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Research article

Determination of extracellular hydrolytic enzyme capabilities of some *Anoxybacillus* isolated from hot spring environments

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Abstract

The development of microbial enzymes was a crucial event in the industrial sectors as a result of the tremendous growth of biotechnology in recent years. Popularity of waste management and bioremediation processes have both made extensive use of microorganisms' whole cells and their enzymes. The pharmaceutical, textile, food, cosmetics, leather, paper, energy, biomaterials, fine chemicals, cellulose, and detergent sectors are some of the uses area of microbial enzymes. Depending on different uses, researchers can search for novel bacterial strains that might exhibit previously unrecognized enzymatic activity. Also for searching for plasmids that could be used as cloning vectors to tackle medication resistance in thermophilic microorganisms. The *Anoxybacillus flavithermus* bacteria, which were isolated from a hot spring in the Turkish city of Afyon, was employed in this investigation. The ability of the identified bacteria to produce extracellular hydrolytic enzymes was tested. For this, the activities of catalase, urease, and lipase as well as the hydrolysis of starch, casein, xylan, and asparagine were researched. Additionally, tests for antibiotic resistance were studied on the isolated bacteria using four different antibiotics (erythromycin, chloramphenicol, rifamycin, and ampicillin). All identified strains fermented starch as carbon and energy sources, and after 24 hours of incubation, amylase activity was detected at 50°C and pH 7.0. All strains were determined to be catalase-positive, and with a few exceptions, the majority of *A. flavithermus* strains were also found to be urease and caseinase positive. Industrial products that can be obtained from bacteria found in extreme environments will be effective in the development of future technology.

Keywords: *Anoxybacillus flavithermus; bacillaceae; casienase; extremozyme; gram-positive*

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 determined by Fisher in 1895 belong to phylum Formicates. Bacillaceae are belong to Gram-positive family, shaped by rod- or coccus, 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 (known as *Bacillus flavothermus*) this strain was first discovered in a hot spring of New Zealand (Heinen et al., 1982). They were identified at the year of 2000 as a relatively new genus. *Anoxybacillus* means small rod living with-out oxygen (Bevilacqua et al., 2016).

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

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bacteria have amylolytic and glucosidic activities and the ability to degradation of carbohydrate (Cihan, 2013). The *Anoxybacillus* can be aerobes, facultative anaerobes, or facultative aerobes. 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 (Ozdemir et al., 2011a).

Recent investigations confirm that the main spore-formers in various types of dairy powders are *B. licheniformis*, *Anoxybacillus*, and *Geobacillus* (Eijlander et al., 2019). *Anoxybacillus* can produce a variety of enzymes that degraded carbohydrates. (Derekova et al., 2008). Solid-state fermentation was employed to produce -amylase from rice husks using *A. flavithermus* that was isolated from a hot spring in Türkiye (Ozdemir et al., 2011b). Amylase enzyme can be used in industrial processes like process of brewing, baking, textiles, paper industry, bioethanol production and detergent (Lama et al., 2009). The whole cells of *Anoxybacillus* are potentially useful as alternative resources for bioremediation and to immune stimulate fish against pathogens and renewable energy generation (Cihan et al., 2013).

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 Türkiye. Also; for separation of heavy metals from aqueous solutions, some species of *Anoxybacillus* are useful and act as a model to developed the biosorption system (Duran et al., 2009). The new studies indicate that lipases enzyme that produced from *A. flavithermus* may be useful for various processes such as medical, food, cosmetic, detergent and leather and textile industries (Hasan et al., 2006).

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. The species that obtained from the various extreme habitats can produce commercially valuable extracellular enzymes (Bischoff et al., 2006).

In current study, the different strains of *Bacillus cereus*, and *A. flavithermus*, isolated from hot spring from Afyon city of Türkiye were analysed for their hydrolytic enzyme capabilities.

2. Materials and methods

Anoxybacillus bacteria, which were isolated, morphological and biochemical tests and 16S rRNA analysis by Ozdemir et al. (2011c) within the scope of the study identified bacteria and isolated locations were given at Table 1.

2.1. Biochemical tests

2.2.1. Gram staining

Before the imaging, isolated bacteria were air dried and fixed on the glass slide by heating and stained by using Gram stain. Samples were covered with few drops of Gentian violet (which is a mixture of methyl violet and crystal violet) for a minute and washed with the tap water. Samples were treated with Gram's Iodine and allowed to effect 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 imaged by using Olympus CX 31 system microscope with Olympus SC 30 Digital color camera. Images were photographed by light microscopy at highest magnification lens (X100) under oil immersion.

Table 1
The name of studied bacteria and their location.

