

T.R.
SIIRT UNIVERSITY
INSTITUTE OF SCIENCE

**DETERMINATION OF EXTRACELLULAR HYDROLYTIC ENZYME
CAPABILITIES OF SOME ANOXYBACILLUS ISOLATED FROM HOT
SPRING ENVIRONMENTS**

MASTER DEGREE THESIS

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Widad Hassan Yarwais JAF



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LIST OF ABBREVIATIONS

<u>Symbols</u>	<u>Description</u>
g	Gram
hr	Hour
α	Alpha
β	Beta
μm	Micrometer
mm	Milli Meter
mg	Miligram
ml	Mililitre
w/v	Wieght Per Volume
g/L	Gram Per Litter
°C	Celsius Degree(temprature)

<u>Abbreviation</u>	<u>Description</u>
NB	Nutrient Browth
NA	Nutrient agar
MIC	Minimum inhibitory concentration
NCBI	National center for Biotechnology Information
PBP	Penicillin-Binding Proteins
RNA	Ribonucleic Acid
USD	American Dollar
ZOI	Zones Of Inhibition
PCR	Polymerase chain reaction

ÖZET

YÜKSEK LİSANS TEZİ

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Bu çalışmada, Türkiye'den Afyon ili kaplıcasından izole edilen *Anoxybacillus flavithermus* bakterileri kullanılmıştır. Bakteriler morfolojik ve fizyolojik açıdan test edildi ve identifikasyonlar için 16S rRNA karşılaştırması ile analiz edildi. Bu tezde, bakteri tespiti için biyokimyasal testler yapılmıştır. Bu amaçla nişasta, kazein, ksilenaz ve asparginaz hidrolizi ve katalaz, üreaz ve lipaz aktiviteleri incelenmiştir. İzole edilmiş bakterilerde ayrıca dört farklı (kloramfenikol, ampisilin, rifamisin ve eritromisin) antibiyotik kullanılarak antibiyotiklere dirençli testler yapılmıştır.

Bakteriler, agar besi ortamında yetiştirildi ve sonuçlara göre; izole edilen tüm suşlar nişastayı karbon ve enerji kaynakları olarak fermente etmiş ve inkübasyondan 24 saat sonra 50 ° C'de ve pH 7.0'da amilaz aktivitesi elde edilmiştir. Tüm suşlar katalaz pozitif olarak değerlendirilmiştir. *A. flavithermus* suşlarının birkaç suşu hariç üreaz ve kazeinaz pozitif olarak elde edildi. Özellikle KJ095001 suşu, hem üreaz ve hem de kazeinaz açısından negatif olarak elde edildi.

Biyoteknoloji son yıllarda oldukça hızlı gelişti ve mikrobiyal enzimlerin üretimi endüstriyel sektörler için gerekli bir proses haline geldi. Mikroorganizmaların enzimleri ve tüm hücreleri, biyoremediasyon işlemleri ve atık yönetimi için çokça kullanılmaya başlandı. Mikrobiyal enzimleri başlıca sanayi, gıda, tekstil, ilaç, deri, kağıt, kozmetik, biyomalzemeler, ince kimyasallar, enerji, selüloz ve deterjan endüstrisinde kullanılmaktadır.

Bu araç, araştırmacıların, daha önce belgelendirilmemiş potansiyel bir enzimatik aktiviteye sahip olabilecek yeni bakteri soylarını taramalarını ve termofilik mikroorganizmada ilaç direncine klonlama vektörleri olarak işlev görebilecek plazmidleri araştırmalarını sağlar.

Anahtar Kelimeler: Bacillaceae, *Anoxybacillus flavithremus*, Extremozyme, Antibiyotik, Biyoteknolojik Sanayi.

ABSTRACT

MSc THESIS

DETERMINATION OF EXTRACELLULAR HYDROLYTIC ENZYME CAPABILITIES OF SOME ANOXYBACILLUS ISOLATED FROM HOT SPRING ENVIRONMENTS

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In this study the *Anoxybacillus flavithermus* bacteria were used which was isolated from hot spring of Afyon city from Turkey. The bacteria were tested for the morphological and physiological aspect and analyzed by 16S rRNA comparison for identifications. In this thesis, the biochemical tests were done for identify the characteristics of the bacterium. For this purpose, starch, casein, xylanase and asparaginase hydrolysis and catalase, urease and lipase activities were examined. Also antibiotic resistant tests were performed to the isolated bacteria by using four different (chloramphenicol, ampicillin, rifamycin and erythromycin) antibiotics.

The bacterium grows in nutrient agar media, and according to results; all isolated strains fermented starch as carbon and energy sources, and amylase activity was obtained at 50 °C at pH 7.0 after 24 h of the incubation. All strains were evaluated as catalase-positive, most of *A. flavithermus* strains were obtained as urease and caseinase-positive except few strains and especially strain no. KJ095001 was founded urease and caseinase negative.

Biotechnology has grown immensely in recent years and the production of microbial enzymes was a necessary event in the industrial sectors. The enzymes and the whole cell of microorganisms have also been much used for bioremediation processes and waste management. The main industries that apply microbial enzymes are the food, textile, pharmaceutical, leather, paper, cosmetics, biomaterials, fine chemicals, energy, cellulose and detergent industries.

This tool enables researchers to screen for novel bacterial strains that may have a potential enzymatic activity which have not been documented before and for looking plasmids that could serve as cloning vectors to drug resistance in thermophilic microorganism.

Key word: *Bacillaceae*, *Anoxybacillus flavithremus*, Extremozyme, Antibio

1. INTRODUCTION

First appearance of the *Bacillaceae* on the Earth before 2 billion years ago, until today, the *Bacillaceae* family are evolved in a dramatic diversification of capabilities and take a majority of niches on our planet (David and Alm, 2011). The *Bacillaceae* was created by Fisher in 1895 belong to phylum Firmicutes. *Bacillaceae* are a family of Gram-positive, rod-or coccus shaped bacteria, heterotrophic, may produce endospores which are oval, round or cylindrical, some members of this family are motile by peritrichous flagella. *Bacillaceae* are aerobic, facultative or strict anaerobes (Vos et al., 2009).

Anoxybacillus genus is belonging to *Bacillaceae* family (formerly known as *Bacillus flavothermus*) this strain was first discovered in a New Zealand hot spring (Heinen et al., 1982). They are a relatively new genus that was proposed in the year of 2000. *Anoxybacillus* means small rod living with-out oxygen (Antonio et al., 2016). The *Anoxybacillus* can be aerobes, facultative anaerobes, or facultative aerobes (Pikuta et al., 2009). Morphology of cells in most species is the same; Gram-positive, straight rods, motile, they form one spore per cell, the spores are round and resistant to freezing and heating and radiation (William et al., 2011). *Anoxybacillus* are moderately thermophilic bacteria, they grow well at 40–70 °C with optimum growth at 55–60°C and many of the genus *Anoxybacillus* species are alkaliphilic they are able to grow at pH 6-10, and the optimum growth pH at 7.5-8. (Garo Antranikian et al., 2011).

Thermophilic microorganisms have been isolated from different habitats such as permanently cold habitats, hot springs, deep ocean-basin cores, deep-sea hydro-thermal vents, shallow marine environments, petroleum reservoirs, and the leachate of a waste pile from a canning factory (Szewzyk et al., 1994; Adiguzel et al., 2009). Because these bacteria are alkali-tolerant thermophiles, they are a good alternative in many industrial applications related to enzyme technology, starch and lignocellulosic biomasses, bioenergy production, and environmental waste treatment (Lasa and Berenguer, 1993). On the basis of 16S rDNA sequence, all species of the *Anoxybacillus* genus are closely related (94.5-99.2%), and the physiological properties indicated by DNA-DNA hybridization (William et al., 2011).

Anoxybacillus genus is one of the best thermophilic bacteria among the other bacilli, in extreme habitats, to produce valuable enzymes biotechnologically, because most of these bacteria have amylolytic and glucosidic activities and the ability to

degradation of carbohydrate (Cihan, 2013). The application of *Anoxybacillus* increased compared to other members of *Bacillaceae* due to their thermo stable enzyme as sources for many biotechnological processes, such as metabolic studies, bioremediation applications, genomic analysis, and biosorption (Lasa and Berenguer, 1993; Özdemir, 2011).

Anoxybacillus flavithermus are Gram-positive, rod shaped, and motile, they are facultative anaerobic, spores' terminal, their colonies appear a round, smooth, yellow color (Heinen et al., 1982). They have chemical features such as catalase and oxidase positive, acetone positive, starch hydrolyze, they used glucose, sucrose, mannose, maltose, arabinose, sorbitol, and rhamnose as carbon source (Sharp et al., 1992), arginine di- hydrolase, tryptophan deaminase and β -galactosidase, lysine decarboxylase, nitrate reduced to nitrite, growth in 2-2.5% NaCl broth (Claus and Berkeley, 1986).

Microbial enzymes are preferred because of their high enzyme yields, stability, continuous availability and great variety of catalytic; it's easy for use in genetic manipulations and for growth of microorganisms which planted on low-cost media and its more safety during the production (Hasan et al., 2006).

The activity of any particular enzyme in soil is a composite of activities associated with various biotic and abiotic components, e.g. proliferating cells, cell debris latent cells, clay minerals, and the soil aqueous phase (Burns, 1982). Some microorganisms have the ability to grow at extreme temperatures due to their enzymes are stable and active at these temperatures (Zuber, 1979). Various types' of carbohydrate-degrading enzymes can produce by *Anoxybacillus* (Derekova et al., 2008). *Anoxybacillus flavithermus* which isolated from a hot spring in Turkey were used as an inoculum for the production of α -amylase from rice husks using solid-state fermentation (Özdemir et al., 2011), amylase is one of the most important industrial enzymes that can be used in a number of industrial processes including brewing, baking, textiles, paper industry, bioethanol production, and detergent (Lama et al., 2009). The whole cells of *Anoxybacillus* are potential useful as alternative resources for bioremediation and to immune stimulate fish against pathogens and renewable energy generation (Kian et al., 2013).

Thermophilic bacteria are able to produce spores during the manufacture of milk powder within the regeneration sections of heat exchangers and in evaporators (Stadhouders et al. 1982; Scott et al. 2007). It is really problem in a milk powder plant and cause contaminating of the final products (Ali Demirci et al., 2015). *Anoxybacillus flavithermus* is important bacteria that cause contamination the dairy industry, even they

are not pathogenic; their presence in dairy products is an indicator of poor hygiene , production of acids or enzymes ,and may be leading to off-flavours of Dairy product(Burgess et al., 2008)

Lipases enzyme have many applications in industrial and organic synthesis such as food, detergent, pulp, and paper industries, waste water treatment, production of biodiesel and synthesis of peptide (Hasan, 2006). The new studies indicate that lipases enzyme that produced from *Anoxybacillus flavithermus* may be useful for various processes such as medical, food, cosmetic, detergent and leather and textile industries. There is new approach in the biotechnological addresses to use the lipases for bioconversions in organic solvents and protection of the activity and stability of this enzyme in organic solvents (Gaur et al., 2008).

Xylanolytic activity was found in several species of *A. flavithermus* (Kambourova et al., 2007), *A. pushchinoensis* (Kacagan et al., 2008), *Anoxybacillus* sp. (Wang et al., 2010), and from many strains isolated from hot spring in Turkey. Also; some species of *Anoxybacillus* useful to remove heavy metals from aqueous solutions and act as a model to developed the biosorption system (Duran, 2009).



2. LITERATURES REVIEW

2.1. *Bacillaceae*

The *Bacillaceae* are a family was created by Fisher in 1895 belong to phylum Formicates, they are a typical Gram-Positive cell wall, rod-shaped, and spore formation that provide high resistance to chemicals, heat, radiation, and drought, allowing these bacteria to survive at extreme conditions for a prolonged period of time. Heterotrophic, some member of *Bacillaceae* are aerobic or facultative anaerobic, motile members of this family are characterized by flagella. Gram-positive bacteria of *Bacillaceae* genera have a cell wall consisting of a plasma membrane and a thick peptidoglycan layer (30–100 nm) containing lipoteichoic acids, teichoic acids, and proteins (Thomas et al., 2010). *Bacillaceae* families are not pathogenic, but *Bacillus* species are known to cause some disease to humans, Insect, plant and other organism. *Bacillaceae* used for production 4 types: enzyme, antibiotic, fine biochemical which including flavor enhancers and food supplements, and insecticides. The family of *Bacillaceae* is consists of more than 19 000 species, subspecies and strain Also new genera and species are continuously being described in this family (Logan et al., 2009).

