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Determination of Extracellular Enzymatic Activities of Bacteria Isolated from Insuyu Cave

Nermin Sarıgül *

Burdur Mehmet Akif Ersoy University, Science and Literature Faculty, Molecular Biology and Genetics Department, Burdur 15010, Turkey

Email: sarigul@mehmetakif.edu.tr

Abstract

Caves are often regarded as extreme environments due to their high humidity, stable low temperatures, limited nutrient availability, and minimal energy input. These unique characteristics attract researchers from various disciplines, including biology, chemistry, geology, and astronomy. Despite the presence of approximately 40,000 caves in Türkiye, microbiological studies in these habitats remain limited. One significant area of research involves the discovery of antimicrobial compounds and enzymes produced by microorganisms in isolated cave environments, driven by interspecies competition. In this study, bacterial strains were isolated from Insuyu Cave to evaluate their extracellular enzymatic activities. Seventy-five isolates were screened for lipase, protease, amylase, catalase, gelatinase, and urease production. Results showed that 39% of the strains exhibited lipolytic activity, 36% protease activity, 36% amylase activity, 91% catalase activity, 13% gelatinase activity, and 45% urease activity. The findings highlight the significant potential of caves as natural resources for industrial enzyme research.

Keywords: Cave; microorganism; extracellular enzymes.

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* Corresponding author.

1. Introduction

Enzymes derived from microorganisms are a significant source of many industrially relevant enzymes [1]. Microbial enzymes are relatively more stable and diverse compared to other enzymes obtained from plants and animals [2]. Enzymes produced by microorganisms can be physiologically and physicochemically controlled with ease, manufactured quantitatively, and extracted at low production costs [1,3]. The industrial use of microbial enzymes is classified as follows:

- Enzymes as final products,
- Enzymes as processing aids,
- Enzymes in food and beverage production,
- Enzymes in genetic engineering,
- Enzymes as industrial biocatalysts [4].

Microbial enzymes are employed across various industrial applications: In the textile industry, they can improve fabric quality; in the pulp and paper industry, they play a role in bio pulping and bleaching processes; in the food industry, they are utilized in fermentation processes for the production of beverages like wine and beer, and they assist in the extraction of substances such as carotenoids and olive oil; in the detergent industry, they exhibit superior cleaning properties and are also used in cosmetics, animal feed, and agricultural industries (Table 1).

Table 1: Industrially important microbial enzymes, producer microorganisms and their areas of use

Enzyme	Industry	Producer microorganisms	References
Amylase	Detergent additive Obtaining glucose syrup as sweetener The starch in the flour is broken down into sugars and the yeast is ready for use.Starch removal in weaving	*Bacillus sp.* *Chryseobacterium sp.* *Streptomyces sp.* *Pseudomonas stutzeri*	[9, 10]
Protease	Detergent additive Clarification of beer Leather Industry	*Bacillus cereus* *B. licheniformis* *Virgibacillus sp.* *Aspergillus terreus* *A. versicolor*	[11, 12, 13]
Lipase	Detergent additive Cheese industry	*Acinetobacter calcoaceticus* *Bacillus sp.* *Staphylococcus arlettae*	[14, 15, 16]
Pullulanase	Obtaining maltose and maltotriose from dextrin in brewing	*Bacillus cereus* *Bacillus halodurans*	[17,18]

Cellulase	Oil production from plants Shredding waste, removing cellulose fibers	*Penicillium sp.*	[19]
Urease	As a diagnostic kit for the measurement of urea In alcoholic beverages As a probe for the detection of heavy metal ions in foods In biosensors of hemodialysis systems for the determination of blood urea	*Proteus mirabilis,* *Staphylococcus saprophyticus* *Helicobacter pylori*	[20, 21, 22]
Catalase	Preventing beverages from spoiling Removal of hydrogen peroxide from foods Conversion of latex to rubber	*A. niger*	[23]
Pectinase	Clarification of fruit juice	*Bacillus sp.* *A. niger*	[24, 25]

2. Material and methods

Insuyu Cave is located within the borders of Burdur province, in the interior of the Western Taurus Mountains. Geographically, it spans an area of approximately 70 km², situated between 30°19’50”–30°26’25” E longitude and 37°35’30”–37°41’50” N latitude. The cave is 597 meters long, with its widest point measuring about 80 meters. This horizontally extending cave consists of nine interconnected chambers. Inside the cave, stalactites and stalagmites have formed over time as a result of the dissolution and erosion of the karstic structure [26].

