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Molecular characterization, identification and enzyme-producing potential of thermophilic bacterial diversity isolated from the Kars sugar factory

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Abstract

The microbial diversity found in the areas contaminated by industrial waste is a great place to drain the tough enzymes that are possible to use for the biotechnological industry. In this study, bacteria were taken from different places in the Kars Sugar Factory. Then, the bacteria were subjected to extensive morphological, biochemical, and molecular examinations. The samples were able to grow at pH of 5.0 to 9.0 and in temperature ranging from 40 to 85 °C. The examination showed that most of the strains belonged to the genera *Bacillus*, *Geobacillus*, *Klebsiella*, and *Acinetobacter*. High frequency of hydrolase production was among the isolates. 60.6% of the isolates were identified as lipase producers, 66.7% as amylase producers, 83.3% as xylanase producers, and 89.4% as protease producers. The greatest part of isolates could produce more than one enzyme at the same time. These results are a further argument in favor of the industrial application of microbial enzymes in waste utilization and biofuel production. The Kars Sugar Factory is a microbially rich niche of great importance.

Keywords Bacillus, Geobacillus, Enzyme activity, Sugar refinery, Thermophilic bacteria

Introduction

Microbial diversity plays a critical role in the development and advancement of industrial tools that exist in the universe [1]. As a result of their high growth rate and unique macromolecular properties, thermophiles are highly suitable for industrial use [2, 3]. These properties make them excellent candidates for use in waste treatment, biofuel production, and food processing.

In biotechnological studies, bacteria that show activity and reproduction ability at temperatures between 40 °C and 80 °C, which are high temperatures, are of interest due to their ability to produce heat-resistant enzymes. The properties of bacteria make them highly valuable in industries [4, 5]. Thermostable enzymes can function in high temperatures and extremely salty environments. Industrial settings are generally extreme environments [6]. Thermostable enzymes are the preferred choice in biotechnology because they can remain active under extreme conditions. Since extreme conditions are not suitable for standard enzymes, processes such as cooling may be required. On the other hand, thermostable enzymes do not require such extra costly processes. Considering all these factors, they enable more efficient management of large-scale processes, particularly in food and waste treatment [7].

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The great amount of organic matter in waste and the elevated temperatures during the processing of sugar create the ideal habitat for thermophilic bacteria [8–11]. These microorganisms are adept at breaking down organic substances in very high heat conditions. Furthermore, the bacteria taken from harsh environments are said to produce the same kinds of enzymes that are used in industrial processes very often [12]. The characterization of bacteria in extreme environments has become a significant area of interest in several industries, including food, healthcare, and bioenergy [3, 13].

The microbial diversity in sugar factories is, in general, quite high. The genera *Bacillus* and *Geobacillus* have often been isolated from these sites [14–18]. Among the microbial species, *Bacillus licheniformis* is the rarest bacteria for the hydrolysis of starch and the production of bioethanol. Besides, the cohabitation of the *Bacillus* and *Geobacillus* strains are the main sources of the heat-resistant enzymes, the so-called thermostable amylases [19, 20].

In fact, the microbial enzymes found in sugar factories are particularly appealing due to their ability to withstand high temperatures, which also makes them suitable for the industrial processes requiring such extreme conditions [21]. For instance, in the case of starch degrading enzymes, their thermostability again is an advantage in the case where the production of fermentable sugars for bioethanol is done; thus, the costs of production are reduced quite significantly [22].

Thermostable proteases are used in detergents to degrade protein stains at low temperatures [23]. In the food industry, proteases contribute to obtaining the desired flavor and texture by the hydrolysis of proteins. Furthermore, lipases and xylanases have shown strong potential for diverse industrial applications [24]. Xylanases are key to lignocellulose degradation, especially in the pulp and paper [25].

The Kars Sugar Factory was selected as the study area because of its high-temperature and organic waste composition conditions. Unlike other sugar factories, cold climates make this region attractive for this study. Both thermophilic and psychrotolerant microorganisms can grow in these environments. The extreme environment of factories provides a valuable environment for the investigation of microbial diversity and enzymes.

From an industrial perspective, numerous studies have been conducted in the literature on the diversity of microorganisms in waste. However, there are very few studies on the diversity of microorganisms in sugar factory waste from cold climates (−20 to −35 °C). This study targeted thermophilic bacterial diversity at the Kars Sugar Factory in eastern Turkey and tested their activities in terms of various enzymes [26]. The study specifically targeted environments where sugar beets processed using hot processes in cold regions are used as the primary nutrient. The study will also fill a significant gap in understanding the bacterial diversity and different enzymatic functions in such environments. The bacteria and their enzymes obtained represent an opportunity for biotechnologically important processes. Although thermophilic enzymes are frequently studied by researchers, the number of studies based on sugar factories remains quite low. In this context, this study has identified thermophilic bacteria originating from the Kars Sugar Factory, which creates an extraordinary industrial environment combining high-temperature processes and the harsh winters of Eastern Anatolia.

The aim of this study was to characterize bacterial strains isolated from the Kars Sugar Factory and determine their potential to produce hydrolases such as amylase, protease, lipase, and xylanase.

Materials and methods

Biological samples and bacterial isolation

The bacteria obtained from water, mud, and sugar beet waste collected from the Kars Sugar Factory in three different seasons constitute the biological material of our study. The spatial distribution of the various origins investigated in this study is illustrated in Fig. 1. Samples were carefully collected from shallow, sediment-rich areas located around the bath zones of thermal springs using sterile capped bottles. Aluminum foil was used to cover the caps in order to protect the samples from sunlight. The temperature and pH of the samples were taken carefully using a hand-held device (Model No. AZ 8685, Taiwan). A short Supplementary Video (Supplementary Video 1) is provided to illustrate the outdoor collection pond (40.565782°N, 43.097986°E) from which the environmental samples were collected. The video captured the physical condition of the industrial waste collection area. Samples were serially diluted using sterile physiological saline (0.85% NaCl) and spread onto Luria-Bertani (LB) agar. The plates were incubated under aerobic conditions at various temperatures ranging from 40 °C to 85 °C for 48–72 h. Colonies with distinct morphological features were repeatedly subcultured to obtain pure isolates [27].