Bacillaceae are naturally occur in soil and they are found in various ecosystems like water, human body, air, sediment, animal systems, foods (including fermented foods) as well as in unconventional environments such as clean rooms in the vaccine-producing company (Vaishampayan et al., 2010). The thermophilic genera of *Bacillaceae* family found in hot springs and hydrothermal vents, while the halophytic genera found in an aquatic habitats like saltems and salt lakes(Jan D.,2015).

Bacillaceae genera are diverse in their:

- Cell wall and membrane lipid compositions.
- Spore shape and placement.
- Abilities to grow anaerobically.
- Motility.
- Sites of isolation
- Production of fermentation products from a variety of sugars.
- Utilization of citrate and/or propionate.
- Hydrolysis of casein, gelatin, and starches.

- PH
- Salt tolerant.
- Growth ranges of temperature. (Vos et al., 2009).

2.1.1. *Bacillus*

Bacilli are the largest genus within the *Bacillaceae* family (Ines et al., 2015). Ferdinand Cohn who was German Botanist first described the *Bacillus* genus in the 1872 (Zhang, 2011). They are rod shaped, Gram- Positive arranged in pairs or chain (Ludwig et al., 2015). Mostly of them are aerobic and facultative anaerobic bacteria (Alexander and Medoza, 2008). They are found in various ecosystems like the human body, soil, water, air (Vos et al., 2009) and occur in diverse environmental ecosystems such as high temperature, high PH, and high salt (Joan and John, 2011).

The lifestyles of all members of the *Bacillaceae* are tightly depending on their ability to form endospores. Spores allow survival under extreme conditions for different time periods, even it reach to thousands of years (Stelow, 2006). *Bacillus* make a single endospore with square rounded end and they are resistant to many adverse condition like high density of cell population, nutrient deprivation and there is also environmental factors affected such as water content, pH, radiation or temperature (Gabriella and Simon, 2002) motile (except *B. anthracis*) by peritrichous flagella, catalase positive, oxidase positive and can metabolize the carbohydrates by fermentation (Thomas et al., 2010).

Bacillus genus are important in the medical significant there is *B. anthracis*, which causes anthrax (Ludwig et al., 2015), and *B. cereus*, that cause food poison for human (Vos et al, 2008). *B. Thuringiensis* can produce a toxin used to kill insect and used as insecticide, some strains of *B. coagulans* may cause food spoilage of highly acidic, tomato-based products, and there is *B. Subtilis* which causing ropines in bread and related food (Ryan, 2004).

Bacillus species are used in many medical, pharmaceutical, agricultural, and industrial processes that take advantage of their wide range of physiologic characteristics and their ability to produce a host of enzymes, antibiotics, and other metabolites. Bacitracin and polymyxin are two well-known antibiotics obtained from *Bacillus* species. Several *Bacilli* sp. is aerobic spore forming rods are gram positive or gram variable. Except for few species the large majorities has no pathogenic potential and have never been associated with disease in man or animals. Members of the genus have significant microbiological uses (Turnbull et al., 1990).

2.1.1.1. *Bacillus cereus*

Bacillus cereus described and named by Frankland in 1887 (Frankland, 1887). *Bacillus cereus* is 1 x 3-4µm in size and they are straight or slightly curved with square ends they present singly or in short chains, motile by peritrichous flagella, spore forming, facultative anaerobes they grow at temperatures between 5°C- 50°C (Drobniewski et al., 1993).

Bacillus cereus are positive for metabolize carbohydrate, amino acid, and protein and they can reduce nitrate to nitrite. In anaerobic respiration, *B. cereus* utilizes fermentation to generate energy. Some strain of *Bacillus cereus* cause spoilage in canned foods and food poisoning which is harmful to humans and cause food borne illness, while other strains are useful for animal (Koneman et al., 1997). *Bacillus cereus* can produce two types of entero-toxins during exponential growth the first one is enterotoxin which causing diarrhea and the second is emetic toxin (Beecher et al., 1995).

2.1.1.2. *Bacillus firmus*

Described and named by Werner, *Bacillus firmus*, they are Gram positive, rod shape (Werner, 1933). Some strains of this species are very alkaline-tolerant and may grow in environments with pH as high as 11 (Arthur et al., 1979), they found in water, soil, sewage and activated sludge (Dias et al, 1966). The purified protease that excreted from *Bacillus firmus* have important role in development of industrial processes that performed under extreme conditions its useful to conversion of marine wastes to generate high value-added products (Neelamegam et al., 2014).

2.1.2. *Anoxybacillus*

Pikuta et al. they was first described *Anoxybacillus* genus from family *Bacillaceae*, the word *Anoxybacillus* means “Bacillus without oxygen” most of the species growth well aerobically but there is some species can grow well aerobically and anaerobically under extreme conditions (Pikuta et al., 2000). They are Gram-Positive bacteria, rod shaped, spore forming, motile (Goh et al., 2014). They are naturally found in geothermal springs, manure. They are facultative thermophilic with growth temperature range 30 -70 °C and an optimum temperature a round 50 -55 °C (Pina et al., 2009), and has a pH range of 6.0–10.0 (optimum at 8.0), *Anoxybacillus* are positive for

methyl red test and nitrate reduction, but negative for gelatin hydrolysis, tyrosine hydrolysis, starch hydrolysis, and lysozyme tests.

Growth was inhibited at NaCl concentrations higher than 3.0% (w/v). facultative anaerobes able to grow on a wide range of carbon sources include: xylose, maltose, mannitol, sucrose, glucose, galactose, fructose, glycerin, mannose, sodium acetate, sodium formate, sodium citrate, tyrosine, sorbitol, sodium succinate, and sodium lactate, sodium oxalate, and inorganic substrates including FeII, SO (Pikuta et al., 2000)

And now contains the following eighteen validly described species:

1. *Anoxybacillus pushchinoensis* (Pikuta et al., 2000).
2. *Anoxybacillus flavithermus* (Heinen et al., 1982).
3. *Anoxybacillus gonesis* (Belduz et al., 2003).
4. *Anoxybacillus contaminans* (De Clerck et al., 2004).
5. *Anoxybacillus voinovskiensis* (Yumoto et al., 2004).
6. *Anoxybacillus kestanbolensi* (Dulger et al., 2004).
7. *Anoxybacillus ayderensis* (Dulger et al., 2004).
8. *Anoxybacillus kamchatkensis* (Kevbrin et al., 2005).
9. *Anoxybacillus amylolyticus* (Poli et al., 2006).
10. *Anoxybacillus rupiensis* (Derekova et al., 2007).
11. *Anoxybacillus bogrovensis* (Atanassova et al., 2008).
12. *Anoxybacillus salvatliensis* (Cihan et al., 2011).
13. *Anoxybacillus thermarum* (Poli et al., 2009).
14. *Anoxybacillus eryuanensi* (Zhang et al., 2011).
15. *Anoxybacillus tengchongensis* (Zhang et al., 2011).
16. *Anoxybacillus mongoliensis* (Namsaraev et al., 2010).
17. *Anoxybacillus kaynarcensis* (İnan et al., 2012).
18. *Anoxybacillus tepidamans* (Schäffer et al., 2004)

Table 2.1.Characteristics of *Bacillus* and *Anoxybacillus*

Phenotypic characteristics	<i>Bacillus</i>	<i>Anoxybacillus</i>
Number of species in genus	141	10
Gram reaction	+/v/-	+
Cell shape:rode	+	+
Cell width o.5-1.0mm	+	+
Motility	+/-	+/-
Spore formation	+/-	+
Spore shape:spherical	+	+
Oxygen requirments:facultative anaerobic	+	+
Growth in media with add NaCl 5%	+	+
Growth at 30°C-60°C	+	+
Growth at PH 5-10	+	+
Catalase	+/-	+/-
Oxidaes	+/-	+/-

*Symbols: +, at least one species within the genus gives a positive reaction; +/-, some species are positive, some are negative; v, varies, Bergey's Manual of Systematics of Archaea and Bacteria Niall A. Logan1, Paul De Vos 2015

Bacillus are gram-positive, or Gram-positive only in early stages of growth, or Gram-negative, most species have little or no pathogenic potential and are rarely associated with disease in humans or other animals; an exception is *Bacillus anthracis*, several other species may cause food poisoning and opportunistic infections, and strains of *Bacillus thuringiensis* are pathogenic to invertebrates. A wide diversity of physiological abilities is exhibited, ranging from psychrophilic to thermophilic, and acidophilic to alkaliphilic; some strains are salt tolerant and some are halophilic.

Anoxybacillus flavithermus are gram positive, non-pathogenic, aerobes, facultative aerobes or facultative anaerobes; alkaliphilic, neutrophilic, moderately thermophilic, chemo organotrophic, with a fermentative or oxygen respiration metabolism (Sneath et al., 1982)

2.1.2.1. *Anoxybacillus flavithermus*

Anoxybacillus flavithermus are Gram-positive, spore forming, motile, and rod-shaped bacteria. They are facultative thermophilic with an optimum temperature for growth of 50–55 °C, facultative anaerobes able to grow on a wide range of carbon sources including D-glucose, D-raffinose, D-sucrose, D-xylose, D-fructose, L-arabinose, maltose, D-mannose and D-mannitol. *Anoxybacillus flavithermus* sources (Pikuta et al., 2009). 'flavithermus' means the dark yellow color of their colonies, and this because of the accumulation of a carotenoid pigment in the cell membrane. Formerly their name was 'Bacillus flavothermus' they can growth in abnormally wide range of pH values 5.5- 10.0 and temperatures 30-72°C.

Thermophilic bacteria have been isolated from different habitats such as hot springs, deep-sea hydro thermal vents, petroleum reservoirs, and the leachate of a waste pile from a canning factory (Szewzyk et al., 1994; Adiguzel et al., 2009).

2.1.2.2. *Anoxybacillus mongoliensis*

They are gram-positive, rod-shape, spore forming, feebly motile, by peritrichous flagella, facultative anaerobic and moderately thermophilic, they grow well at 30–70 °C with optimum growth at 60°C. *Anoxybacillus mongoliensis* is facultative alkaliphilic grow well at pH 5–10, with optimum growth at pH 8.0. *Anoxybacillus mongoliensis* is also found in soil, water, hot spring, and first isolated from alkaline hot 80 °C, pH 9.8 springs Tsenher, central Mongolia (Namsaraev et al., 2010).

2.1.2.3. *Anoxybacillus kestanbolensis*

Gram-positive, rod shape, motile, spore forming, they isolated from mud and water samples of the Kestanbol hot springs in Turkey. The water temperature of these hot springs is around 60–70 °C, and PH around 6-11 (Dulger et al., 2004).

2.2. Enzyme

2.2.1. Enzyme definition

Enzymes are proteins having catalytic capability to convert substrate in to another at a high reaction rate. all living organism to control their living system and maintaining their life they needs enzymes because the occurrence of all cell metabolic path ways depend on the participation of at least one enzyme (Khan et al., 2015) which is a protein

molecule and this true nature was discovered by James B. Smner in 1926 (Nobel Prizes and Laureates 2015). Enzymes have the ability to control the speed of chemical reactions in our body like digestion, breathing, growth, reproduction, disease, healing, and blood coagulation.

2.2.2. Enzymes characteristics

The important one is the rate of reaction, in the presence of enzyme the reaction increased and characteristic that enzymes change their state of low activity to high activity and vice versa and specificities enzyme act with only one reactant substrate to produce products (Keith and John, 2000).