The study material consists of soil, water, and sediment samples isolated from Insuyu Cave in Burdur. Sampling was conducted from both illuminated and dark areas across 15 different stations within Insuyu Cave. The sampling locations are detailed in Table 2.

Table 2: Insuyu Cave sampling stations

No	Sampling Site	Material
1	Big Lake/Dark	Soil
2	Big Lake/ Bright	Soil
3	Big Lake – Stalactite / Bright	Water/Swab
4	Big Lake path- Cave Wall / Bright	Soil
5	Big Lake path- Cave Wall / Dark	Soil
6	Upper wall/ Bright	Swab
7	Visitor path -Cave Wall / Bright	Soil
8	Visitor path – Cave Ceiling/ Bright	Water/Swab
9	Visitor path - Cave Wall / Bright	Soil
10	Visitor path- Soil / Bright	Soil
11	Visitor path- Soil / Bright	Soil
12	Visitor path- Soil / Bright	Soil
13	Cave Wall / Dark	Water/Swab
14	Wish lake/ Bright	Water/Swab
15	Cave Wall / Dark	Water/Swab

2.1. Culturing and purification of microorganisms

1 ml of water or 1 g of soil sample was homogenized in 9 ml of sterile 0.9% saline solution, and a series of dilutions (up to 10^-4) were prepared. All samples were inoculated onto 0.1x Nutrient Agar, M9, and R2A media under both dark and light conditions using the spread plate method, and incubated at 28°C. After incubation, colony morphology was examined, and characteristic colonies were identified. To obtain pure isolates, streak plating was performed on 0.1x Nutrient Agar, and the plates were incubated at 28°C for 48-72 hours. The purified strains were then inoculated into 0.1x Nutrient Broth and incubated at 28°C for 48 hours. Cultures showing growth were preserved as stocks at -20°C in a medium containing 40% glycerol.

2.2. Gram staining

Gram staining was performed to determine the cell wall characteristics of the isolates. Samples were inoculated onto 0.1x Nutrient Agar using the streak plate method and incubated at 28°C for 48 hours. A droplet of 0.09% NaCl solution was added to a slide using a Pasteur pipette. Colonies showing growth were picked and spread on the slide to dry. Once dried, the slide was heat-fixed by passing it through a flame five times. After fixation, the slide was stained with Crystal Violet Solution for 1 minute, followed by rinsing with water. The slide was then stained with Gram's Iodine Solution for 1 minute and rinsed with water. Subsequently, the slide was treated with 70% ethanol for 15 seconds and washed with water. Finally, the slide was counterstained with Safranin Solution for 30 seconds, rinsed, and excess dye was removed. After washing, the slides were dried with blotting paper. Bacteria that appeared purple under the microscope were considered Gram-positive, while those that appeared

pink/red were considered Gram-negative .

2.3. Protease

The protease test was performed using Nutrient Agar containing 1% casein and 2% gelatin. Samples were inoculated using the spot method and incubated at 28°C for 48 hours. A transparent zone around the microbial culture was observed as a positive result [27].

2.4. Catalase

A small amount of sample was taken using a sterile loop and transferred to a clean petri dish containing 0.1x Nutrient Agar. Hydrogen peroxide (3%) was added to the sample using a Pasteur pipette. The release of gas was considered a positive result.

2.5. Lipase

The lipase test was performed using Tween 80 Agar. Samples were inoculated by the spot method and incubated at 28°C for 48 hours. The formation of a white precipitate around microbial growth was considered a positive result for lipase activity [28].

2.6. Amylase

For the amylase test, 0.1x Nutrient Agar containing 2% starch was used. Activated pure culture was inoculated onto the medium using the spot method and incubated at 28°C for 48 hours. After incubation, Iodine Mordant Solution was added to the agar plates using a Pasteur pipette, covering the surface. The plates were left for approximately 1 minute, and the transparent zone around the microbial culture was considered a positive result for amylase activity [29].

2.7. Gelatinase

The gelatinase test was performed using 0.1x Nutrient Broth containing 10% gelatin. The pure culture was inoculated into the medium and incubated at 28°C for 48 hours. After incubation, the samples were placed in a refrigerator at +4°C for 1 hour. Afterward, the tubes were observed for solidification. Solidification indicated a negative result for gelatinase activity, while a liquid appearance was considered a positive result [29].

2.8. Urease

The urease test was performed using Christensen's Urea Agar. The activated pure cultures were inoculated into the medium using a micropipette and incubated at 28°C for 48 hours. A change in the color of the medium from orange to red was considered a positive result for urease activity [30].