Sample collection and contamination control

Careful attention was paid to aseptic techniques when collecting samples to minimize the risk of contamination. Sterilized glass jars and collection tools were used throughout the study. Ten water, ten sediment and ten organic waste samples were collected from four different locations in the sugar factory: (i) an outdoor pulp waste site, (ii) an outdoor collection pond, (iii) an indoor sugar processing waste area, and (iv) an indoor sugar-water waste channel. Specifically, eight samples were collected



Fig. 1 Location of the study area: **A**: Indoor sugar processing waste area (40.576591°N, 43.097270°E), **B**: Outdoor pulp waste site (40.575392°N, 43.096937°E), **C**: Outdoor collection pond (40.565782°N, 43.097986°E), and **D**: Indoor sugar-water waste channel (40.586397°N, 43.098941°E)

from the outdoor pulp waste site, seven from the outdoor collection pond, eight from the indoor sugar processing waste area, and seven from the indoor sugar-water waste channel, totaling 30 samples. Field blanks (sterile water samples treated in the same way as the real samples) were also included to monitor environmental contamination at the study locations. The sampling strategy was chosen to represent different microhabitats.

Bacterial isolation strategy and diversity control

Sampling areas were selected over a wide range to ensure that all areas of the factory could be included. The aim was to obtain both thermophilic and mesophilic bacteria from the environment. Samples were taken from areas of the sugar factory where temperatures are particularly high.

The study made use of Luria-Bertani (LB) medium as the general culture. This allowed for a wide range of bacterial species to flourish and thus represented the microbial diversity in the ecosystem. The collection points for the samples were both the inside and outside areas of the factory. the bacteria were consequently cultured in the laboratory at varying temperatures of 40 °C to 85 °C. The maximum diversity possible was obtained by conducting the experiments thrice in each case. In addition, control groups were maintained throughout the study to eliminate the risk of any external contamination. Ultimately, the microbial groups collected underwent preliminary testing based on standard tests like colony morphology and Gram staining [28].

Phenotypic and morphological characterization

Morphological assessment

To observe the characteristics and movements of the colonies, we relied on light microscopy (Bestscope BS-2030t + BLC – 450). The morphological aspects were identified

through Gram and endospore staining, while motility was assessed according to the specified protocols [29].

Motility examination

According to the established protocols, motility was assessed with the help of phase-contrast microscopy on wet mount preparations. Moreover, the shape of the cells was additionally determined through Gram and endospore staining [30].

Gram staining

The bacterial cultures were subjected to overnight incubation under the temperature condition of 40 to 85 °C with Luria-Bertani (LB) agar as the medium. Afterward, colonies for Gram's staining were selected and treated with the standard methods of crystal violet, iodine, ethanol, and safranin (Harley and Prescott, 2002). The staining color seen under light microscopy was the basis for classifying bacteria as Gram-positive or Gram-negative [31].

Endospore staining

The endospore staining was done with carbol fuchsin and methylene blue and followed the Klein method, which is considered a substitute for the Schaeffer-Fulton technique. The latter is the method more frequently used. The Klein method, however, has become common in the microbiological literature for endospore visualization through these dyes in light microscopy, even though it is still not widely adopted [32].

Physiological characteristics assessment

Physiological properties such as temperature, pH and NaCl tolerance were tested in order to examine the conditions that the isolates could reach [27]. The growth response to pH was assessed across values from 4.0 to 12.0, in increments of 1.0. The salt-tolerant medium was supplemented with NaCl concentrations up to 10% (w/v). In addition, the suitability of various compounds as carbon sources was investigated at 1% concentration [33].

Control experiments were conducted to verify the validity of biochemical and enzymatic analyses. A sterile petri dish without bacterial inoculation was used as a negative control to monitor contamination during the enzyme assays. Thus, the accuracy of the enzymatic production experiments was checked, and it was shown that the activity was only due to bacterial isolates.

Biochemical properties

This research was conducted mainly for the purpose of qualitatively screening the hydrolytic enzyme production by the bacterial isolates which were selected for 16 S rRNA gene sequencing. The study aimed to assess the microorganisms' ability to produce amylase, lipase, protease, xylanase, catalase, and oxidase activities during in vitro experiments. The isolates were first subcultured from stock cultures stored at −80 °C onto nutrient agar plates and incubated at 37 °C for 24 h. Single colonies were then used to prepare inoculum suspensions in sterile saline, adjusted to an optical density (OD) of 0.5 at 600 nm. Plates were incubated in an incubator with controlled humidity (approximately 60–70%) to ensure optimal growth conditions [34].

Amylase test

Nutrient agar plates containing starch were used to test for amylase production. The amylase test is performed to determine whether the bacteria can break down starch. 8 g of NB medium is weighed and placed in durham glass, then 10 g of starch and 12 g of agar are weighed and added to the durham glass. Complete dH_20 so that the final volume is 1000 ml. Bacteria were inoculated on the prepared 1% starch NA medium and incubated for 72 h. At the end of incubation, lugol solution was dropped onto the petri dishes. Clear zones showed amylase production when iodine solution was added after incubation [35].

Catalase test

The catalase enzyme is responsible for breaking down hydrogen peroxide, which is harmful to bacteria, into water and free oxygen. To prepare the medium, 8 g of nutrient broth (NB) is weighed, followed by adding 12 g of agar, and the volume is completed to 1000 ml with dH_20 in a Durham tube. It is then sterilized in an autoclave at 121 °C and 1.5 atm pressure for 15 min. Subsequently, the nutrient agar is poured into petri dishes and divided into quarters for inoculation. After incubating the plates for 24 h in NB agar medium, 3% hydrogen peroxide (H_2O_2) is dripped onto the isolates. The formation of bubbles in the petri dish is interpreted as a positive result for catalase [36].

Oxidase test

For the oxidase test, specialized strips incorporating N, N-dimethyl-1,4-phenylenediammonium dichloride (Bactident Oxidase) were employed. Bacterial isolates, collected using a loop, were inoculated onto the tip of the strip. Positive results were identified by observing color alterations [34].

Protease test

Protease production was assessed using skimmed milk agar. Protease test 2 g of NB, 3 g of agar and 25 g of skim milk are weighed. 250 ml of dH_20 is added to the medium placed in Durham glass. Autoclave sterilized NB medium containing 10% volume of skim milk is poured into sterile petri dishes. Line inoculation was made on the medium

which was not transparent in color in the petri dishes and incubated for 72 h. After incubation, a clear zone around the bacterial growth indicated casein hydrolysis [37].

Lipase test

Lipase production was evaluated using butter-supplemented nutrient agar. Accordingly, a nutrient agar containing fat will be prepared. In the prepared nutrient agar, 1.25 g of peptone, 2.5 g of butter, 0.75 g of meat extract, and 3.79 g of agar were weighed and placed into Durham tubes, and the volume was completed to 250 ml with dH_2O before being sterilized in an autoclave. Line inoculation from the bacterial isolate was performed onto the agar poured into petri dishes. Incubation was carried out for 72 h. Following $CuSO_4$ treatment, the appearance of clear zones or a greenish tint around the colonies was considered indicative of lipase activity [27, 38].