2.2.3. Enzyme benefits

To use any enzyme in any industrial area must depended on the enzyme must be not toxic or have any allergic effect, low cost ,and then being able to use it in various areas (Wiseman et al.,1987).Today, the enzymes that used in many field of industry are derived from micro-organisms. And this after comparison between enzymes originating from plant and zoological sources, they notice that the enzymes derived from microorganisms have high catalytic activities, excess quantities, and there is no create waste (Ozdemir et al., 2015).

Industrial biotechnology means the used of biotechnology application for industrial purposes, including biomaterials, manufacturing, and bioenergy .After biotechnology revolution industrial biotechnology became more interested and have evolved dramatically and significantly and the wide application of enzymes in industrial sectors such as detergent, food, animal-feed, chemical, pulp and paper industries, textile, agriculture, leather.

Recent advances in recombinant-DNA technology, protein engineering and process development have begun to influence the industrial enzyme market in a very positive way by producing purified enzymes with new activities and new process conditions in various Industrial biotechnologies (Zhu et al., 2011).

The used of enzyme in industrial in the world increased it was 1 billion dollarsin 1995, but in 2000 it reaches to 1.5 billion dollars (Kirk et al., 2002).The industrial enzymes market is estimated to be valued at USD 4.61 Billion in 2016 (markets, October 2016).

By usage of enzyme in industrial processes can eliminate the use of organic solvents, high temperatures, and extremes of pH, at the same time yield pure product, increased reaction specificity, and the environmental impact reduced. The industrial enzymes in growing and this growing dependent on constant innovation by recombinant DNA ,increasing database of natural enzyme diversity and fermentation technologies to reduce cost, protein modification, and improve performance to fit the world enzyme market (Cherry and Fidantsef ,2003) .

Amylase is very important enzymes in biotechnology and its benefits in many industrial applications which constitute approximately 25% of enzymes in the world market (Rajagopalan and Krishnan, 2008). Different species of microorganisms can produced α -Amylase especially from *Bacillus* genus like: *Bacillus stearothermophilus*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis* used for commercial applications and processes such as fermentation, textiles, food, and paper industries (Konsoula and Liakopoulou-Kyriakides 2007).

The most widespread applications of α -amylases are in the starch industry, starch is one of the most important component of the human food and is used enzymatically and chemically processed into different products such as glucose syrups, maltodextrin derivatives, starch hydrolysates, fructose or cyclodextrin,which can used in the food industries(Agrawal et al., 2005). Potato, maize, wheat, and tapioca are major sources in starch industry, but because of thermal resistance, high tendency towards retrogradeation, low shear resistance, and thermal decomposition limiting their usage in food industry applications (van der Maarel et al., 2002).

Lipases are a class of enzymes which catalyze the hydrolysis of long chain triglycerides. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology. Lipases constitute the most important group of biocatalysts for biotechnological applications such as food, fat modification, and enhancement flavor in food processing, fats and oils hydrolysis, detergent, pulp and paper industries, and chemical analyses (Sharma, 2001).

Fine chemicals are very important in pharmaceutical and cosmetic industries by adding pure enantiomer compounds, many enzymes can be used to alternatives the chemical catalysts and use of specific enzymes at milder condition reaction to solve many

problem such as large amounts of raw materials, solvents, and energy consume during process, many intermediate reactions, and waste generation (Kuhn et al., 2010).

For more than 35 years; enzymes are an eco-friendly solution that has been used to improve the cleaning efficiency, and these enzymes are staple ingredients in liquid and powder detergents, stain removers, medical cleaning, automatic dishwashing detergents. Production of detergent from nature by using enzyme such as lipase, amylase, protease, and cellulose to digest fats, protein, carbohydrates, and cellulose, enzymes make cleaning easier, energy save, and reduce the aquatic pollution (Nielsen and Skagerlind, 2007).

2.2.4. Extremozymes

Extremozymes (heat stable enzymes) produced by thermophilic bacteria, which normally found in extreme condition (pH, temperature, water activity, radiation,), its great in industrial interest used in the field of textile, detergent, cosmetic, food and molecular biology (Van den, 2003) The species that obtained from the various extreme habitats can produce a wide range of commercially valuable extracellular enzymes (Bischoff et al., 2006).

There are many advantages of conducting industrial processes at high temperature for improved solubility of substrates, increased the bioavailability, lower viscosity, minimizes contamination risk and more faster in reaction rate (Mishra and Pascale, 2009). Thermophilic bacteria that generate and refractory from hot spring water can produce unique biocatalyst under extreme condition called thermo stable enzyme (Lasa and Berenguer, 1993), Turkey is a country rich in geothermal heat water sources and these geothermal have large varying in their temperatures ranging from 45 to 100 °C and pH ranging from 5-11, so many of *Anoxybacillus* species are isolated from these locations to produce thermophilic enzymes and used it in biotechnological industrial (Canakci et al., 2007).

2.2.5. Some types of enzyme used in biochemical tests

2.2.5.1. Amylases enzyme

By amylase enzyme the starch breaks up to produce glucose molecules, dextrin, oligosaccharide (Mukherjee et al., 2009). α -Amylases are part of endoamylases which hydrolyzes the glycosidic bond of starch. Hydrolases enzyme which is produced by

bacteria can catalyze the splitting of organic molecules (like starch) to small molecules with the presence of water. It's known that, the starch molecule made up of two parts: amylose (un branched glucose polymer 200 to 300units) and amylopectin (a large branched polymer). By amylases enzyme, bacteria hydrolyze both amylose and amylopectin to yield glucose, dextrin, and maltose (Harley, 2002). Amylases are significant enzymes for their specific use in the industrial (Nigam and Singh, 1995). Also amylase enzyme has important role in the conventional technology (Gupta et al., 2003). α -amylases that prepared from microbial used in food, chemistry, detergent industry, textile, pharmacy (Mukherjee et al., 2009).

Amylases enzyme that isolated from thermophilic bacteria is thermostable enzyme and active at high temperature. Some of thermophilic bacteria are known for the production of starch-hydrolyzing enzymes like *Anoxybacillus* and *Bacillus* species such as *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Geobacillus stearothermophilus*, *Bacillus alvei*, and *Bacillus licheniformis* (Agüloglu et al, 2000).

An extensive and excellent review on the α -amylase enzyme of *Anoxybacillus flavithermus* was published recently such as study of Sadin Özdemir et al., 2013 which reports that *A. flavithermus* is able to produce high levels of thermostable α -amylase and the optimum enzyme activity was determined as 55 °C and pH as 7.0.

2.2.5.2. Xylanase enzyme

Xylan is a major structure component of the cell wall of plant cell. The xylan backbone is β -1, 4-D-xylopyranosides (Jänis et al., 2007; Kumaret al., 2012) and for biodegradation this structures it's required several enzymes (Blanco et al., 1999). Like endoxylanase, xylan 1,4- β -xylosidase, and α -glucuronidase , and side-chain cleaving enzymes: α -arabinofuranosidase and acetylxylan esterase (Ahmed et al., 2009; Choi ID et al., 2000). In a study presented by Kambourova et al., (2007) who obtained Xylans from different sources were hydrolyzed at high degree at 70°C by co-action of a xylanase from the thermophilic bacterium *Anoxybacillus flavithermus* (Kambourova et al., 2007). These xylanase enzyme activities have also been reported by Beldüz et al., (2011) on *Anoxybacillus* from some hot springs in Turkey

Xylan constitute about (20–40) % of the total biomass of plant, chemical hydrolysis of xylan an important industries, although it is, accompanied with the

formulation of hazardous to the environment and toxic components (Biely et al., 1985), but the microbial enzymes have specific inaction and its save on environment, this lead to use the xylanases enzyme (that produced by microorganism) in many application like clarification of juices, bioconversion of lignocellulosic material to fermentative products, and the digestibility of animal feed stock etc. (Wong et al.,1988). For production of enzymes many of agro-industrial wastes are used now as alternative substrates, due to represent an alternative source of low commercial value and local availability especially when the aim of the production these enzymes on a large scale (Hernández et al., 2006). Hemicellulosic low-cost substrates like corn cobs, wheat bran and sugarcane bagasse are used for the production of hydrolases by certain microorganisms (Nagar et al., 2011).

2.2.5.3. Caseinase enzyme

The protein casein is a large polymer of amino acids incapable of enter to the plasma membrane of bacteria. So, it must be degraded by proteolytic exoenzymes (caseinases and peptidases) to small chains of amino acids, dipeptides, and polypeptides to use it as source of carbon and energy. To complete this process the bacteria must secret proteolytic enzymes to catalyze the hydrolysis of casein to amino acids then transported into the cell and catabolized (Harly et al., 2002).

In human milk the casein content is approximately 20-45% of the total protein and 80% of the protein in cow's milk (Kunz and Lonnerdal, 2011). The enzyme caseinase is secreted out of the cells (an exoenzyme) that hydrolyze the milk protein casein (Karin and Robert. 2015). Also published data from Pikuta et al., (2000) indicated that the detection of casein by *A. flavithermus* DSM 2641T was positive.

2.2.5.4. Asparaginase enzyme

Asparaginase enzyme (L-asparagine aminohydrolase-EC3.5.1.1) hydrolyzes the asparagine to NH_4 and aspartate, which transaminated to oxaloacetate (Jeremy Berg et al., 2001). Asparaginase enzyme; that extracted from plant different in structure and evolution origin from bacterial asparaginases. Bacterial asparaginases are two types intracellular and extracellular, intracellular (cytosolic) has a lower affinity for asparagine, while extracellular (periplasmic) has a high substrate affinity for asparagine (Michalska and Jaskolski, 2006). Asparaginase from bacterial origin is more useful because it cans easily culture and the extraction and purification is convenient and facilitates large scale

production (Savitri et al., 2002). *Bacillus* genus such as *Bacillus mesentericus* (Tiul Panova, 1972) and *Bacillus polymyxa* (Nefelova, 1978) are most economical and used for production Asparaginase enzyme (Karolina and Mariusz, 2006).

Asparaginase enzyme it induces an immune response in a significant percentage of treated patients (Woo et al., 2000). Asparaginase acts as an anti-tumor agent, which can inhibit the growth of cancer cells. It is used mainly as an enzyme drug for acute lymphoblastic leukemia in children (Schemer and Holcenberg, 1981). Some groups of researchers found the L-asparaginase in *Bacillus sp.* (Mohapatra et al., 1995) and *Bacillus mesentericus* (Tiul Panova, 1972).

Recently; production of L-asparaginase using thermal microbial has attracted much attention because of cost-effective and its natural product act on a broad spectrum of antitumor activity, Pritsa et al., (2001) who detect the L-asparaginase in *Thermus thermophilus*.

2.2.5.5. Urease enzyme

On the basis of the similarities in sequence and reaction kinetics, it is reasonable to assume that the known ureases have a common structure and catalytic mechanism (Karplus et al., 1997). The primary role of ureases is to allow the organism to use external and internally generated urea as a nitrogen source. The product ammonia can be taken up and utilized by soil microbes and plants, which explains the wide use of urea as a nitrogen fertilizer and also its low cost and high nitrogen content. In plants, urease probably participates in systemic nitrogen transport pathways and possibly acts as a toxic defense protein (Mobley and Hausinger, 1989).

2.2.5.6. Lipase enzyme

Lipase (E.C. 3.1.1.3)] belong to a group of enzymes whose biological function is to catalyze the hydrolysis of triacyl glycerols into diacyl glycerols, monoacyl glycerols, free fatty acids (FFA) and glycerol. Then, the glycerol and free fatty acid molecules can be taken up by the bacterial cell. Lipids are high molecular weight compounds possessing large amounts of stored energy. The two common lipids catabolized by bacteria are the triacyl glycerols and phospholipids (Ertugrul et al. 2007).

Lipase enzymes are highly usage in industrial application such as dairy food, paper, detergent industries, textile, leather, waste water treatment, pharmaceuticals,

production of fine chemicals, cosmetics, and synthesis of surfactants and polymers (Park et al., 2005).