Sample	Accession Number - Identified Bacteria	Name Hot spring/phase
Seq1	KJ434779 *Anoxybacillus*	Ömer/Soil
Seq2	KJ434780 *A. flavithermus*	Ömer/Soil
Seq3	KJ434781 *A. flavithermus*	Ömer/Soil
Seq4	KJ094998 *A. flavithermus*	Gecek/Soil
Seq5	KJ434782 *Bacillus firmus*	Ömer/Soil
Seq6	KJ434783 *Anoxybacillus* sp.	Ömer/Water
Seq7	KJ434784 *A. flavithermus*	Ömer/Soil
Seq8	KJ434785 *A. flavithermus*	Ömer/Soil
Seq9	KJ434786 *A. flavithermus*	Gecek /Water
Seq10	KJ094999 *A. mongoliensis*	Ömer/Soil
Seq11	KJ434787 *A. flavithermus*	Ömer/Water
Seq12	KJ434788 *A. flavithermus*	Gecek/Soil
Seq13	KJ095000 *A. flavithermus*	Gecek/Soil
Seq14	KJ434790 *A. flavithermus*	Gecek/Soil & Water
Seq15	KJ434791 *B. cereus*	Ömer/Soil
Seq16	KJ434792 *A. flavithermus*	Gecek/Soil & Water
Seq17	KJ434793 *A. kestanboliensis*	Ömer/Soil
Seq18	KJ095001 *A. flavithermus*	Gecek/Soil & Water
Seq19	KJ434794 *Bacillus* sp.	Ömer/Soil

2.2.2. 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-day incubation at 50°C. Following incubation, at the rate of 0,001% Rhodamine 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).

2.2.3. Xylan hydrolysis test

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

2.2.4. 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).

2.2.5. Starch hydrolysis test

Nutrient agar isolates (1% soluble starch) were planted by loop in the form of an intense line. Petri dishes were incubated for a day at 50°C. After the incubation, Lugol solution was added on the medium. Zones were observed around the colony, clear zones consider as positive starch digestion and purple zones accepted as negative starch digestion was assessed in areas that the starch hydrolyzed (Aygan et al., 2008).

2.2.6. Urease test

Nutrient agar plates containing 0.3% uric acid was sowed from alkalophilic isolates in a 20 mm diameter area. Petri dishes were left at 50°C for 24-48 h incubation. The transparent zone formed around the planted area following incubation was evaluated as positive for urease production (Honarbakhsh et al., 2014).

2.2.7. Asparaginase test

Nutrient agar plates containing 1% L-asparaginase milk dust were inoculated on halophilic isolates in 20 mm diameter petri dishes. Petri dishes were left at 50°C for 24-48 h incubation. Following incubation, 0.001% phenol was added and the pH was adjusted to 6.5. Color change from yellow to red around the colony was considered positive for L-asparaginase enzyme production (Gulati et al., 1997; Ogun, 2020).

2.2.8. Catalase test

Bacteria strains were isolated for 18-to-24 hours and samples were taken from the culture. Samples placed onto the microscope slide and drop of 3% H_2O_2 was added on it. The readability is improved by observing for bubble development on a dark background.

2.2.9. Antibiotic resistance test

The agar diffusion method with filter paper disk was used for antibiotic resistance test. Individual disk was containing a content concentration of a different antibiotic. The specific 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.

3. Results

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 were shown positive results with gram stain test. Also, the cells are often arranged in pairs or chain, straight, violet color and rod-shaped (Fig. 1A). 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 (Fig. 1B)

The xylan hydrolysis test is the composed of xylanolytic enzyme system of hydrolytic enzymes, the function of these enzymes is conversion of xylan to 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 (Fig. 1C).

Casein is a large protein molecule made of amino acids. It is the main protein found in milk and accounts for its white colour. Caseinases, which hydrolyse protein in stages to amino acids, were secreted outside of bacteria cells. If a clear zone, a zone of casein hydrolysis, evaluated it as positive. In this study 4 strains show negative result including Seq11, Seq12, Seq16, Seq18 and *Staphylococcus* and other isolates shows positive (Fig. 1D).

These forms are bonded by 1,4-glycosidic amylase and oligo-1,6-glucosidase. Enzymes are able to hydrolyse starch by breaking the glycosidic linkages between the sugar subunits. For detection of hydrolyse; Iodine reagent reacts with starch and produces a blue or dark colour; therefore, a clear zone surrounding the growth was revealed as any microbial starch hydrolysis. In this study, all strains show positive result (Fig. 2A). Ammonia, CO_2, and water are the by-products of urea's nitrogen and carbon bond assault by the urease enzyme. In this study, 15 strains show positive result including Seq2, Seq4, Seq5, Seq6, Seq7, Seq8, Seq9, Seq10, Seq11, Seq12, Seq13, Seq15, Seq17, Seq18, and Seq21 and other isolates shows negative (Fig. 2B).