Xylanase test

Xylanase enzyme, which plays an effective role in the degradation of cellulose-rich wastes such as sugarcane. All isolates were inoculated onto an agar medium containing 10 g xylan, 2 g tryptone, 15 g agar, 2 g yeast extract, 2 g KH_2PO_4, and 0.5 g $MgSO_47H_2O$ using the streaking method. Xylanase production was detected on xylan-containing agar. Clear zones around colonies were taken as positive results [27, 39].

Statistical analysis

Statistical analyses were run through IBM SPSS, v. 26.0 and GraphPad Prism, v. 10.4.1. Pearson Chi-square test was applied to frequency data and Independent Samples T-test to quantitative data. Rstudio version 2024.04.2, and pheatmap library were the tools for dendrogram analyses and visualization [40]. Hierarchical clustering analysis was carried out using the frequencies of amylase, lipase, protease, and xylanase enzyme expression in bacteria isolated from each location to evaluate functional similarities among the different sampling locations. For each location, a data matrix was constructed with values between 0 and 1, where the ratio of isolates expressing the relevant enzymes to the total number of isolates was calculated. Functional differences among the locations were measured using Euclidean distance as a distance metric. Clustering was done based on average linkage method. The heat map shows the sampling locations in the rows and the enzymes in the columns. The color scale is a gradient from purple (0) to yellow (1), depicting the frequency of each enzyme's expression at the respective location. For all statistical analyses, $P < 0.05$ was considered statistically significant.

Molecular identification

Genomic DNA isolation

Genomic DNA was extracted using a modified Sambrook method with phenol: chloroform: isoamyl alcohol [41, 42]. Cultures grown in LB broth were processed through standard lysis, extraction, and ethanol precipitation steps, with minor modifications to optimize yield and purity [27].

Gel electrophoresis

In order to get a 1% agarose gel, 2.2 g of agarose were measured and taken. 220 ml of 1xTAE (Tris Acetic Acid EDTA) solution was measured and poured into the agarose, then it was dissolved completely using a microwave oven. The mixed solution was placed in the fume hood and cooled down. Ethidium bromide was added at the rate of 1 µL per every 20 ml of gel. The gel was then cooled, poured into the cassette, and allowed to solidify. The solidified gel was placed in the electrophoresis tank filled with 1x TAE buffer and the combs were taken out. Marker (2 µL dye + 4 µL dH_2O + 1 µL) and then DNAs (2 µL dye + 5 µL DNA) were loaded in the first well of the gel by adjusting the loading amounts respectively [27].

Gene amplification and sequencing of isolates

The 16 S rRNA gene region of the bacterial isolates was amplified using universal primers (27 F and 1385R) [27]. The PCR products were purified and sequenced using standard protocols [43]. Amplicon sizes were verified using agarose gel electrophoresis and sequencing. [https://blast.ncbi.nlm.nih.gov/Blast.cgi]. The NCBI database received sequencing results, and accession numbers were generated in accordance with these results [44].

Phylogenetic analysis

Phylogenetic analysis was conducted using the MEGA11 software, and sequence quality control was performed by checking for ambiguous base calls and trimming low-quality regions before alignment. Only high-confidence sequences have been used for phylogenetic tree construction [45]. The internal and external groups and 16 S gene regions of the identified bacteria were determined, and a phylogenetic tree was constructed to reveal their evolutionary relationship [46]. The Neighbor-Joining method was employed to infer evolutionary relationships (Saitou and Nei, 1987), resulting in the identification of the optimal tree with a sum of branch lengths of 0.43955091 [27, 47]. The bootstrap value of replicating trees (consisting of related taxa) was provided beside the branches [48]. The tree was constructed by scaling the tree with branch lengths representing the evolutionary distances used to infer relationships between different species. These evolutionary distances were calculated using the p-distance method [49].

Results and discussion

Locations of investigation

Soil/sediment, mud, sugarcane, and water samples were collected from different areas of the sugar factory. The location of the study area; A: Indoor sugar processing waste area (40.576591°N, 43.097270°E), B: Outdoor pulp waste site (40.575392°N, 43.096937°E), C: Outdoor collection pond (40.565782°N, 43.097986°E) and D: Indoor sugar-water waste channel (40.586397°N, 43.098941°E) (Fig. 1).

Characterization of bacterial isolates

Bacteria recovered from the sugar factory were incubated aerobically at different temperatures ranging from 40 to 85 °C to determine their heat resistance. The features of the isolates under the microscope and their forms were examined using light microscopy along with Gram and endospore staining techniques. Such evaluations resulted in most of the isolates being identified as bacilli and cocci. The whole thermal tolerance profile of each isolate was determined in order to find out the best growth conditions. The isolates exhibited maximal growth between 40 °C and 85 °C, within a pH spectrum of 5.0 to 9.0, and demonstrated NaCl tolerance up to 9%.These findings indicated that the majority of the isolates were capable of surviving at elevated temperatures and across a broad range of pH and salinity conditions (Table 1).

Diversity of thermophilic bacterial isolates

Amplification using universal primers for the 16 S rRNA gene sequences was confirmed by gel electrophoresis. All isolates identified by BLAST analysis had nucleotide sequences of 807–1557 base pairs, and their accession numbers are given in the NCBI database (Supplementary Table 1). The bacteria identified have all been shown to exist as thermophiles and are able to flourish at a range of elevated temperatures ranging from 40 to 85 °C. The data were compared with the GenBank data, interspecies relationships were determined, and phylogenetic analyses were performed. As a result of these analyses, different bacterial strains were obtained. These are: *Acinetobacter baumannii*, *Aeribacillus composti*, *Aeribacillus pallidus*, *Aeribacillus* sp., *Anoxybacillus* sp., *Bacillus altitudinis*, *Bacillus circulans*, *Bacillus flexus*, *Bacillus haynesii*, *Bacillus hisashii*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus safensis*, *Bacillus* sp., *Bacillus zhangzhouensis*, *Brevibacillus borstelensis*, *Brevibacillus thermoruber*, *Enterococcus durans*, *Enterococcus faecium*,, *Geobacillus* sp., *Geobacillus thermodenitrificans*, *Klebsiella pneumoniae*, *Klebsiella* sp., *Prolinoborus fasciculus*, *Sphingobacterium thermophilum and Ureibacillus thermosphaericus* (Fig. 2).