Lipases enzyme which produced from microorganism have more attention in industry mainly because of easy cultivation of microbes on inexpensive media, the availability of a wide range of hydrolytic and synthetic activities, ease of genetic manipulation high yields, regular supply due to absence of seasonal fluctuations (Hasan et al., 2006). There is some important lipase producing bacterial genera include *Bacillus*, *Burkholderia* and *Pseudomonase* (Vakhlu et al., 2006), in a study presented by Bakir and Metin, (2016) that an intracellular lipase from *Anoxybacillus flavithermus* HBB 134 was found and purified. The lipase enzyme was stable between pH 6.0-11.0 at 25°C- 40°C, and 50°C for 24 h and the maximum activity of the enzyme was at pH 9.0 and 50°C. Recently, other groups have reported similar results which showed that lipase positive of *Anoxybacillus flavithermus* WK1 strain (Shahinyan et al., 2017). The demand for the biocatalysts with specific properties such as temperature, specificity, pH stability is increasing day by day (Bornscheuer et al., 2002).

2.2.5.7. Catalase enzyme

The enzyme of catalase mediates the breakdown of hydrogen peroxide into free oxygen and water. Bacteria thereby protect themselves from the lethal effect of Hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism.



Catalase production and activity can be detected when hydrogen peroxide added to the inoculum a bacterial isolate culture the rapid elaboration of oxygen bubbles represent a positive catalase test. These are extremely toxic because they are powerful oxidizing agents and destroy cellular constituents very rapidly. A bacterium must be catalase O₂ products of superoxide to destruction the hydrogen peroxide; facultative anaerobes usually contain the catalase enzymes superoxide (Harly, 2002).

Catalase enzyme described by several researchers and the published data previously reported catalase positive (Heinen et al., 1982; Claus and Berkeley, 1986; Sharp et al., 1992; Rainey et al., 1994; Pikuta et al., 2000).



3. MATERIAL AND METHODS

3.1. Preparation of Media and Isolation of Bacteria

3.1.1. Preparation of nutrient broth medium(g/L)

0.5% Gelatin Peptone

0.3% Beef Extract

Gelatin peptone and Beef extract provide nitrogen, vitamins, minerals and amino acids essential for growth. Final pH 7 at 25°C

Preparation:

- Suspend 8 grams of the medium in one liter of distilled water.
- Heat this mixture while stirring to fully dissolve all components
- Boil for one minute until complete dissolution.
- Dispense into sterilize bottle in autoclave (HICLAVE 50L) at 121°C for 15 minutes.

3.1.2. Preparation of nutrient agar (solid medium)

Composition of Nutrient agar

0.5% Peptone

0.3% Beef extract/yeast extract

1.5% Agar

0.5% NaCl

Distilled water: pH is adjusted to neutral (7.4) at 25 °C.

Agar is a complex carbohydrate obtained from certain marine algae. It is used as a solidifying agent for media and does not have any nutritive value.

Preparation:

- Suspend 20g of nutrient agar powder in 1 liter of distilled water.Heat this mixture while stirring to fully dissolve all components.
- Autoclave the dissolved mixture at 121 degrees Celsius for 1 hour.
- Once the nutrient agar has been autoclaved.

- Prepare sterile petri dish and Pour nutrient agar into each plate and leave plates on the sterile surface on Laminar cabinet (NÜVE LN 090) until the agar has solidify.

3.1.3. Isolation of bacteria

- Weight 10 g of clay and soil samples.
- Put it in flask containing 90 ml of sterile water to obtain 10^{-1} dilutions.
- Homogenized the mixtures by shaking in shaker (JERO TECH SI-600), by repeating this process provided dilutions of 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} .
- Transferred 1 ml of the suspension, which were diluted at different rates, to sterile NB (Nutrient Broth) medium.
- Cultivations of all dilutions which performed in petri dishes.
- Spread the liquid with a sterile glass rod, and homogenizations were provided.
- To treatment the water samples that taken from hot spring in Turkey, and diluted suspensions that subjected it to heat at 80°C for 10 minutes before isolating the thermophilic microorganisms and the result of this process, the microorganisms that form spores survive, whereas other micro-organisms that do not form spores will die.
- Put all the petri dishes (that contain isolated thermophilic microorganisms) in incubation at 50°C for 24 hours, after 24 hours of incubation, different colonies will be formed.
- Transfer the colonies to solid medium (Nutrient agar) to obtain single cultures.
- With the help of platinum loop, the pure cultures of bacteria were transfer to NB liquid medium and incubated it for 24 hours at 50°C and at 110 rpm on a shaker.
- After the performing of some biochemical and molecular test, the bacteria were diagnosed. Also the 16 s ribosomal RNA diagnoses of bacteria were made by Özdemir et al., the Axes number and location of bacteria were given in Table 3.1.

Table 3.1. The name of studied bacteria and their location

Sample	Access Number (NCBI)	Name Hot spring/phase
BankIt1693947 Seq1	KJ434779 <i>Anoxybacillus</i>	Ömer/Soil
BankIt1693947 Seq2	KJ434780 <i>Anoxybacillus flavithermus</i>	Ömer/Soil
BankIt1693947 Seq3	KJ434781 <i>Anoxybacillus flavithermus</i>	Ömer/Soil
BankIt1688173 Seq4	KJ094998 <i>Anoxybacillus flavithermus</i>	Gecek/Soil
BankIt1693947 Seq5	KJ434782 <i>Bacillus firmus</i>	Ömer/Soil
BankIt1693947 Seq6	KJ434783 <i>Anoxybacillus sp</i>	Ömer/Water
BankIt1693947 Seq7	KJ434784 <i>Anoxybacillus flavithermus</i>	Ömer/Soil
BankIt1693947 Seq8	KJ434785 <i>Anoxybacillus flavithermus</i>	Ömer/Soil
BankIt1693947 Seq9	KJ434786 <i>Anoxybacillus flavithermus</i>	Gecek /Water
BankIt1688173 Seq10	KJ094999 <i>Anoxybacillus mongoliensis</i>	Ömer/Soil
BankIt1693947 Seq11	KJ434787 <i>Anoxybacillus flavithermus</i>	Ömer/Water
BankIt1693947 Seq12	KJ434788 <i>Anoxybacillus flavithermus</i>	Gecek/Soil
BankIt1688173 Seq13	KJ095000 <i>Anoxybacillus flavithermus</i>	Gecek/Soil
BankIt1693947 Seq14	KJ434789 <i>Bacillus cereus</i>	Ömer/Water
BankIt1693947 Seq15	KJ434790 <i>Anoxybacillus flavithermus</i>	Gecek/Soil&water
BankIt1693947 Seq16	KJ434791 <i>Bacillus cereus</i>	Ömer/Soil
BankIt1693947 Seq17	KJ434792 <i>Anoxybacillus flavithermus</i>	Gecek/Soil&water
BankIt1693947 Seq18	KJ434793 <i>Anoxybacillus kestanboliensis</i>	Ömer/Soil
BankIt1688173 Seq19	KJ095001 <i>Anoxybacillus flavithermus</i>	Gecek/Soil&water
BankIt1689720 Seq20	KJ095002 <i>Geobacillus stearothermophilus</i>	Gecek/Soil&water
BankIt1693947 Seq21	KJ434794 <i>Bacillus sp</i>	Ömer/Soil

3.1.4. Preparation of young bacteria

On the sterile surface in the laminar hood and near the Bunsen burner flames, the bacteria cultures were inoculated by each bottle media with one of the pure bacteria samples (Table 3.1). In a sterile loop, the pure bacteria cultures were taken and simply dipped into the bottle that contain liquid medium. Replace the cover of each bottle and put all the bottles that inoculated in the shaking incubator at 50 °C for 2 days for growing young bacteria. Finally also, Staphylococcus were inoculated and incubated at 37°C for 2 days.

Number 14 and number 20 samples were can't use because of their contamination with mold.



Figure 3.1. Laboratory studies

3.2. Biochemical Tests

3.2.1. Gram staining

Prior to imaging, purified bacteria were air dried and heat fixed on the glass slide and stained using Gram stain method. Fixed sample were covered with few drops of Gentian violet (which is a mixture of methyl violet and crystal violet) for a minute before it was rinsed with the tap water. Samples were treated with few drop of Gram's Iodine and allowed to act for a minute. Rinsed and dried. Then samples decolorized in absolute ethyl alcohol for about 30 seconds, rinsed and dried in air and heat fixed.

The stained samples were imaging using Olympus CX 31 system microscope equipped with Olympus SC 30 Digital color camera. Light microscopy imaging was done under oil immersion lens for highest magnification (X100).

Lugol solution: 1 g crystalline iodine, 2 g KI, 300 ml dH₂O, Çotuk and Küçükler, (1992).

3.2.2. Tween 80 hydrolysis test

Nutrient agar isolates which contained 3% Tween 80 were planted as an intense line with help of loop. Petri dishes were left for 1 days incubation at 50°C. Following incubation, at the rate of %0,001 Rhodamin B was added so as to enclose the surface of the petri dishes. Zones which occurred around the colonies were considered as positive for lipase activity (Karnetova et al., 1984).

Rhodamines B dye: Rhodamine B (0.001%) dye was used to determine the lipase test.

3.2.3. Xylene hydrolysis test

Nutrient agar isolates which contained 1% Xylene were planted in an intense line with the help of loop. Petri dishes were left for 1 days incubation at 50°C. After incubation zones which occurred around the colonies were considered as positive for xylene activity (Karnetova et al., 1984).

3.2.4. Skim milk powder hydrolysis test

Isolates were planted in Nutrient agar which contained 1% Skim milk powder in the form of an intense line with help of loop. Petri dishes were left for 1 day's incubation at 50°C. After incubation, a clear zone around the colonies was accepted as positive for protease activity (Yin et al., 2010).

3.2.5. Starch hydrolysis test

Nutrient agar isolates, which had 1% soluble starch, was planted in the form of an intense line with help of loop. Petri dishes were left for 1 days incubation at 50°C. Following the incubation, lugol solution was added in such a way that it overlaid on the medium. Clear zones, which were around the colony, as positive starch digestion and purple zones accepted as negative starch digestion were assessed in areas where the starch hydrolyzed (Aygan et al., 2008).

3.2.6. Urease test

Nutrient agar plates containing 0.3% uric acid will be sowed from alkalophilic isolates in a 20mm diameter area. Petri dishes will be left at 50 ° C for 24-48 h incubation.

The transparent zone formed around the planted area following incubation will be evaluated as positive for urease production (Honarbakhsh et al., 2014).

3.2.7. Asparaginase test

Nutrient agar plates containing 1% L-asparaginase milk dust will be inoculated on halophilic isolates in 20 mm diameter petri dishes. Petri dishes will be left at 50 ° C for 24-48 h incubation. Following incubation, 1% phenol red will be added to cover the surface of petri dishes. Transparent zone formation around the colonies will be evaluated as positive for L-asparaginase enzyme production (Krishnapura, 2015).

3.2.8. Catalase test

A small amount of organism were taken from a well-isolated 18- to 24-hour colony and placed it onto the microscope slide by a sterile inoculating loop and place 1 drop of 3% H₂O₂ onto the organism on the microscope slide. Observing for the formation of bubbles against a dark background enhances readability.

3.2.9. Antibiotic resistance test

This method is also called disk diffusion method, the procedure followed is simply that a filter disk impregnated with an antibiotic is applied to the surface of an agar plate containing the organism to be tested and the plate is incubated at 37°C for 24-48 hours. As the substance diffuses from the filter paper into the agar, the concentrations were decreases as a function of the square of the distance of diffusion. At some particular distance from each disk, the antibiotic is diluted to the point that it no longer inhibits microbial growth. The effectiveness of a particular antibiotic is shown by the presence of growth-inhibition zones (ZOIs) appear as clear areas surrounding the disk from which the substances with antimicrobial activity diffused. The agar diffusion method uses commercially available filter paper disks, each containing a defined concentration of a specific antibiotic. The relative effectiveness of different antibiotics provides the basis for a resistance spectrum of the organism. In this study the Oxoid mark of antibiotics were used, Chloramphenicol (30 mg), Ampicillin (10 mg), Rifamcyin (30 mg) and Erythrosine (10 mg) were used for anti-biogram.

