Table 5.6 Comparison of laccase enzyme of Bacterium BS72 with other laccases.

Enzyme	Organism	Optimum pH	Optimum temperature (^{o}C)	Reference	Application
Laccase	*Bacterium BS72*	8	50		
EC 1.10.3.2 - laccase	*Bacillus licheniformis*	7	85	Koschorreck, 2008	Dimerization of phenolic acids
EC 1.10.3.2 - laccase	*Bacillus pumilus*	4-7	70	Reiss, 2011	Synthesis, versatile biocatalyst
EC 1.10.3.2 - laccase	*Bacillus sp.*	5	40	Talked, 2011	Textile industry
EC 1.10.3.2 - laccase	*Bacillus sp.*	3	35	Kashia, 2013	Biodegradation of synthetic dyes

In general, most of the enzymes isolated in this thesis have shown enzyme activity at thermophilic temperatures (alpha amylase- 50 ^{o}C, lipase- 80 ^{o}C, laccase - 50 ^{o}C, xylanase- 50 ^{o}C). One enzyme purified has shown activity at mesophilic temperature (protease- 25 ^{o}C). In terms of pH, partially purified xylanase and laccase enzyme showed maximum activity at pH 8, alpha amylase and protease enzyme showed maximum activity at pH 6 while lipase enzyme showed maximum activity at neutral pH (pH 7).

Future studies need to be done on isolated bacteria (constructed small bacteria bank) and enzymes partially purified and characterized in this thesis. With first results obtained in this thesis, enzymes that have industrial usage potential have been obtained from bacteria isolated from different soil samples in our country. With future studies done one further characterization on these enzymes, searching the thermostabilities, shelf lives and improving these characteristics, they can be used in production of industrially important enzymes in larger scales. In addition, as stated in this thesis, some enzyme genes are not discovered in some bacteria species (putative genes) isolated in this study. So, with cloning studies that can be done for these putative enzymes, there is

an opportunity to explore novel enzymes and get the patent special for our country. Since Turkey is foreign-dependent to enzyme production, this will add value to our economy as well.

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APPENDIX A

GROWTH MEDIA USED IN SCREENING OF ENZYME ACTIVITIES ON AGAR

L Medium	M Medium	A1 Medium	A2 Medium	La Medium	Rennin Agar
LB Broth (17,5 g) Tributyrin (6,78 ml) Agar (10,5 g)	LB Broth (17,5 g) Skim milk (7 g) Agar (10,5 g)	Starch (7 g) NaCl (3,5 g) Peptone (7 g) Yeast extract (3,5 g) Agar (10,5 g)	Peptone (2,1g) KH_2PO_4 (0,42 g) $ZnSO_4$ (0,0014 g) $FeSO_4$ (0,0007g) $MnSO_4$ (0,07 g) Starch (7 g) K_2HPO_4 (0,28 g) $MgSO_4$ (0,714 g) Agar (14 g)	LB Broth (17,5 g) Agar (10,5g) Guaiacol (124,6 µl)	NaCI (2,5 g) Yeast extract (2,55 g) Peptone from casein (5 g) Skim milk (5 g)
Total volume (700 ml)	Total volume (300 ml)	Total volume (700 ml)	Total volume (700 ml)	Total volume (700 ml)	Total volume (500 ml)

APPENDIX B

CHEMICALS, SOLUTIONS, KITS AND REAGENTS

NAME	BRAND
xylan	Sigma, X4252
skim milk	Merck, 115363
tributyrin	Sigma, 91015
guaiacol	Sigma, G5502
Casein Hydrolysate	Sigma, 22090
Peptone from soybean	Sigma, 70178
Beef extract (Meat Extract)	Merck, 103979
Starch soluble	Merck, 101252
Calcium chloride	Merck, 102382
Tween 80	Merck, 822187
Agar-Agar (ultra pure) granule	Merck, 101613
Sodium chloride	Merck, 106404
POTASSIUM DIHYDROGEN PHOSP.GR for analyis	Merck, 104873
SODIUM DIHYDROGEN PHOSP. Emprove	Merck, 106345
AMMONIUM CHLORIDE for analysis emsure	Merck, 101145
AMMONIUM SULFATE for analysis emsure	Merck, 101217
Phosphate buffered saline	Sigma, P4417
COPPER (II) SULFATE PENTAHYD.for analysis.emsure	Merck, 102790
Yeast Extract Agar	Merck, 103750
Peptone for Bacteriology (from meat pancreatic) granule	Merck, 107214
ZINC SULFATE HEPTAHYDRATE GR for analysis	Merck, 108883
IRON (II) SULFATE heptahydrate GR for analysis	Merck, 103965
MANGANESE(II) SULFATE spray dried emprove	Merck, 105999

Magnesium sulfate heptahydrate	Merck, 105886
LB broth (MILLER) (Luria Bertani)	Merck, 110285
Sodium Nitrate cryst.extra pure	Merck, 106535
Congo Red (C.I. 22120) Indıcator Ph 3,0	Merck, 101340
Tributyrin Agar	Sigma, 91015
Potassium phosphate dibasic	Sigma, P3786
Tris-HCl	Promega H5121
Potassıum Chlorıde Gr For Analysis	Merck, 104936
Tryptone (Peptone from Casein pancreatic) free from sulfonamide	Merck, 102239
Ammonıum Peroxodısulfate Extra Pure	Merck, 101200
Acetıc Acıd (Glacıal) 100 % Anhydrous Gr For Analysis Emsure	Merck, 100063
Methanol	Merck, 106007
albumin from bovine serum	Sigma, A2153
Bradford Reagent	Sigma, B6916
N,N,N,N-Tetrameyhyl Ethylenedıamıne temed	Merck, 110732
HYDROCHLORIC ACID EXTRA PURE %37	Sigma, 7102
di-Sodium hydrogen phosphate anhydrous for analysis EMSURE®	Sigma, 106586
3,5-Dinitrosalicylic acid	Sigma, D0550
Glycıne Chryst.Ph.Eu	Merck, 100590
Sodium acetate trihydrate	Sigma, 25022
trizma hcl, biotechnology performance*ce	Sigma, SB.T5941
Hydrochlorıc Acıd 37 % Gr For Analysis	Merck, 100317
Trıchloroacetıc Acıd Gr For Analysis	Sigma, 100807
Sodium Potassium Tartrate	Merck, 108087
endo-1,4-β-Xylanase from Trichoderma longibrachiatum	Sigma, X2629
Dialysis Bag	Biobasic, TX0111
Gene Jet DNA purification kit	Thermo Scientific, K0721