Phylogenetic analysis

Phylogenetic relationships were inferred using the neighbor-joining method in MEGA11 (one outgroup, 22 type strains) [50]. In this case, a tree structure was built to indicate the relationship between species and their DNA or genetic data (Fig. 3). *Aeribacillus* sp. and *Geobacillus* sp. were shown to be interrelated, reflecting their ancestry. To improve readability, the phylogenetic tree is also presented in A3 size (Supplementary Fig. S6). The bootstrap values (numbers next to the branches) strongly endorse this arrangement. Bootstrap values of 99 or 100 provided strong support for a shared evolutionary origin. *Geobacillus* sp. are thermophiles, which could explain why they are closely associated with *Aeribacillus*. Then it is shown that there is a relation between *Anoxybacillus* and *Ureibacillus* sp. with bands of high bootstrap support (99 and 100). These genera also exhibit thermophilic characteristics, implying that their ecological adaptations are responsible for their evolutionary closeness. *Brevibacillus* sp. were placed into a separate cluster with high levels of bootstrap support (such as 100), indicating a clearly established evolutionary relationship. This cluster contains members such as *Brevibacillus borstelensis* and *Brevibacillus thermoruber*, both of which are thermophilic and live under harsh conditions. The tree indicates that *Bacillus* sp. occupies a large portion of its clade and divides it into numerous subgroups. In particular, branches that include *Bacillus licheniformis*, *Bacillus safensis*, and *Bacillus altitudinis* have strong support for their separation from other *Bacillus* sp. The presence of different *Bacillus* sp. strains at a given location indicates that they are evolutionarily related. Distinct clades are formed by *Klebsiella pneumoniae* and *Acinetobacter baumannii*, which are both considered pathogenic in a clinical setting. *Klebsiella* genus including for instance *Klebsiella pneumoniae* Type Strain and several other strains show one hundred per cent bootstrap support from the resulting cladogram implying that the strains share a common ancestor. *Sphingobacterium thermophilum* and *Enterococcus faecium*/*durans* undergo bifurcation. The relationships within the tree indicate that they do not belong to the *Bacillus* and *Geobacillus* groups, and thus are evolutionarily distant. The position of *Enterococcus* and *Sphingobacterium* relative to the central *Bacillus* group corresponds to their evolutionary history. *Prolinoborus fasciculus* is a strain that seems to branch out much younger in the tree and does not belong to the *Bacillus* group. Its peripheral position in tree projections indicates effective evolutionary isolation. The tree representation of the phylogenetic relationships among thermophiles and pathogenic bacteria provides an effective representation of evolutionary divergence. The high bootstrap values in many branches develop confidence regarding the declared relationships. The interspecific

Table 1 Identification and characteristics of bacterial isolates

Identification	Isolate Code	pH	NaCl (%)	Temperature (°C)/T_{opt} (°C)	Aerobic growth	Movement	Morphology	Chain	Endospore	Gram test
Bacillus safensis	UA41, UA59, UA70, UA87	5–9	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Bacillus licheniformis	UA3, UA12, UA57, UA62,	5–9	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Brevibacillus borstelensis	UA2, UA8	5–9	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Geobacillus sp.	UA5, UA72	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Brevibacillus thermoruber	UA10, UA17, UA18, UA19, UA20,	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Klebsiella pneumoniae	UA21, UA25, UA31, UA32, UA35, UA36, UA38, UA81, UA82	5–8,5	≤ 5	40–85/72	+	-	Rod/Bacil	-	-	-
Acinetobacter baumannii	UA54, UA55, UA56,	5–8,5	≤ 5	40–85/72	+	-	Coco Bacil	-	-	-
Bacillus sp.	UA1, UA4, UA27, UA42, UA44, UA47, UA50, UA52, UA74, UA78, UA79	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Aeribacillus sp.	UA23, UA28, UA29, UA80, UA83	5–8,5	≤ 9	40–85/72	+	+	Bacilli	-	+	+
Bacillus altitudinis	UA26	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Aeribacillus pallidus	UA30	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Bacillus circulans	UA45	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Bacillus flexus	UA66, UA71, UA73	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Bacillus haynesii	UA69	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Sphingobacterium thermophilum	UA9, UA11	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Bacillus zhangzhouensis	UA22	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Anoxybacillus sp.	UA86	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Bacillus pumilus	UA61	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Geobacillus thermodenitrificans	UA43	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Enterococcus faecium	UA65	4,5–9	≤ 9	40–85/72	+	-	Coccus	+	-	+
Ureibacillus thermosphaericus	UA7	5–9,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Klebsiella sp.	UA33, UA37	5–8,5	≤ 9	40–85/72	+	-	Rod/Bacil	-	-	-
Bacillus hisashii	UA13	6–9	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Aeribacillus composti	UA84	6–9,5	≤ 9	40–85/72	+	+	Bacilli	+	+	+
Enterococcus durans	UA63	4,5–9	≤ 9	40–85/72	+	+	Coccus	+	-	+
Prolinoborus fasciculus	UA64	5–8,5	≤ 9	40–85/72	+	+	Bacilli	+	-	-

T_{opt} Optimum temperature

grouping not only demonstrates genetic relatedness, but also ecological and functional attributes, such as thermophiles or pathogens. This tree provides a clear view of how these bacterial species evolved and their relative positions on an evolutionary scale based on 16 S rRNA sequence data.

Enzyme production profile

All the isolates were subjected to enzyme production tests to determine their amylase, lipase, protease, and xylanase enzyme production capabilities (Supp. Tables 2 and Supp. Fig. S1, S2, S3, S4 and S5 "A, B, C, D, E, F, G, H, I, J, K and L"). The figure shows the distribution of enzyme production among bacteria. Protease was the most common enzyme (89.4%) followed by xylanase (83.3%), amylase (66.7%), and lipase (60.6%) (Fig. 4). To assess the association between the production of different hydrolytic enzymes among the bacterial isolates, chi-square tests were performed.

Statistical analysis

All statistical tests were performed in accordance with standard assumptions, and categorical variables were analyzed using non-parametric tests where appropriate.