4. RESULTS

4.1. Culture for *Anoxybacillus*

Anoxybacillus bacteria were transplanted on the Nutrient agar medium. In the first application, the bacteria cultures were incubated for 24 hr., however they were showed fast growing and covered all the plate, so cultures were transplanted again and incubated for 6 hr. The result showed creamy color, smooth, and circular without any pollution and the colonies appear in middle of each side of the two compartment petri dishes.

Table 4.1 The axes number of the final bacteria used for study.

Samples	Axes Number (NCBI)
Seq1	KJ434779 <i>Anoxybacillus flavithermus</i>
Seq2	KJ434780 <i>Anoxybacillus flavithermus</i>
Seq3	KJ434781 <i>Anoxybacillus flavithermus</i>
Seq4	KJ094998 <i>Anoxybacillus flavithermus</i>
Seq5	KJ434782 <i>Bacillus firmus</i>
Seq6	KJ434783 <i>Anoxybacillus</i> sp
Seq7	KJ434784 <i>Anoxybacillus flavithermus</i>
Seq8	KJ434785 <i>Anoxybacillus flavithermus</i>
Seq9	KJ434786 <i>Anoxybacillus flavithermus</i>
Seq10	KJ094999 <i>Anoxybacillus mongoliensis</i>
Seq11	KJ434787 <i>Anoxybacillus flavithermus</i>
Seq12	KJ434788 <i>Anoxybacillus flavithermus</i>
Seq13	KJ095000 <i>Anoxybacillus flavithermus</i>
Seq 15	KJ434790 <i>Anoxybacillus flavithermus</i>
Seq 16	KJ434791 <i>Bacillus cereus</i>
Seq17	KJ434792 <i>Anoxybacillus flavithermus</i>
Seq18	KJ434793 <i>Anoxybacillus kestanboliensis</i>
Seq19	KJ095001 <i>Anoxybacillus flavithermus</i>
Seq21	KJ434794 <i>Bacillus</i> sp
S	<i>Staphylococcus</i>

4.2. Gram Stain Test

The first test was performed as gram test. The test is study on staining of bacteria with gram dye. In this study all strains of bacteria was shown positive results with gram stain test. Also, the cells are violet color, rod-shaped and straight, often arranged in pairs or chain.

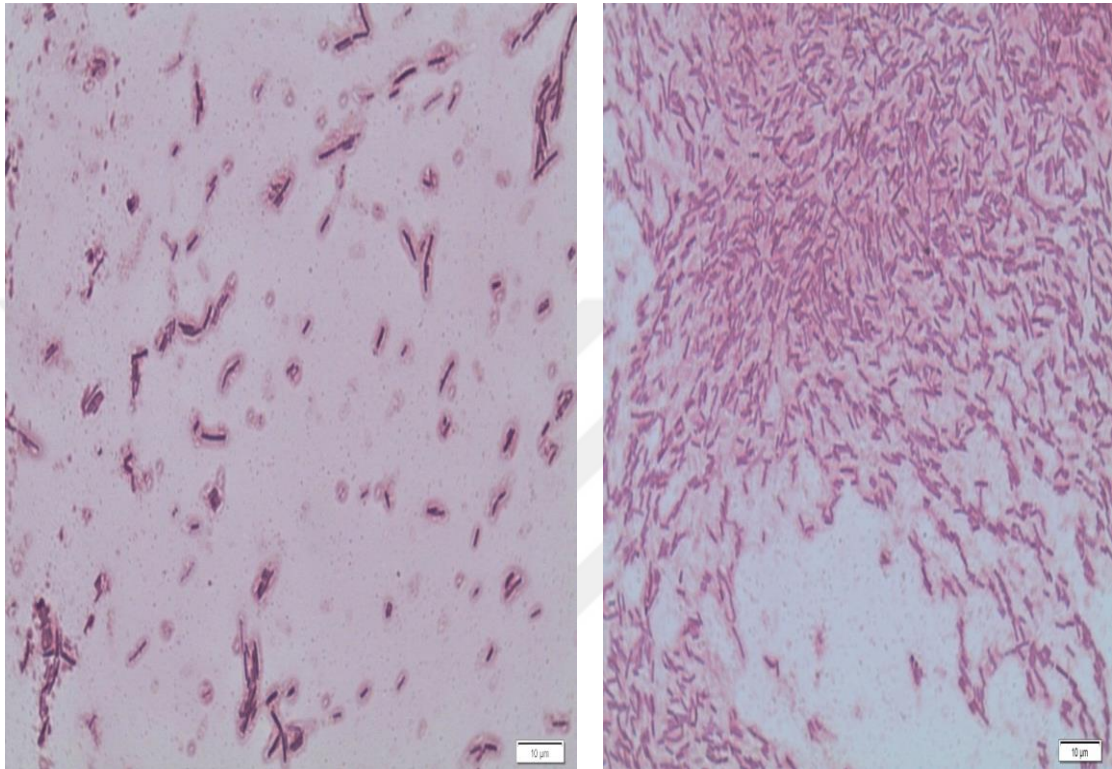


Figure 4.1.The samples of Gram stain

4.3. Lipase Hydrolysis Test

Some bacteria have the ability to hydrolyze lipids (fats) to glycerol and fatty acids, as they possess the lipolytic enzyme ‘lipase’. These bacteria are called lipolytic bacteria. The bacteria were checked for lipase test. In this study, Tween 80 was used for medium; all the bacteria that we used show negative results.

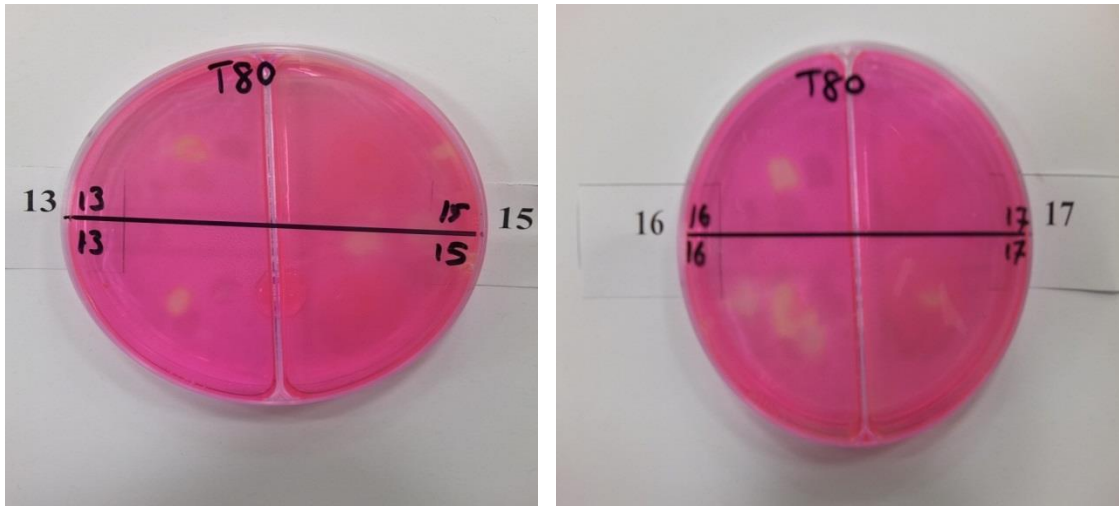


Figure 4.2.Samples of lipase hydrolase test

4.4. Xylenase Hydrolysis Test

The xylenolytic enzyme system that carries out the xylene hydrolysis is normally composed of a repertoire of hydrolytic enzymes, All of these enzymes act cooperatively to convert xylan into its constituent sugar. According to our results There is no zones appear around the colonies of our bacteria that we used in this study, so the result is negative for this test.

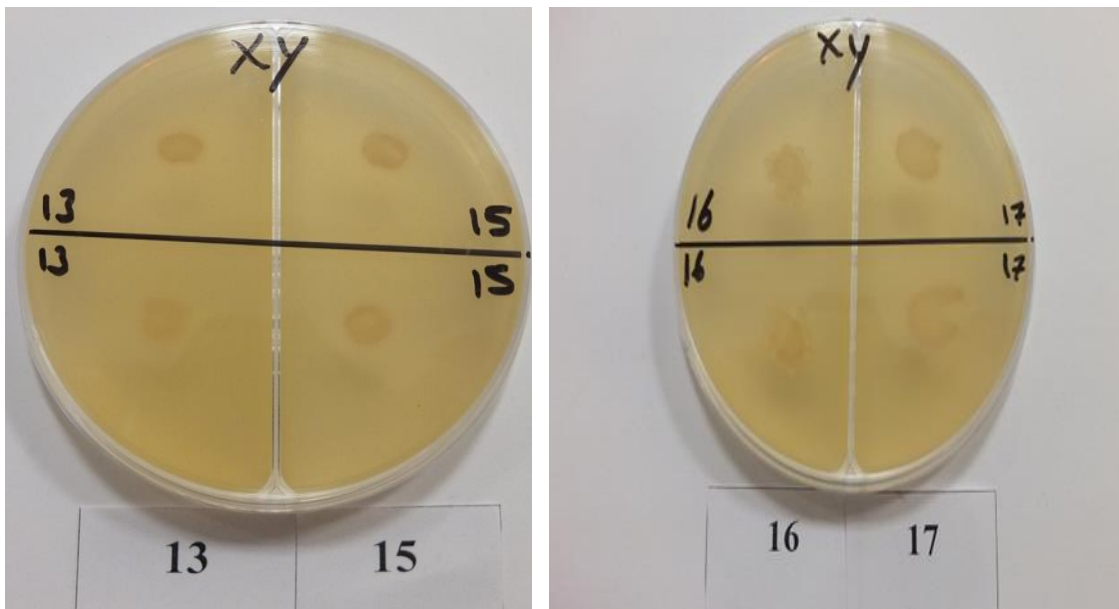


Figure 4.3.The samples of xylenase hydrolysis test.

4.5. Skim Milk Hydrolysis Test

Gasein is a large polymer of amino acids that make around 85% of the protein found in milk as well as the white color of milk. Bacteria cells secrete Gaseinases outside of the cell that hydrolyze the protein in steps to amino acids. If a clear zone, a zone of casein hydrolysis, evaluated it as positive. Otherwise, score it as negative.

In this study 16 strains show positive result including KJ434779, KJ434780, KJ434781, KJ094998, KJ434782, KJ434783, KJ434784, KJ434785, KJ434786, KJ094999, KJ095000, KJ434790, KJ434791, KJ434792, KJ434794 and Staphylococcus and other isolates shows negative.

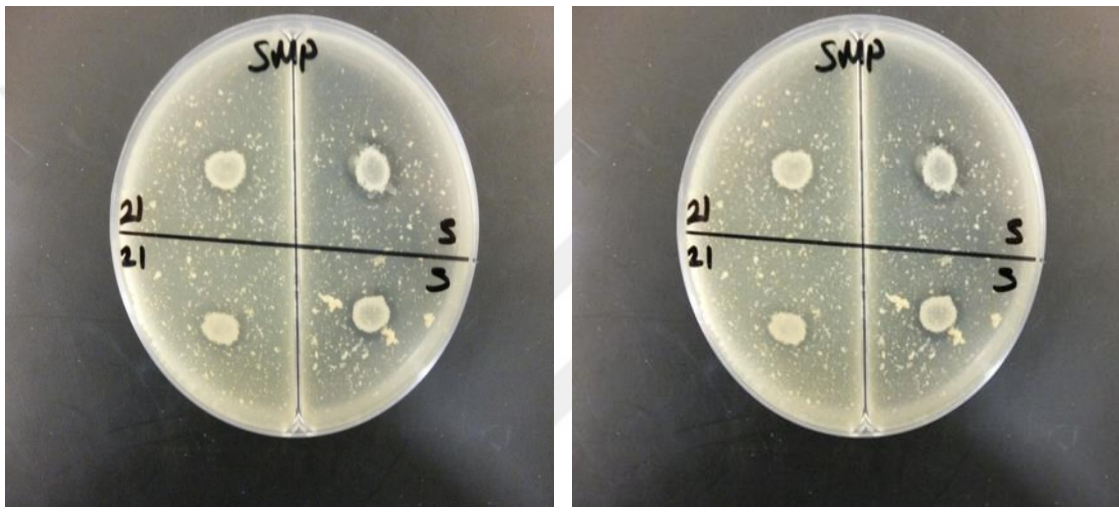


Figure 4.4. The samples of skim milk test

4.6. Amylase Hydrolysis Test

Starch is a polysaccharide made up of -D-glucose subunits. It exists in two forms amylose and amylopectin are bonded by 1,4—glycosidic, amylase and oligo-1,6-glucosidase are able to hydrolyze starch by breaking the glycosidic linkages between the sugar subunits and Iodine reagent reacts with starch and produces a blue or dark color; therefore, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth. In this study, all strains show positive result.