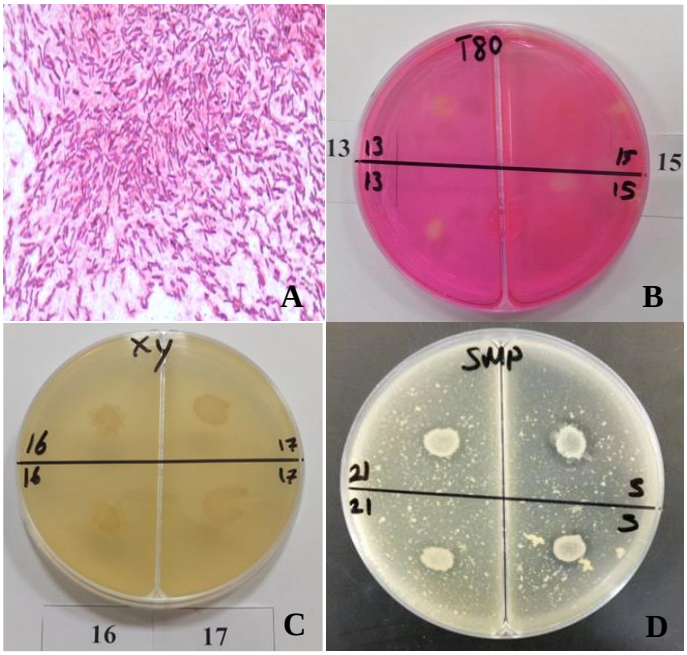

Fig. 1. Samples from (A) Gram staining, (B) Tween 80 hydrolysis test, (C) Xylan hydrolysis test, (D) Skim milk powder hydrolysis test.

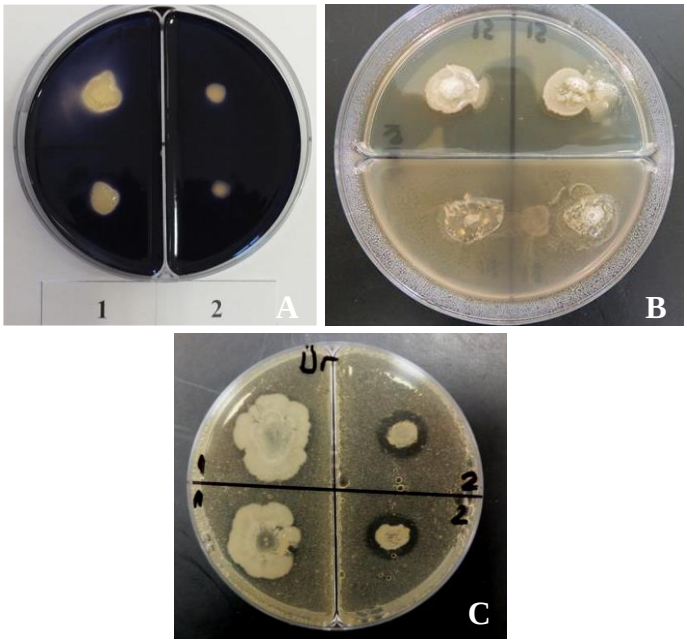

Fig. 2. A samples from (A) Starch hydrolysis test, (B) Urease test, (C) Catalase test.

Catalase test was applied to all bacteria strains that we isolated it in this study and all shows bubbles of O_2, so the result is catalase test positive (Fig. 2D).

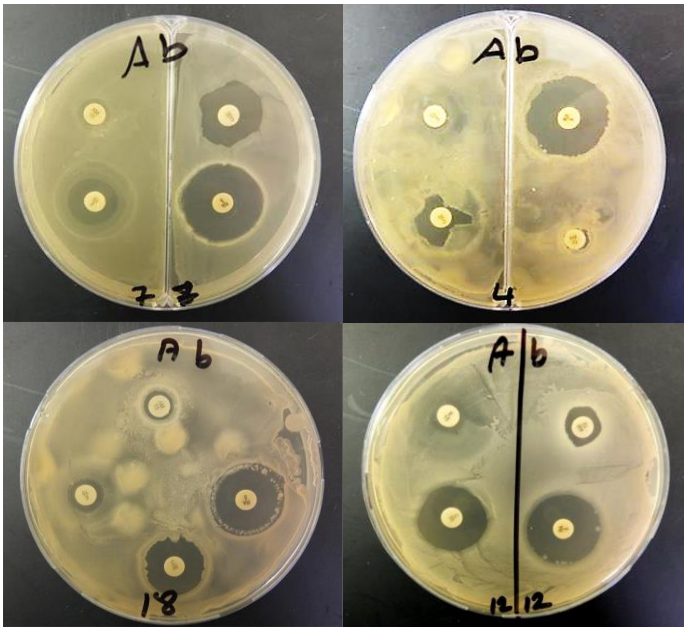

Fig. 3. The samples of antibiotic test exposed to *Anoxybacillus* strain.