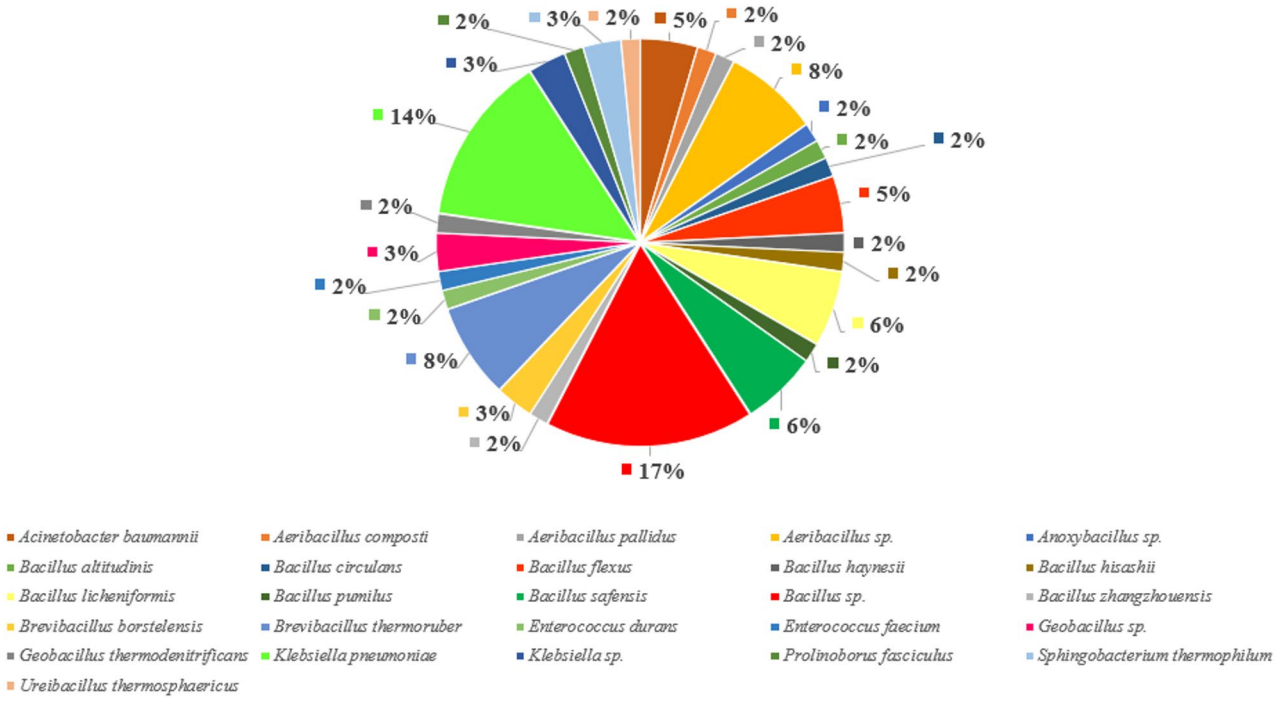


Fig. 2 Pie chart showing the diversity of bacterial genera and species based on 16 S rRNA gene sequencing of isolates from various sampling sites in the Kars Sugar Factory. Several diverse bacterial taxa were identified using BLAST analysis. The relative abundances of the bacterial taxa studied are shown

A total of 66 bacteria were identified at the species level at the Kars Sugar Factory. *Bacillus* spp. were the most common group (16.7%), followed by *Klebsiella pneumoniae* (13.6%), *Aeribacillus* sp. (7.6%), *Brevibacillus thermoruber* (7.6%), *Bacillus licheniformis* and *Bacillus safensis* (6.1% each). Isolates were obtained from four different sources: outdoor residual bagasse (28.8%), indoor sugar processing waste (27.3%), indoor sugar water waste channels (25.8%), and outdoor collection pools (18.2%). Enzyme production tests showed that 89.4% of the isolates were able to produce protease, 83.3% xylanase, 66.7% amylase, and 60.6% lipase. The most commonly detected enzymes were protease and xylanase, indicating that the bacteria have a high potential to degrade proteins and lignocellulosic structures. This study demonstrated the existence of vast bacterial diversity in the Kars Sugar Factory and the capacity of these bacteria to synthesize several hydrolases such as amylase, protease, lipase, and xylanase (Supplementary Table 2).

Statistical evaluation of the relationship between enzyme production and sampling locations

Significant differences were found between amylase, lipase, and xylanase production and sampling locations ($p < 0.05$), whereas protease production was high regardless of location ($p = 0.200$). Lipases and xylanases were mostly produced by isolates from bagasse and indoor waste ($p < 0.05$) (Table 2). In addition, bacteria isolated from acidic environments predominated, and this distribution was statistically significant in relation to pH ($p = 0.000$) (Fig. 5).

When the relationship between the pH levels of the sampling collection locations and enzyme production status was analyzed, it was determined that lipase and xylanase enzymes changed significantly depending on the pH of the environment (Table 3). While acidic environments were effective for lipase production, lipase production was significantly decreased in basic environments ($p = 0.000$). Xylanase was also observed more frequently in acidic environments ($P = 0.006$). However, there were no significant differences in amylase and protease production depending on the pH level ($p > 0.05$).

In the analysis of bacterial species isolated from different sampling sites in terms of all enzyme types, *Bacillus*, *Geobacillus*, *Klebsiella*, and *Brevibacillus* species had the capacity to produce all four hydrolase enzymes (Supplementary Table 3). Outdoor bagasse and indoor sugar processing waste zones were the areas where most enzyme-producing species were found. This indicates that the organic matter content and microbial diversity in these areas are important for enzymatic production.

Although biochemical procedures investigate enzymatic activities relevant for biotechnological evaluation, morphological characterization serves as a preliminary differentiation. Because phenotypic methods often have limitations, precise identification at the species level is achieved through 16 S rRNA sequencing, which also provides clues for differentiating closely related taxa. In