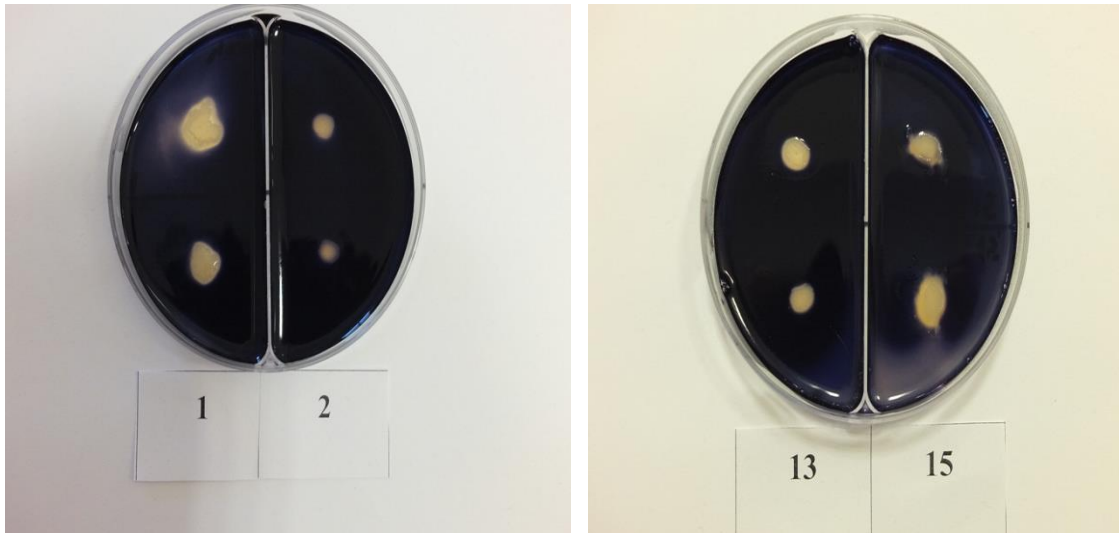


Figure 4.5.The samples of amylase hydrolysis test

4.7. Urease Hydrolysis Test

Urease enzyme attack nitrogen and carbon bond in compounds such as urea, forming the end products ammonia, CO₂, and water. In this study, 15 strains show positive result including KJ434780, KJ094998, KJ434782, KJ434783, KJ434784, KJ434784, KJ434785, KJ434786, KJ094999, KJ434787, KJ434788, KJ095000, KJ434790, KJ434792, KJ434793, and KJ434794. and other isolates shows negative.

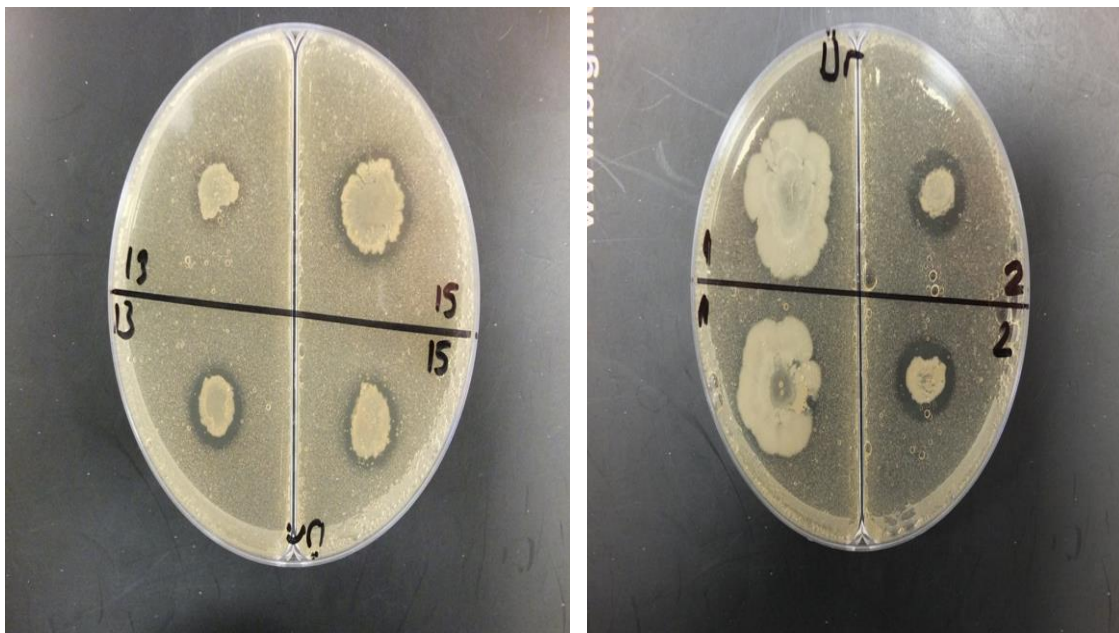


Figure 4.6.The samples of urease hydrolysis

4.8. Asparaginase Test

Asparaginase enzyme hydrolyzes the amino acid asparagine to NH_4 and aspartate, in this study, the result was negative.

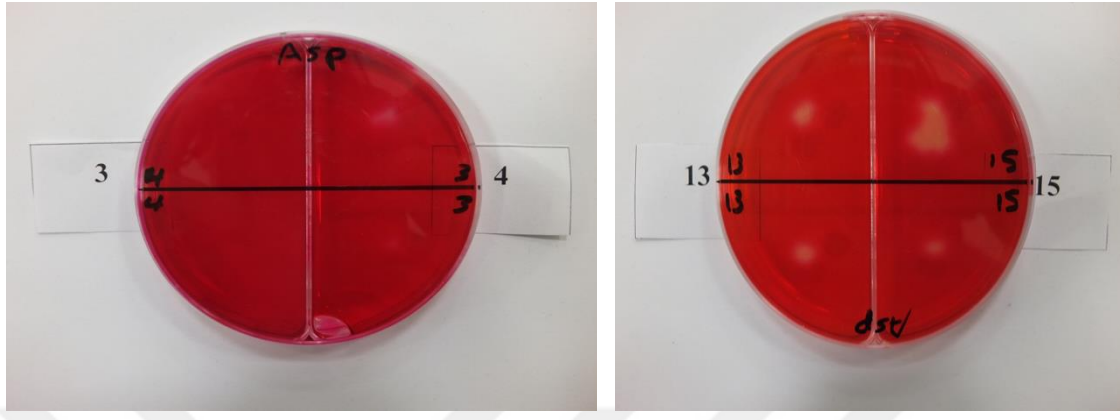
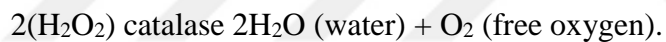


Figure 4.7. The samples of Asparaginase test

4.9. Catalase Test

Some bacteria contain flavin-proteins that reduce O_2 , resulting in the production of hydrogen peroxide (H_2O_2) or superoxide (O_2^-).



Catalase activity can be detected by adding the 3% H_2O_2 , catalase test was applied to all bacteria strain that we isolated it in this study and all shows bubbles of O_2 , so the result is catalase test positive.

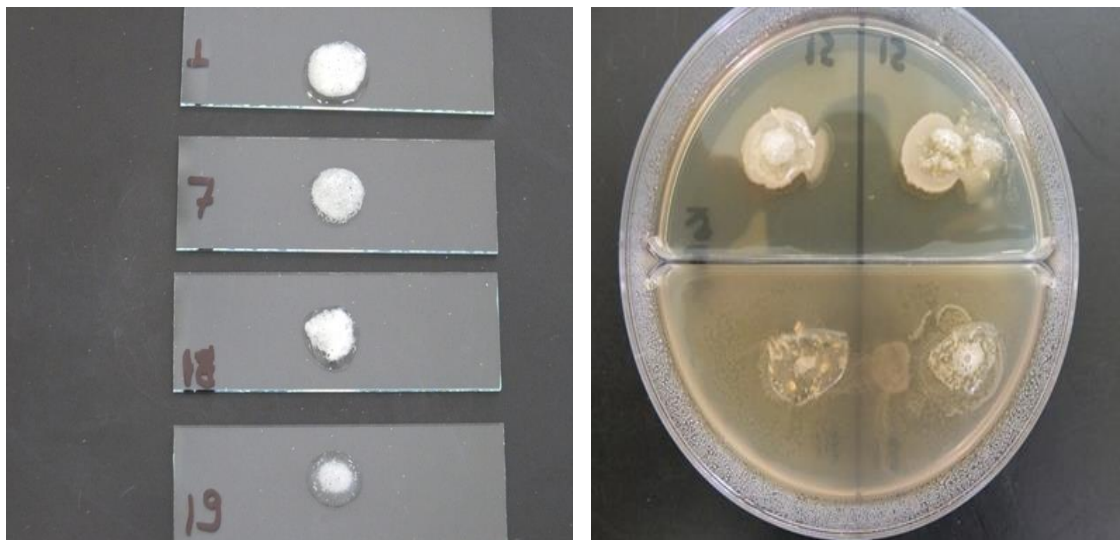


Figure 4.8. The samples of catalase test

4.10. Antibiotic Resistant Test

Antibiotics are natural antimicrobial agents produced by microorganisms. This test used to measure the effectiveness of antibiotics on microorganisms. A clear zone will appear around the disk where growth has been inhibited, the size of this zone of inhibition depends upon the sensitivity of the bacteria to the antibiotic and the point at which the chemical's minimum inhibitory concentration (MIC) is reached. The zone of inhibition appears surrounding the 4 types of antibiotic disk, were used in this study which are Chloramphenicol, Ampicillin, Rifamcyin and Erythromycine. Table 4.2.