When the growth of bacteria has been inhibited, a clear zone was appeared around the paper disk, the diameter of this zone related with the sensitivity of the bacteria to the antibiotic and minimum inhibitory concentration (MIC) is reached at this point. The zone of inhibition appears surrounding the 4 types of antibiotic disk that were used in this study which are Chloramphenicol, Ampicillin, Rifamcyin and Erythromycin (Fig. 3).

4. Discussion

Thermophilic organisms; It is widely preferred in scientific researches because it is used in the production of enzymes, antibiotics, various biochemical substances and insecticides. *Bacillus* genus is one of the most studied thermophilic bacterial groups. These organisms are usually gram-positive, aerobic, rod-shaped bacteria that form endospores. Formation of endospores makes this breed resistant to unfavourable conditions. Among the reasons why *Bacillus* genus is preferred in the production of industrial enzymes: rapid and easy reproduction, using a wide variety of carbon and nitrogen sources and different substrates, and creating high yield products can be counted. In addition, organisms included in this group can be isolated from soil, fresh water and seas, plant rhizospheres, foods, intestinal systems of some living things, some insect larvae and even from areas with extreme pH and temperature (Tarakcioglu, 2016).

In this study, the starch hydrolysis test for 21 strains isolated from hot spring in Türkiye showed positive result for all strains and this result agrees with the concept of a study conducted previously in Afyonkarahisar, Türkiye by a team of researchers, who found that amylase enzyme positive of the *A. flavithermus* (Ozdemir et al., 2015). Also a similar result was obtained from a study in Ramadi (Dhafer, 2007).

This study showed that all 19 strains which isolated from hot spring in Türkiye positive result for catalase test and this result are in agreement with most published data (Heinen et al., 1982; Claus and Berkeley, 1986; Rainey et al., 1994; Pikuta et al., 2000; Belduz et al., 2003; Sharp et al., 2021). It's also agrees with the concept previously reported by several researchers, who found that catalase enzyme positive of the *A. flavithermus* (Ozdemir et al., 2015; Dhafer, 2007; Dai et al., 2011).

In current result, negative result were obtained for sample Seq1, Seq3, Seq19 of *A. flavithermus* and this result supported the published data, which described the urease enzyme negative (Pikuta et al., 2000; Belduz et al., 2003). In contrast to these, positive results were obtained for urease enzyme in *A. flavithermus* numbered as; Seq2, Seq4, Seq7, Seq8, Seq9, Seq11, Seq12, Seq13, Seq15, Seq17. And positive result to *A. mongoliensis* sample Seq10 and *A. kestanboliensis* sample Seq18. The result of this study agree with the concept previously reported by Filippidou et al., (2015) who found urease enzyme positive for *A. geothermalis* Strain GSsed3.

The positive result of casein indicated in *A. 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 Seq11, Seq12, and Seq19 of *A. flavithermus* for casein hydrolysis. The current results coincide with the results obtained by Shahinyan 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 Seq11, Seq12, Seq19 sub strain of *A. flavithermus*. The consensus sequences among bacterial species are needed for identification of bacteria. 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 technology allows researchers to search for novel bacterial strains that may have previously unreported enzymatic activity.

A. 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 economic importance for the conversion of plant biomass into fuels and chemicals.

In this study, 19 strains isolated on Nutrient agar at 50°C from samples from hot springs from Afyon city, 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. Antibiotic resistance capacity is due to transfer of antibiotic resistant genes from the others and its get conveyed by plasmids and this dissemination is rapidly occur and even among bacteria that are distantly related, horizontal gene transfer occurs frequently (Amábile-Cuevas and Chicurel, 1992). This process is important because it caused to increasing of drug resistance when one bacterial cell acquires resistance, resistance genes can quickly transfer to numerous species (Raghunath, 2008).

5. Conclusion

In the 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. *A. flavithermus* is a relatively new species and the knowledge of the physiology, metabolism, and metabolomics of the *A.*

flavithermus is incomplete, quite limited, and seldom compared with other members of Bacillaceae that well-studies. The majority of the available information indicates that this strain produces intriguing enzymes that are thermostable and tolerant of alkaline pH. The importance of bacteria used in industry has increased. Because they can use 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, the stability natural enzymes 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, Türkiye were screened. In order to identify these strains, some biochemical tests and molecular identification based on 16S rRNA gene were performed. As a result, it was identified as a strain of *A. 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 1-2 days: growth at 50°C and pH 7. Isolates were also screened for the extracellular enzyme hydrolysis at 50°C such as starch, xylan, casein and asparagine hydrolysis and catalase, lipase and urease activities were examined. 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.

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Conflict of interest: The authors declare that they have no conflict of interests.

Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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