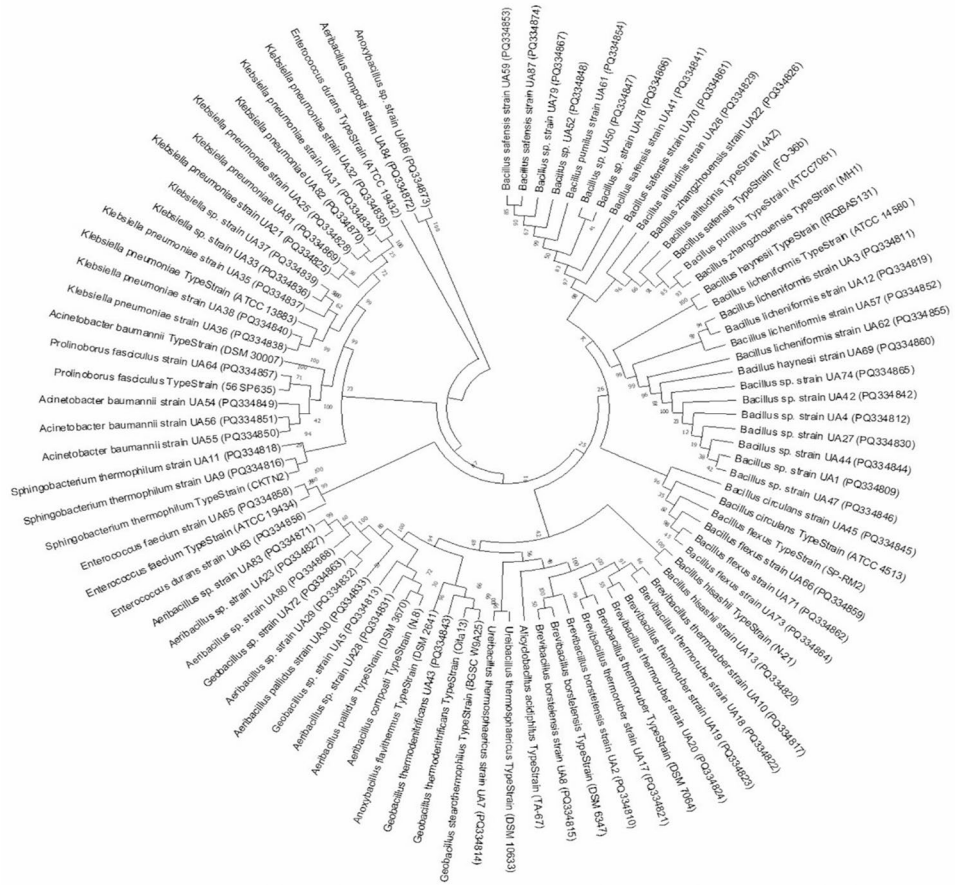


Fig. 3 The phylogenetic tree shows the evolutionary relationships of bacterial species based on 16 S rRNA sequence data. Thermophilic bacteria such as *Aeribacillus* and *Geobacillus* species show a structure reflecting their close relatedness and previous common ancestral links. Other thermophilic genera such as *Anoxybacillus* and *Ureibacillus* are similarly related, with high bootstrap support. *Brevibacillus* species, as thermophilic bacteria living in harsh conditions, are in a distinct evolutionary relationship. *Bacillus* sp. species form a broad clade, while pathogenic species such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* are clearly within distinct clades. This tree helps us to understand how bacterial species evolve and interrelate according to their genetic and ecological characteristics

turn, phylogenetic analysis has elucidated evolutionary relationships [51]. The methods used and their reproducibility ensure the reliability of the results obtained. These assessments demonstrate the accuracy of the findings.

In the alignment and sequencing of sequence analysis data, 16 S-based approaches can sometimes cause sequencing errors or alignment errors. Such errors can also prevent the emergence of different taxa. Additional strain identification through 16 S rRNA gene sequencing revealed isolates resembling *Bacillus*, *Geobacillus*, *Klebsiella*, *Acinetobacter*, and *Brevibacillus*. Likewise, *Geobacillus* spp. are known for their thermostable enzyme production, which is valuable in industrial processes such as starch hydrolysis and biofuel production [52, 53]. Phylogenetic analysis distinguished *Geobacillus* from *Aeribacillus*, confirming previous findings [54].

Most isolates produced proteases and xylanase enzymes. These enzymes contribute to nutrient acquisition and survival under environmental stress conditions. In contrast, the lipase production varied. Lipase enzyme production is probably influenced by the amount of substrate present in the environment. Although the limited number of samples makes it difficult to generalize, our findings reveal an understudied microbial diversity in sugar factory wastes and highlight the high enzyme activity between the thermophilic and mesophilic isolates. Although enzyme screening in cold regions is common in the literature, studies on thermophilic bacteria isolated from industrial wastes in cold climates are very limited. The extreme environmental conditions of the factory, such as high temperature, variable pH, and chemical content of the waste, may favor the selection of stress-resistant and enzyme-producing microorganisms. Amylase is widely used in starch processing, textile, and paper industries [55]. The rate of amylase production (66.7%) recorded in the current study suggests that the isolates obtained from the Kars Sugar Factory may have potential in industries that require starch hydrolysis, although further validation is necessary. Although amylase production did not present statistically significant differences

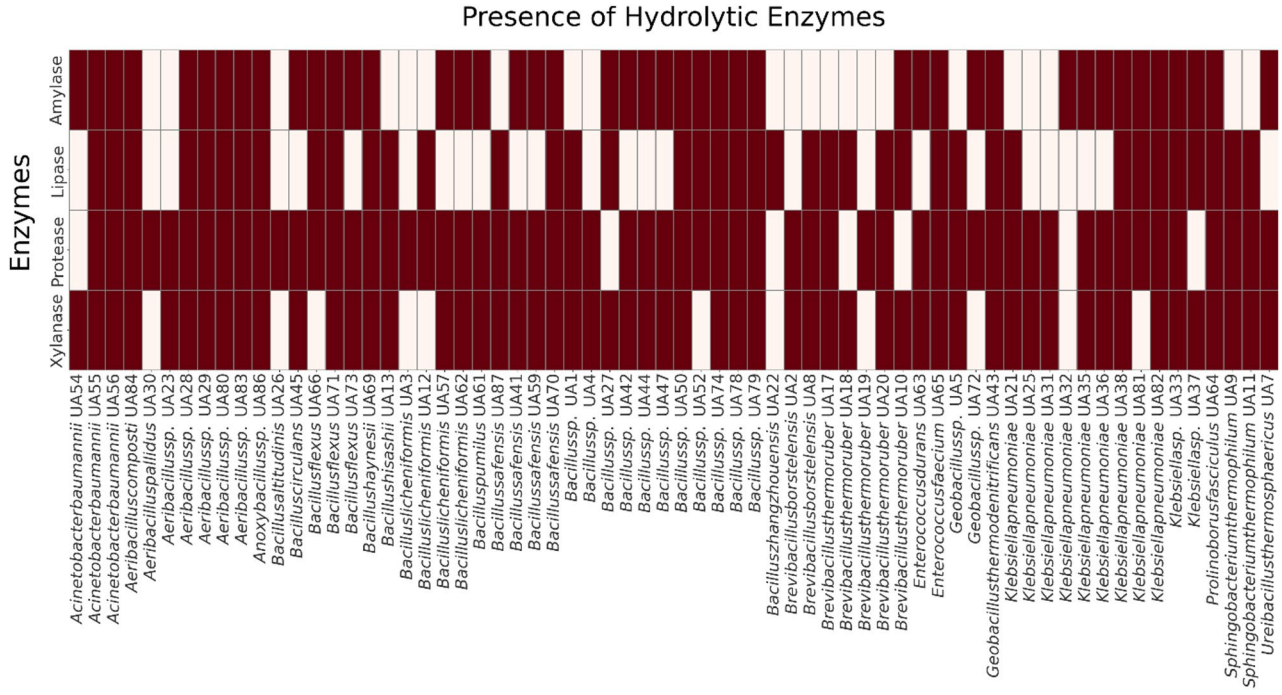


Fig. 4 The figure illustrates the presence (red) and absence (white) of four hydrolase enzymes: amylase, lipase, protease, and xylanase in bacterial isolates identified from the Kars Sugar Factory