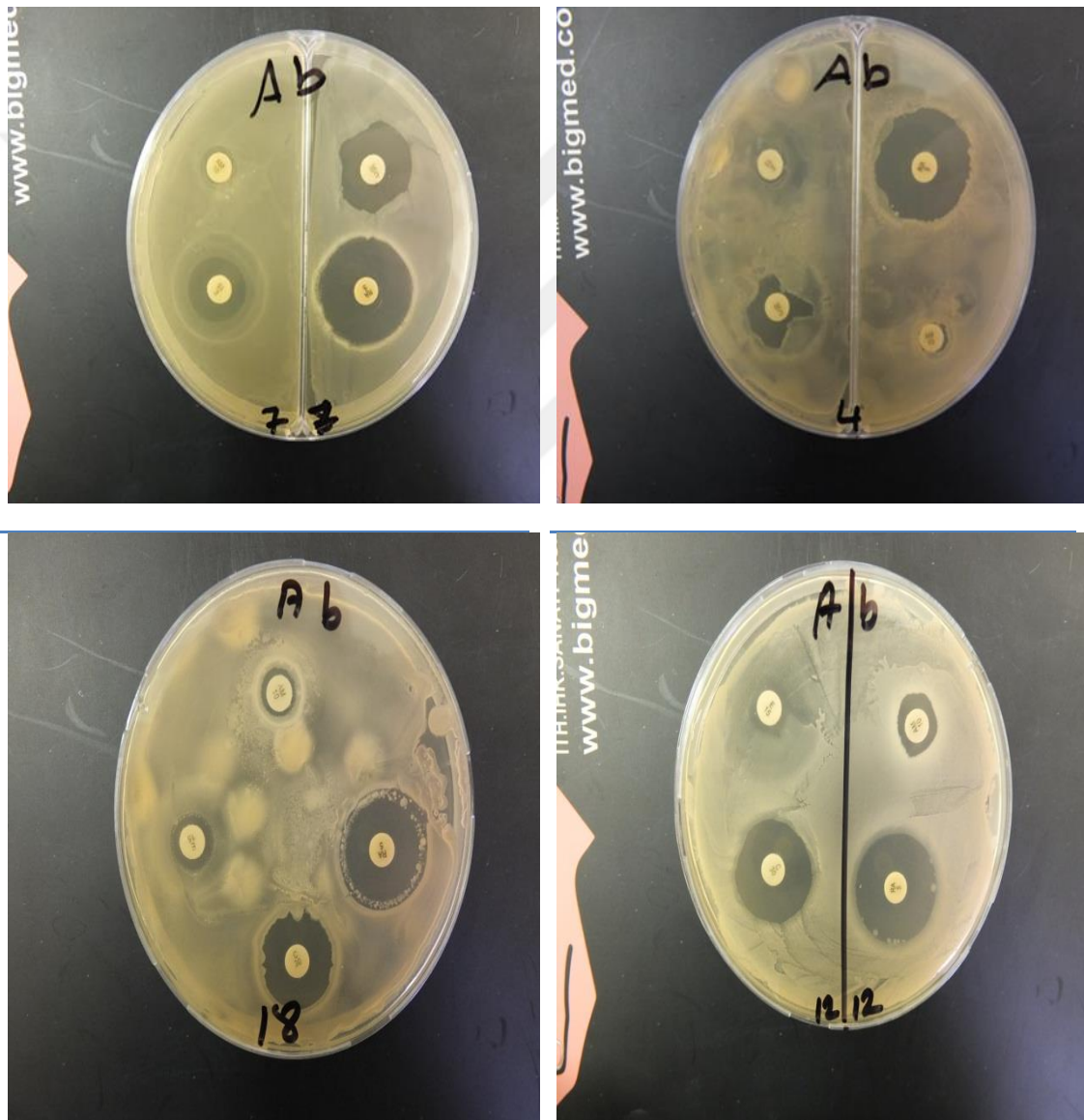


Figure 4.9. The samples of antibiotic test

Table 4.2. The results of antibiotic resistances tests

	Bacteria's name and no.	Chloramphenicol (mm)	Ampicillin (mm)	Rifamycin (mm)	Erethromycin (mm)
1	<i>Anoxybacillus flavithermus</i> KJ434779	15	10	21	17 *
2	<i>Anoxybacillus flavithermus</i> KJ434780	23	12	25	18
3	<i>Anoxybacillus flavithermus</i> KJ434781	17	10	22	12
4	<i>Anoxybacillus flavithermus</i> KJ0949	15	7	24	20 ●
5	<i>Bacillus firmus</i> KJ434782	12	8	24	11
6	<i>Anoxybacillus</i> sp. KJ434783	19	10	22	10
7	<i>Anoxybacillus flavithermus</i> KJ434784	20	8	24	15
8	<i>Anoxybacillus flavithermus</i> KJ434785	21	8	23	9
9	<i>Anoxybacillus flavithermus</i> KJ434786	16	7	23	8
10	<i>Anoxybacillus mogoliensis</i> KJ094999	20	6	24	8
11	<i>Anoxybacillus flavithermus</i> KJ434787	20	7	20	7
12	<i>Anoxybacillus flavithermus</i> KJ434788	21	12	23	12
13	<i>Anoxybacillus flavithermus</i> KJ095000	17	8	24	11
15	<i>Anoxybacillus</i> flavithermusKJ434790	20	9	21	10
16	<i>Bacillus cereus</i> KJ434791	21	8	16	20
17	<i>Anoxybacillus flavithermus</i> KJ434792	24	15	24	11
18	<i>Anoxybacillus kestanboliensis</i> KJ434793	19	18	19	10
19	<i>Anoxybacillus flavithermus</i> KJ095001	22	8	18	11
21	<i>Bacillus</i> sp. KJ434794	19	11	20	12
S	<i>Staphylococcus</i>	22	12	20	18

(Two zone occur around the bacteria; *first diameter is 10 mm.; ● is 9 mm.

5. DISCUSSION

In order to identify and characterize biotechnologically interesting bacterial strains, samples were taken from extreme environments of hot spring from Afyon City from Turkey. Following golden standard molecular identification, 16S RNA analysis, 21 bacterial isolates were identified. These strains were further characterized for both antibiotic and biochemical tests.

For many decades ago, scientists have been intrigued by the wonderful organisms that inhabit extreme environments and at extreme conditions. These organisms, known as extremophiles, they can life and thrive under conditions that, from a human point of view, are clearly extreme or even lethal. Different types of microorganisms are adapted to living in extreme conditions such as salt solutions, hot niches, ice, pressure, as well as chemical concentration and have shown grow, thrive, tolerate, and survive to these conditions. Some of these organisms may grow in heavy metals, toxic waste, organic solvents. Extreme tolerant organisms; which can tolerate extreme values of one or more physicochemical parameters though may grow optimally at normal conditions. These Extremophiles include members of bacteria of *Bacillaceae* family (Pabulo Henrique, 2013).

Bacillaceae Gram-Positive cell wall, rod-shaped, and spore formation that provide high resistance to chemicals, heat, radiation, and drought, allowing these bacteria to survive at extreme conditions for a prolonged period of time. They are heterotrophic, some members of *Bacillaceae* are aerobic or facultative anaerobic, motile members of this family are characterized by flagella. The *Bacillaceae* family members bacteria have important role in bioprocessing and biotransformation, and because of these bacteria are alkali-tolerant thermophiles, their enzymes are useful for many industrial applications (Colin, 1989).

Extremozymes are specialized enzymes that are highly stable, can remain active when other normal enzymes would typically fail. Extremozymes are extremely thermostable and usually resistant to the action of chemical denaturants, detergents, chaotropic agents, organic solvents as well as to the exposure to extreme values of pH. They speed up chemical reactions and can make major contributions to industry, biomedical research, etc. (Çanakcı et al., 2007)

A test for the morphological and physiological identification of the obtained isolation was conducted. Gram, and spore staining methods and motility tests were used in order to determine the characteristics of the bacterium. Through biochemical tests

(starch, gelatin and casein hydrolysis, catalase, urease and lipase activities, etc.) some characteristics of the isolates were determined and comparison was made.

Ribosomal RNA (r RNA) 16S is the constituent RNA of the small ribosomal sub-unit of prokaryote 30S. r RNA encoding by gene is called 16S r RNA gene (Clarridge, 2004). It is different in number of copies but it present in all bacterial species (Petti, 2007). It is composed of 1500 nucleotides and contains 9 hyper variable regions. This relationship between the conserved regions and variable regions theoretically allows to using this gene to detect and identify all bacterial species. Before 30 years ago the scientists determined the phylogenic relationships of living beings by comparing of their nucleic sequences and used it to study the evolution, taxonomy and phylogeny of the bacteria (Woese CR., 1987).

Different strains of bacteria identify and detect depending on phenotypic characteristics such as staining, ability to grow on some culture media, morphology, biochemical test, but there is some bacteria are inaccurate identified phenotypic characters for various reasons: some bacteria are difficult to cultivate, a few number of phenotypic characters are expressed for strain or sub strain, old culture and stress may have altered the phenotypic characters and the absence of databases for rare bacteria of systems available on the market, therefor, PCR targeting the gene encoding 16S ribosomal RNA it's a broad-range technique, accurate and reliable, for amplification and sequencing and comparison the result with the sequence that obtained from databases have proved their value for bacterial identification (Petti et al.,2005).

After performing of molecular test (16 s r RNA) from Özdemir et al., the 16S rRNA gene sequence analysis showed that bacteria number 1,2,3,4,6,7,8,9,10,11,12,13,15,17,18,19 were identified as representatives of the same genus which is *Anoxybacillus* genus. And bacteria no.5, 16, 21 were diagnosed to *Bacillus* genus; these bacteria were identified according to NCBI (National Center for Biotechnology Information).

But molecular identification (based on 16S RNA) can't be the only to screen for biotechnologically interesting strain because of some problem like: its expensive Cost and lack of automatization technique was a limiting factor for its global use (Kiratisin et al.,2003), large volume of the test sample tested need for detection threshold for end-point PCR may lead false negative results (Petti, 2007) and if any contaminations occur during working and preparation of reagent mix, DNA extraction or gene amplifications it make it difficult to explain results and response false positive results (Sontakke et al., 2009).

Antibiotics are the most commonly prescribed in the world. When they were first developed in the 1940s, antibiotics were hailed as a miracle breakthrough in the treatment of previously incurable bacterial infections. but the over-prescription and misuse of antibiotics resulted in the development of resistant strains of various pathogens (Raghunath, 2008). So, the incessant research for discovering new class of antibiotic from bacteria at different locations with extreme conditions, they found the soil is one of the best explored locations for bioprospecting. In recent times, symbiotic associations and hypersaline environments have been investigated for novel antimicrobial compounds (Girish and Lakshmi, 2016).

Thermophilic microorganisms which isolated from hot springs are less discovered compared with other terrestrial samples (Auguet et al., 2010). It demonstrates extreme environmental conditions such as higher temperature and alkaline pH (Farmer, 2000). The industrial biotechnology discusses to use the genetics in the production of medical pharmaceuticals and other products. It includes medical biotechnology (protein production system, therapeutic proteins, antibiotics, and biopharmaceutical markets), enzymes, bioremediation, biofuels, primary metabolites, biopolymers and bio-plastics, and agricultural biotechnology (Preeti and Arnold, 2016).

Chloramphenicol is an antibiotic first isolated from cultures of *Streptomyces venequelae* in 1947. It's semisynthetic, broad-spectrum, primarily bacteriostatic activity but may be bactericidal in high concentrations. Chloramphenicol act by interfering with bacterial protein synthesis, it diffuses through the bacterial cell wall and reversibly binds to the bacterial 50S ribosomal subunit and these bindings interferes with peptidyl transferase activity, leading to prevent the transfer of amino acids to the growing peptide chains and finally blocks peptide bond formation, blocks protein synthesis and impede proliferation of bacteria cell.

(<https://pubchem.ncbi.nlm.nih.gov/compound/chloramphenicol>).

Chloramphenicol have serious side effect to human for example aplastic anemia and bone marrow damage it used in treatment of serious and life-threatening infections like cholera; it destroys the *vibrios* and decreases the diarrhea, and typhoid fever. Chloramphenicol used in treatment the superficial infections caused by susceptible organisms, such as *Staphylococcus aureus*, *Escherichiacoli*, *Klebsiellapneumonia*, *Hemophilusinfluenza*, *Pseudomonasaruginosa*, *Proteus* species and *Enterobacteraero genes* (Thomson, 2004).

Ampicillin is a penicillin beta-lactam antibiotic, a broad-spectrum, semi-synthetic, derived from the *penicillins*, bactericidal activity (<http://www.drugbank.ca/drugs/DB00415>). It's active against gram-positive bacteria includes *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Enterococcus* and gram-negative bacteria includes *Neisseria meningitidis*, *Haemophilus influenzae*, *Enterobacteriaceae* (Hauser AR, 2013). Ampicillins binds to penicillin-binding proteins (PBP) which located in the inner membrane of the bacterial cell wall and inactivated it, inactivation of PBPs interferes with the cross-linkage of peptidoglycan chains necessary for bacterial cell wall rigidity and strength, this interrupts in the cell wall synthesis and then the cell wall of the bacteria become weak and causes cell-lysis (<https://ncit.nci.nih.gov/ncitbrowser/ConceptReport.jsp>). Ampicillin is comparatively less toxic than other antibiotics it causes severe side effects such as angioedema, anaphylaxis, diarrhea, nausea, vomiting and rash, itching (Davis. 2017).

Rifamycins are semisynthetic antibiotics , first isolated in 1957 from a fermentation culture of *Streptomyces mediterranei* at the laboratory and based on 16S ribosomal RNA sequences, Bala et al. renamed the species in 2004 *Amycolatopsis rifamycinica*. Rifamycin inhibits the synthesis of bacterial DNA-dependent RNA (Calvori et al., 1965) which appears to occur as a result of drug binding in the polymerase subunit deep within the DNA/RNA channel, facilitating direct blocking of the elongating RNA (Campbell et al.,2001).