3. Results

For the inoculation of isolated samples, media with low nutrient content, such as 0.1X Nutrient Agar, M9, and R2A, were selected. After incubation, isolates with similar morphological characteristics were discarded, and 75 isolates showing distinct differences were chosen for the study. The selected colonies were purified on 0.1X Nutrient Agar. After activating the pure cultures, the Gram staining method was applied. The Gram staining characteristics and cell morphologies of the samples were examined using a light microscope. Of the 75 bacterial isolates examined, 50 were Gram-negative, and 25 were Gram-positive. The results are given in Table 3 and 4.

The results of the biochemical tests showed that approximately 91% of the bacterial isolates from İnsuyu Cave could produce catalase, 36% produced protease and amylase, 45% produced urease, 13% produced gelatinase, and 39% produced lipase.

Table 3: Gram staining, cell morphology characteristics and biochemical test results of bright environment isolates

Code	Gram staining	Cell morphology	Catalase	Urease	Protease	Lipase	Amilase	Gelatinase
2C	Gr (-)	Coccus	+	-	+	-	-	+
2D	Gr(-)	Bacil	+	-	-	-	-	-
2F	Gr(+)	Coccobacil	+	-	+	+	+	+
2G-2	Gr(-)	Bacil/spiral	+	+	+	+	-	-
2I	Gr(-)	Coccobacil	ND*	-	-	-	ND	-
2J	Gr(+)	Coccus	+	-	-	-	-	-
2L	Gr(+)	Streptobacil	+	-	+	+	-	-
2M-2	Gr (-)	Coccus	+	ND	-	-	-	-
2G-1	Gr(-)	Coccus	+	+	-	+	-	-
4B	Gr(-)	Coccus	+	-	-	-	-	ND
4D	Gr(+)	Coccobacil	-	+	-	-	-	ND
4E	Gr(+)	Coccus	+	-	ND	+	+	+
4F	Gr(+)	Coccus	+	-	+	-	+	-
4O	Gr(+)	Coccus	+	+	ND	+	-	ND
4H	Gr(-)	Coccus	+	+	ND	ND	-	+
4M	Gr(-)	Coccus	+	-	-	-	-	+
6B-1	Gr(-)	Bacil	+	+	-	+	-	-
6D	Gr(-)	Coccobacil	+	ND	-	-	+	ND
6I-1	Gr (+)	Bacil	+	+	-	+	-	-
7A	Gr (+)	Bacil	+	-	-	-	-	-
7B	Gr(-)	Bacil	-	+	-	+	-	+
7C	Gr (-)	Bacil	+	-	-	-	-	-

7D-1	Gr (+)	Streptobacil	+	-	-	-	ND	ND
7J	Gr(-)	Bacil	+	-	+	-	-	+
9A	Gr(+)	Coccus	+	-	+	+	-	+
9D	Gr(-)	Coccus	+	-	-	+	-	ND
10B	Gr (+)	Bacil	+	-	-	+	-	-
11L	Gr(+)	Coccus	+	-	ND	-	-	-
11M	Gr(+)	Coccus	+	-	-	+	-	ND
12C	Gr (+)	Coccus	+	-	-	-	+	-
12F	Gr (-)	Streptobacil	+	-	-	-	-	-
12G	Gr (+)	Bacil	+	+	-	-	-	-
12M	Gr (-)	Bacil	+	+	-	-	-	-
13A	Gr (-)	Streptobacil	+	+	-	-	ND	-
13C-1	Gr (-)	Coccus	+	+	-	+	-	ND
13G	Gr (-)	Bacil	+	-	-	-	-	-
13H	Gr (-)	Coccus	+	+	-	-	-	-

*: Not Detected

Table4: Gram staining, cell morphology characteristics and biochemical test results of dark environment isolates