Table 2 Distribution of enzyme production status of bacteria isolated from the Kars sugar factory according to sampling locations and environmental pH values (pearson Chi-square test results). Statistically significant values are indicated by an asterisk (*) ($p < 0.05$)

(*N*-%)	The external environment is now pulp	Outdoor collection pool	Indoor sugar processing wastes	Indoor sugar water waste duct	Total	*p*
Amylase (-)	9–13.6	9–13.6	1–1.5	3–4.5	22–33.3	***0.000****
Amylase (+)	10–15.2	3–4.5	17–25.8	14–21.2	44–66.7	
Lipase (-)	1–1.5	8–12.1	0–0.0	17–25.8	26–39.4	***0.000****
Lipase (+)	18–27.3	4–6.1	18–27.3	0–0.0	40–60.6	
Protease (-)	2–3	3–4.5	2–3	0–0	7–10.6	0.200
Protease (+)	17–25.8	9–13.6	16–24.2	17–25.8	59–89.4	
Xylanase (-)	0–0	8–12.1	2–3	1–1.5	11–16.7	***0.000****
Xylanase (+)	19–28.8	4–6.1	16–24.2	16–24.2	55–83.3	
Acidic (pH < 7.0)	19–28.8	0–0	18–27.3	0–0	37–56.1	***0.000****
Basic (pH > 7.0)	0–0.0	12–18.2	0–0	17–25.8	29–43.9	

among the sampling sites ($p > 0.05$), the reasonably high observed frequency (66.7%) coincides with the findings from earlier studies on sugar factory waste, where starch residues are known to serve as natural inducers.

Proteolytic enzymes or proteases are naturally occurring proteins that play a major role in the degradation of foods, human health and bacteria control, and thus they are the most widely used enzymes in food, pharmaceutical, and detergent industries [56]. Our 89.4% rate of the thermophilic strains producing proteases confirms the thermophilic nature of the bacteria in the compost and supports the earlier reports on thermophilic protease producers [57]. The isolates from the outdoor collection pond and indoor processing waste also significantly correlated with protease production ($p = 0.017$), suggesting that environments enriched with nitrogen may have selected the bacteria producing protease. The results obtained indicated the high protease activity which is a great advantage for the industry. On the other hand, it is essential to conduct further studies to evaluate [58, 59].

Lipase activity, quantified at 60.6%, aligns with previous scholarly findings concerning isolates derived from industrial wastewater. Strains such as *Bacillus licheniformis* and *Geobacillus* sp. present considerable potential for applications in biodiesel and detergent production [60, 61]. The variability in lipase production among the isolates was pronounced and did not demonstrate statistically significant differences across the various sampling locations ($p > 0.05$), which may indicate the extensive

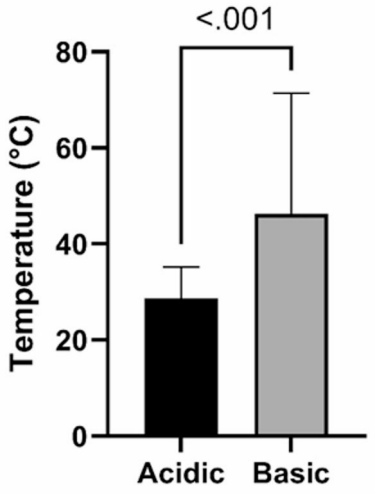


Fig. 5 Boxplot comparing sample temperatures obtained from acidic (pH < 7.0) and basic (pH > 7.0) environments at different collection sites. The results indicated that samples from acidic environments had significantly lower temperatures (*$p < 0.001$, Independent Samples T-test)

Table 3 Relationship between enzyme production and pH levels (acidic/basic) of sampling sites (*Pearson's chi-squared test). Statistically significant results are indicated by an asterisk ($P < 0.05$)

(*N*-%)	Acidic (pH < 7.0)	Basic (pH > 7.0)	Total	*p*
Amylase (-)	10–15.2	12–18.2	22–33.3	0.220
Amylase (+)	27–40.9	17–25.8	44–66.7	
Lipase (-)	1–1.5	25–37.9	26–39.4	***0.000****
Lipase (+)	36–54.5	4–6.1	40–60.6	
Protease (-)	4–6.1	3–4.5	7–10.6	0.951
Protease (+)	33–50.0	26–39.4	59–89.4	
Xylanase (-)	2–3.0	9–13.6	11–16.7	***0.006****
Xylanase (+)	35–53.0	20–30.3	55–83.3	

metabolic diversity inherent in lipase-producing strains operating under thermophilic conditions.

Xylanases are the key enzymes in the degradation of hemicellulose-rich materials [25, 62, 63]. The proportion of xylanase production (83.3%) indicated in this study is the highest so far and suggests that these isolates can be applied as biocatalysts in the lignocellulose degradation processes. The correlation between xylanase production and isolates from indoor processing environments was statistically significant ($p = 0.021$) indicating that the organic-rich, high-temperature conditions were the most influential factor for the potential of enzyme production.

These findings provide insights that could be relevant for industrial biotechnology, particularly in waste resource engineering, biofuels, and green manufacturing, although further validation in real-world applications is required [64]. Enzymes made by extreme surroundings bacteria have a variety of industrial applications, with organic waste elimination and chemical industry being the main ones. The above-mentioned facts clearly indicate the increasing preference of microorganisms as sources of enzymes in extreme conditions. The future direction of research should be to improve the environmental conditions for the growth and enzyme production of these isolates. The entire spectrum of enzymes produced by the isolates and their capacity to produce multiple enzymes at once imply that they are truly great candidates for industrial biotechnology [65]. The isolates mentioned here might have a good potential as waste management agents because they are already proving to be very active against organic waste. They could facilitate an eco-friendly waste management system involving biomass as a resource and a circular economy. It will take more research to determine the large-scale enzymes' usability. The industrial applications of the defined bacterial isolates offer a great deal in terms of enzymes, yet more research is needed, particularly in field or large-scale industrial conditions. It should be the case that future studies of industrial enzymes focus on large-scale production. The chosen methods, particularly the sampling strategies (selective media and incubation conditions such as 40–85 °C), could have limited the detection of all bacterial isolates.