Erythromycin is a macrolide antibiotic, it was first isolated in 1952 from *Saccharopolyspora erythraea*, Antibacterial activity was confined to gram-positive and very few gram-negative bacteria (Kibwage et al., 1985). Erythromycin inhibits protein synthesis of the bacteria by binding to the 50S ribosomal subunits; binding inhibits peptidyl transferase activity and this interferes with the production of functionally useful proteins, which is the basis of this antimicrobial action (Vedas, 2000).

In this study, 21 strains (isolated on Nutrient agar at 50°C from samples from hot springs from Avyon city ,Turkey) were tested against different antibiotic to screen for candidate isolates that may serve as a candidate one for further characterization and evaluating the possibility of possessing a novel plasmid that may be used as cloning vector, In this study, six isolate were obtained as resistant against chloromephnecol ;five of these isolated was *Anoxybacillus flavithermus* no.1, 4, 9, 3, 13, which their result(15, 15, 16, 17, 17) mm consecutive, and the sample no. 5 which is *Bacillus firmus* was more resistance among chloromephnecol with result 12mm. For ampicillin antibiotic, three

ampicillin resistance were obtained from *Anoxybacillus flavithermus*, which numbered as sample no. 4, 9, 11 which their result was 7mm, *Anoxybacillus mongoliensis* was more resistance than *A. flavithermus* with result 6mm and *A. kestanboliensis* was sensitive to ampicillin with wide range of zone about 18mm. In case of erythromycin there is four strains was erythromycin antibiotic-resistant and their numbers are 8, 9, 10, and 11 with results 9, 8, 8, 7 consecutive. These thermophilic bacteria differed morphologically as well as microscopically, the resistant occurred perhaps due to presence of resistance genes to this antibiotic possibly expressed in that particular isolate or the mutation in the gene may have occurred. The spreading of antibiotic resistance commonly due to transfer of antibiotic resistant genes and its get conveyed by plasmids and this dissemination is rapidly occur and the horizontal gene transfer is common among bacteria even distantly related ones (Amabile and Chicurel, 1992) This process is thought to be an important cause of increased drug resistance when one bacterial cell acquires resistance, it can quickly transfer the resistance genes to many species (Raghunath, 2008).

Several vectors developed the Molecular cloning systems in *Bacillus subtilis* (Ehrlich, 1978), some of these vectors can transfer into other *Bacillus* species; such as pUB10 was introduced into *Bacillus megaterium* by protoplast transformation (Vorobjeva et al. 1980) and into *Bacillus pumilus* and *Bacillus licheniformis* by transduction (Keggins et al., 1978 and Bingham et al., 1979), they isolated from thermophilic bacilli 4 plasmids just one of those plasmids could transform *B. subtilis*, conferring tetracycline resistance on the host bacterium. There is a little of drug resistance plasmids in thermophilic bacilli and these specific plasmid which isolated from the natural environment is worthy of attention. Further exploitation of these plasmids and their application as shuttle vectors is now in progress, for this, it provides a motive to looking and search for other plasmids that could serve as cloning vectors and these search must be supported by another approach to explain the transformation system in thermophilic bacilli.

Amylases enzyme

It's known that, the starch molecule made up of two parts: amylose (un branched glucose polymer 200 to 300 units) and amylopectin (a large branched polymer). By amylases enzyme, bacteria hydrolyze both amylose and amylopectin to yield glucose, dextrin, and maltose After Adding Gram's Iodine, the complete breakdown all the starch and it is appear by the clear (white) halo around the colony ,this indicate Positive hydrolysis (Harley, 2002).

In this study, the starch hydrolysis test for 21 strains isolated from hot spring in Turkey showed positive result for all strains and this result agrees with the concept of a study conducted previously in Afyon karahisar, Turkey by a team of researchers, who found that amylase enzyme positive of the *Anoxybacillus flavithermus* Ozdemiret al., 2015. Also a similar result was obtained from a study in Ramadi, Iraq which found same result (Dhafer, 2007).

Catalase enzyme

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments. Catalase is the enzyme that breaks hydrogen peroxide (H₂O₂) into H₂O and O₂ to neutralize toxic forms of oxygen metabolites; H₂O₂. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor.

Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor. Catalase was one of the first enzymes to be purified to homogeneity, and has been the subject of intense study (Paul and Janet, 2008).

The current study showed that all 21 strains which isolated from hot spring in Turkey positive result for catalase test and this result are in agreement with most published data, which described the catalase (Heinen et al. 1982; Claus and Berkeley, 1986; Sharp et al. 1992; Rainey et al. 1994; Pikuta et al., 2000; Belduz et al., 2003). It's also agrees with the concept previously reported by several researchers, who found that catalase enzyme positive of the *Anoxybacillus flavithermus*. (Ozdemir et al., 2015; Dhafer, 2007; Jun Dai et al., 2011)

Urease enzyme

Urease (urea amido-hydrolase; E.C.3.5.1.5) is a highly efficient catalyst for the hydrolysis of urea with a rate approximately 10¹⁴ times the rate of the non-catalyzed reaction. Urease played a decisive role in proving the protein nature of enzymes. The presence of sulfhydryl groups in a protein was first described for urease (Sumner, 1951). Urease has been isolated from various bacteria, fungi, and higher plants. The primary role of ureases is to allow the organism to use internally and externally generated urea as a nitrogen source. The product ammonia can be taken up and utilized by soil microbes, which explains the wide use of urea as a nitrogen fertilizer, its high nitrogen content and

low cost (Mobley and Hausinger, 1989). Recently, urease molecular structure and catalytic mechanism were reviewed by applications in chemical and clinical analysis, water reclamation in spacecraft. Biochemical reaction engineering, environmental protection, biomedical engineering, and feed production (Mobley and Hausinger 1989, Zerner, 1991, Jabri et al. 1995, Karplus et al. 1997 and Ciurli et al. 1999).

In current result, negative result were obtained for sample no. 1,3,19 of *Anoxybacillus flavithermus* and this result supported published data, which described the urease enzyme negative (Pikuta et al., 2000; Belduz et al., 2003). In contrast to these, positive result were obtained for urease enzyme in *Anoxybacillus flavithermus* numbered as; no. 2, 4, 7, 8, 9, 11, 12, 13, 15, 17. And positive result to *Anoxybacillus mongoliensis* sample no.10 and *Anoxybacillus kestanboliensis* sample no.18. The result of this study agree with the concept previously reported by Filippidou et al., (2015) who found urease enzyme positive for *Anoxybacillus geothermalis* Strain GSsed3.

Casein enzyme

Bacteria cells secrete proteolytic exoenzymes that utilize of casein. Casein hydrolysis is tested by growing an organism on a skim milk agar plate (providing nutrients and the casein) and then checking the plates for hydrolysis. This test used to identifying bacteria that grow in milk, and differentiating among *Bacillaceae*, *Enterobacteriaceae*, and several other families.

The positive result of casein indicated in *Anoxybacillus flavithermus* of this study agree with the concept previously reported by several researcher who found casein positive (Pikuta et al., 2000; Belduz et al.,2003; Yavuz et al., 2004).

Also, negative results were obtained for sample no.11, 12, and 19 of *Anoxybacillus flavithermus* (Table 4.1) for casein hydrolysis. The current results coincide with the results obtained by Grigor et al., (2017) who showed that casein negative for *A. flavithermus* DSM 2641. A possible explanation for this finding is that may be the mutation in the gene may have occurred, or the sample no. 11, 12, 19 sub strain of *Anoxybacillus flavithermus*.

This tool for bacterial identification is merely dependent on the consensus sequences among bacterial species. However, it does not reflect the physiological variations among bacterial strains. Since microbial adaptive physiology to environment is highly variable among the same species. Therefore, biochemical tests represent a reliable approach that reflects the actual bacterial physiology. Furthermore, this tool

enables researchers to screen for novel bacterial strains that may have a potential enzymatic activity which have not been documented before.

Recently, academic and industrial laboratories demonstrated that the big potential of extremophiles in the industrial biotechnology by study the new isolated extremophilic microorganism and analysis their genomes and investigations of their enzymes (Egorova and Antranikian, 2005). Because of the exceptional characteristic of thermophile, their potential applications in industry and medical biotechnology are far reaching, ranging from production bio-battery to production of substances of medical use. Thermophilic microorganisms can produce thermostable enzymes which have a great commercial application area (Fincan and Enez 2013). Due to their enzymes are stable and not being denatured they have been arousing so much interest (Cordeiro et al., 2002). Among these thermophilic microorganisms, *Anoxybacillus* was the most of the reported data has revealed that to produce interesting enzymes. Because these bacteria are alkali-tolerant thermophiles, they are benefits for many industrial applications related to enzyme technology, environmental waste treatment, starch and lingo-cellulosic biomasses, and genomic analysis.

Anoxybacillus flavithermus isolated represent a promising candidate for many kind of biotechnology approach. The extracellular xylanase crude extract produced by this bacterium with the best inducer substrate was further characterized for their optimal temperature, pH, and stability (Goh et al., 2013). The ability of xylanases to produce xylose from commercial xylan, has an enormous economic potential for the conversion of plant biomass into fuels and chemicals (Coughlan and Hazlewood, 1993). Its complete hydrolysis is important in order to obtain, in high yields, monosaccharides like d-xylose and l-arabinose which could find applications in the food and fuel industries (Kim and Oh, 2003). The ability of *Anoxybacillus flavithermus* to remove dyes from a textile polluted effluent by remediation strategy consisting of a sequential biological and physical process is proposed (Álvarez et al., 2013). Biosorption of pesticides by *Anoxybacillus flavithermus* (Ozdemir et al., 2011). *Anoxybacillus flavithermus* played a role in enhancing the settle ability of sludge from sewage (Zitomer et al., 2007).

CONCLUSION

In present study, all of the identified bacteria belonging to the family *Bacillaceae*, *Bacillus* and *Anoxybacillus* genus, they are thermophilic bacteria can live and survive at high temperature and pH and under extreme environmental condition. *Anoxybacillus flavithermus* is a relatively new species and the Knowledge of the physiology,

metabolism, and metabolomics of the *Anoxybacillus flavithermus* is incomplete, quite limited, and seldom compared with other members of *Bacillaceae* that well-studies. Most of the reported data has revealed that this strain produce interesting enzymes that are thermostable and tolerant to alkaline pH. The importance of these bacteria has increased as the source of thermostable enzymes, thermal stability enables these enzymes to be active in the presence of chemical denaturants and to resist harsh process conditions, and these features are thus mutually beneficial for the industry and biotechnology in different areas.

In this study, hydrolytic enzymes of some *Anoxybacillus* strains from hot spring samples from Afyon, Turkey were screened. In order to identify these strains, some biochemical tests and 16S rRNA gene analysis were performed. As a result, it was identified as a strain of *Anoxybacillus flavithermus*. Isolates were first Gram stained and examined under the light microscopy. Catalase tests were performed. The isolates were then subjected to some physiological tests on nutrient agar plates for 1–2days: growth at 50C and pH 7. Isolates were also screened for the extracellular enzyme hydrolysis at 50°C such as starch, xylanase casein and asparaginase hydrolysis and catalase, lipase and urease activities were examined. Also antibiotic resistant tests were performed to the isolated bacteria by using four different (chloramphenicol, ampicillin, rifamycine and erythromycin) antibiotics.

RECOMENDAL

- Increase education on biotechnology and enzymatic processes and increase openness on production the enzymes and used it in the industry.
- More research for looking plasmids that could serve as cloning vectors to drug resistance in thermophilic bacilli and these searches must be supported by another approach to explain the transformation system in thermophilic microorganisms.
- The study of characteristics of antibiotic plasmids isolated from extreme condition lead to study the expression of gene at high temperature, and also to amplify the production of their thermostable enzymes.
- It's very important to have the information as how thermophilic bacteria contaminated and forms biofilms with in a milk powder manufacturing plants.
- Because of extensive use of heat as a preservation technology, Thermophilic bacteria used in wide range in Biotechnology industry.

- The potential of Hot-springs microbes as source of antibiotic drugs seems to be promising had shown broad spectrum antibacterial activity including some infections caused by pharmaceutical bad bugs.



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