Code	**Gram staining**	**Cell morphology**	**Catalase**	**Urease**	**Protease**	**Lipase**	**Amilase**	**Gelatinase**
K-1A	Gr (+)	Coccus	+	-	-	-	+	ND*
K-1C	Gr (+)	Streptobacil	+	-	+	-	+	-
K-1E	Gr (+)	Bacil	+	-	-	+	+	-
K-2A	Gr (+)	Coccus	+	+	+	+	+	+
K-2B	Gr (-)	Coccus	+	-	+	-	+	-
K-2E	Gr (+)	Coccus	-	-	-	+	+	ND
K-2G	Gr (-)	Coccobacil	-	-	-	-	+	-
K-2H	Gr (-)	Bacil	-	-	-	+	+	-
K-2I	Gr (-)	Coccus	+	+	+	+	+	+
K-2J	Gr(-)	Bacil	+	-	-	-	+	ND
K-2N	Gr (-)	Bacil	+	+	+	-	+	-
K-4B	Gr(+)	Coccus	-	-	+	-	-	-
K-4C	Gr(-)	Coccus	+	-	-	-	-	-
K-4E	Gr (-)	Coccus	+	ND	ND	ND	ND	ND
K-4G	Gr(-)	Coccus	+	-	-	-	-	-
K-4H	Gr(-)	Coccus	+	ND	ND	ND	ND	ND
K-4F	Gr(-)	Coccus	+	-	+	+	+	ND

K-7B	Gr (-)	Bacil	+	-	+	-	-	-
K-7K	Gr (+)	Bacil	+	-	-	-	+	-
K-9M	Gr(-)	Coccus	+	-	-	+	+	ND
K-11A	Gr(-)	Coccus	+	-	-	-	+	ND
K-11D	Gr(-)	Coccobacil	+	-	-	-	+	ND
K-11G	Gr(-)	Bacil	+	-	-	-	+	ND
K-12E	Gr(-)	Coccus	+	-	+	ND	ND	ND
K-13B	Gr(-)	Bacil	+	+	+	+	+	-
K-13J	Gr(-)	Bacil	ND	ND	ND	ND	ND	ND
K-13I	Gr(-)	Bacil.	ND	ND	ND	ND	ND	ND
K-15A	Gr(-)	Streptobacil	+	+	+	-	-	-
K-15C	Gr (-)	Bacil	+	+	-	+	-	-
K-15F	Gr(-)	Coccus	+	-	-	+	-	ND
K-15G	Gr(-)	Coccus	+	ND	-	ND	ND	ND

*: Not Detected

4. Discussion and Conclusion

Research on newly discovered microorganisms is increasing globally, especially in caves, as they offer a unique opportunity to explore microbial diversity. These studies aim to isolate and characterize bacteria that could have biotechnological significance. In this regard, our study was conducted with 75 bacterial isolates from İnsuyu Cave. Gram staining revealed that 50 of the 75 bacterial isolates were Gram-negative, and 25 were Gram-positive. Biochemical tests for catalase, lipase, protease, amylase, urease, and gelatinase showed that approximately 91% of the isolates produced catalase, 36% produced protease and amylase, 45% produced urease, 13% produced gelatinase, and 39% produced lipase.

A study by Tomova and his colleagues. in Bulgaria investigated the diversity and biosynthetic potential of aerobic heterotrophic bacteria isolated from Magura Cave. The study found that 81% of the isolates were Gram-negative, with enzyme production including protease (87%), xantham lyase (64%), lipase (40%), β-glucosidase (40%), and phytase (21%) [31]. In our study, most of the isolates were Gram-negative bacteria, and approximately 39% of them tested positive for lipase production, similar to the findings of Tomova and his colleagues.

Another study conducted in the Gilindire Cave in Mersin examined the biochemical characterization of 30 isolates. Catalase, amylase, and urease tests were performed, and it was found that all but four isolates tested positive for catalase. Among the isolates, 13 were negative for urease, and 17 were negative for amylase [32]. Similarly, in our study, the highest positive results were observed in the catalase test, with approximately half of the isolates showing positive results for urease production. Catalase is known to play a key role in breaking down hydrogen peroxide, a byproduct of aerobic respiration, thus protecting the cell from oxidative stress. It is a common enzyme found in most organisms [33].

In another study conducted in Alanya, Turkey, the enzymatic profile and antimicrobial activity of bacteria isolated from Kadıini Cave were investigated. The study found that the isolates exhibited high proteolytic activity [34]. A study conducted in 2016 in Sarawak characterized ureolytic bacteria isolated from karstic caves and examined their potential for industrial use. The findings suggested that the isolated ureolytic bacteria have the potential to be used as microbial-induced calcite precipitation (MICP) agents for biochemical applications [35].

In our study, the lowest enzyme activity was observed for gelatinase (13%). Since gelatin is an animal-derived protein, it is expected that microorganisms isolated from caves would not produce gelatinase, which is consistent with our results.

In conclusion, the findings of this study suggest that the bacterial isolates from Insuyu Cave could be valuable sources for industrial applications. These isolates are thought to possess biotechnological potential for further research and industrial use.

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