A metagenomic or high-throughput approach should provide a more holistic view of microbial diversity [66]. The sample size was sufficient to support the preliminary findings; however, more comprehensive studies are required for generalization. More complete sampling over multiple time points and environmental conditions will certainly help achieve a more accurate representation of microbial diversity in future studies. This study is confined to culturable thermophilic bacteria, as they are grown under specific laboratory conditions (LB agar medium, aerobic incubation, and temperatures between 40 and 85 °C). Therefore, the bacterial diversity described herein may not fully reflect the total microbial diversity of the sample sites. The use of selective media and incubation temperatures is likely to bias isolation toward strains that would be dominant or faster-growing, and exclude slow-growing or unculturable taxa. In addition, phenotypic and biochemical characterization provides valuable information, but has limitations in species-level identification, unless confirmed by molecular techniques. All of these limitations should be borne in mind when interpreting the results. Overall, this study reinforces the existing knowledge while bringing in new data from a cold-region sugar factory that is rarely studied, thus highlighting the biotechnological potential of the isolates.

Conclusions

This study provides novel insights into the thermophilic microbial diversity found in the Kars Sugar Factory, and the possibility of using these microorganisms for industrial enzyme production. Following a thorough isolation

and characterization of bacterial strains, it was found that most of the isolates which mainly belonged to the genera *Bacillus*, *Geobacillus*, *Klebsiella*, and *Acinetobacter* possessed remarkable enzymatic capabilities, including amylase, protease, lipase, and xylanase production. Protease and xylanase producers were the dominant producers, whereas amylase production was less common. The distribution pattern of enzymes among individual isolates suggests the potential for further exploration of biomass degradation and protein hydrolysis applications. Phylogenetic analysis also proved that many of these bacteria are thermophilic or thermotolerant as they are adapted to high temperatures, which is an added benefit for industrial processes that utilize enzymes that would need to be active at high temperatures. The facts that 89,4% of the isolates produce protease indicate great promise for its use in the manufacture of detergents, food processing, and in waste management. Likewise, the high activities of amylase, lipase, and xylanase suggest the importance of these isolates in the production of biofuels and the manufacture of textiles and paper. This study subscribes to numerous investigations that have recognized the role of thermophilic and mesophilic microorganisms in industrial biotechnology. The discussion of the results in relation to the available literature indicates the likely presence of many enzyme producing bacteria in sugar factory environments. One of the possibilities for the future research is to find methods that not only improve but also greatly increase the production of these enzymes and at the same time to use gene manipulation techniques to improve the efficiency of these isolates. Overall, the bacterial isolates from Kars Sugar Factory appear to be a great asset to the industries, especially the enzymes producing ones, that prefer to use eco-friendly and cost-effective ways as they are the main beneficiaries. The results obtained in this study provide a foundation for further research on microbial biotechnologies with industrial relevance. Genetic engineering may improve the enzyme yield and stability. Metabolic studies may clarify thermophilic adaptation.

This study has revealed that bacteria isolated from the Kars Sugar Factory have high enzyme production potential. However, evaluating the quantitative properties of these enzymes and their stability under industrial conditions will be an important focus of future studies.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04532-0.

Supplementary Material 1.

Supplementary Material 2: Supplementary Fig. S1 A: UA19, B: UA35, C: UA10, D: UA26, E: UA7, F: UA12, G: UA44, H: UA25, K: UA41, L: UA30, J: UA72, and I: UA4 Petri dish images of amylase test results of the coded isolates. Supplementary Fig. S2 A: UA2, B: UA4, C: UA25, D: UA36, E: UA62, F: UA70, H: UA50, and G: UA82 Petri dish images of catalase test results of the coded isolates. Supplementary Fig. S3 A: UA9, B: UA13, C: UA29, D: UA38, E: UA19, F: UA66, H: UA50, and G: UA82 Petri dish images of lipase test results of the coded isolates. Supplementary Fig. S4 A: UA5, B: UA20, C: UA29, D: UA41, F: UA19, I: UA22, H: UA50, and G: UA62 Petri dish images of protease test results of the coded isolates. Supplementary Fig. S5 A: UA18, B: UA36, C: UA56, and D: UA81 Petri dish images of the xylanase test results of the coded isolates. Supplementary Fig. S6 A3-sized PDF format, allowing for a more detailed visualization of phylogenetic relationships. The phylogenetic tree shows the evolutionary relationships of bacterial species based on 16S rRNA sequence data. Thermophilic bacteria, such as *Aeribacillus* and *Geobacillus* species, show a structure that reflects their close relatedness and previous common ancestral links. Other thermophilic genera, such as *Anoxybacillus* and *Ureibacillus*, were similarly related, with high bootstrap support. *Brevibacillus* species are thermophilic bacteria that live in harsh conditions and have a distinct evolutionary relationship. *Bacillus* sp. species form a broad clade, whereas pathogenic species such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* are clearly within distinct clades. This tree helps us to understand how bacterial species evolve and interrelate according to their genetic and ecological characteristics.

Supplementary Material 3: Supplementary video 1 The outdoor collection pond of the Kars Sugar Factory (40.565782°N, 43.097986°E).

Supplementary Material 4.

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Authors' contributions

OU and UA Conceptualization, Methodology, Validation, Investigation, Resources, Formal analysis, Writing - Original Draft, Writing - Review & Editing; OU, Methodology, Investigation, Resources, Supervision. OU, Conceptualization, Methodology, Investigation, Resources. All authors have read and approved the final manuscript.

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Data availability

The datasets generated and/or analyzed during the current study are available from supplementary information files and the corresponding author upon reasonable request. The 16S rRNA sequence data that support the findings of this study have been deposited in the NCBI GenBank database under the accession numbers;"PQ334809–PQ334874" (https:/www.ncbi.nlm.nih.gov/nuccore/?term=PQ334809:PQ334874%5Baccn%5D). A complete list of accession numbers corresponding to each isolate is provided in the Supplementary Table 1.

Declarations

Ethics approval and consent to participate

This study did not involve human participants, human data, or animals, and thus, ethical approval and consent to participate were not required. All experimental protocols and methodologies adhered to relevant institutional and international guidelines for research.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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