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Characterization of Endolithic Extremophilic Bacterial Diversity from Sandstone of Keroo, Jodhpur, Rajasthan

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Abstract: ***Extremophilic bacteria thrive in extreme environment like hot springs, deep-sea vents and acidic lakes. Their unique adaptation makes them valuable in biotechnology for enzyme production, bioremediation and pharmaceuticals. Studying these organisms provides insight into life's limits and potential applications in industries requiring robust enzymes or ecofriendly solutions. In the present investigation extremophilic bacteria from mine stones were collected from various microbiologically unexplored locations within the Keroo, Jodhpur (26^{O}33'81.26" N; 72^{O} 90'54.87" E).Total 41 Isolated extremophilic bacteria were examined based on their morphological, physiological, biochemical and molecular characteristics. Under adverse conditions, these strains were produced enzymes - cellulase, protease, amylase and laccase. Using sequence analysis and 16S rRNA gene amplification, the four selected specific strains—E1B-LT, E2A-LT, E1D-HT, and E2A-HT were further explicated at the molecular level. The identified strain E2A-HT and E1D-HT demonstrated their identity with the type strains Bacillus sp. strain NG4-2 and Geobacillus sp. Strain G4-1 respectively. The strains E1B-LT and E2A-LT had identical with the type strain Pseudarthrobacter phenanthrenivorans Sphe3, respectively. These closest strains were isolated from habitats in cold and marine environments, which support their evolutionary significance of habitat. While E1D-HT and E2A-HT can withstand temperatures as high as 60°C, strains E1B-LT and E2A-LT can only withstand temperatures as low as 10°C. Because the isolates have the ability to degrade dyes, bioremediation can be approached from a variety of perspectives.***

Keywords: *Bacilli*, *Bioremediation*, Extremophile, *Geobacillus*, Keroo, *Pseudarthrobacter*.

1. Introduction

Extremophiles that thrive in harsh environments, can be found in deserts, hot or acidic springs, saline or alkaline lakes, and ocean bottoms (Rothschild et al. 2001; Kumar et al. 2010; Goswami and Das, 2016). Because of unique mechanisms and the creation of an abundance of secondary metabolites, only extremophiles can survive in harsh environments (Merino et al. 2019). The geography of North-Western India consists of plains, sand dunes, and rocky hill outcrops. The Marwar supergroup (Jodhpur), which includes a portion of North-West Rajasthan, is a late Neoproterozoic to early Cambrian lithostratigraphic unit in North-West India.

It is estimated that the sandstones and limestones in the Marwar region date back around 780 million years. Different shallow marine to non-marine environments were where it was deposited (Pandey and Bahadur, 2009). Microbial mat-induced sedimentary formations with a range of colours from white to brownish are found in the Girbhakar Sandstone (Lohawat and Phalodi) and Sonia Sandstone (Sursargar, Mandore, Kaliberi, Keroo, and Balesar areas) (Parihar et al. 2012). The second largest city in Rajasthan state is Jodhpur (26^{O}23'8" N; 73^{O}02'43" E), which is well-known for its vast range of meteorological conditions, including temperatures between 0°C to 50°C and wind velocities between 20 to 130 km/h (Sharma and Mehra, 2009). Jodhpur is also a district that excels in quarrying and mining.

Furthermore, the soil has minimal fertility and very little organic matter and other nutrients. The various soil and climate circumstances suggest the presence of a variety of microorganism species, which could serve as a possible source of beneficial metabolites (Parihar et al. 2020). Numerous sandstone and lime mines exist, including Kaliberi, Sudho ki Dhani, Fidusar, Bambor, and others (Borana et al. 2018). Finding new and adaptable bacterial strains that can benefit both the environment and people will be aided by investigation on microbial diversity in harsh environments. Based on more microbiological data about how life may survive in harsh environments.

Thus, in order to investigate the bacterial population from the east site of the Keroo mine No. 47 in Jodhpur, Rajasthan, an attempt was made to isolate such bacteria at various depths as part of the current study. About forty-one pure colonies of extremophiles have been isolated. Four selected bacteria were identified: E1D-HT and E2A-HT as thermophiles with the ability to produce laccase for dye degradation, and E1B-LT and E2A-LT as psychrophiles.

2. Methodology

1) Selection of sample collection area and sample collection

Rajasthan experiences year-round, erratic weather, including periods of intense cold, intense heat, and precipitation. Jodhpur, Rajasthan is home to several stone mines, including Bambor, Fidusad, Kaliberi, Keroo, and Sudho ki Dhani, from which stones were extracted for use in building construction. Thus, mines of Keroo (26^{O}33'81.26" N; 72^{O}90'54.87"E), Jodhpur, Rajasthan was chosen for the research of microbial diversity because of its harsh climate. Samples of stone were taken in September

2021 at different depths from various locations within Mine No. 47 in Keroo (Table No. 1) in an aseptic environment conditions with a temperature range of 31°–38°C, pH of 8–10, humidity of 11–88%, and wind speed of 8 km/h during the time of collection. Within a day after being gathered, the stone samples were taken in a sterile container, transported to the laboratory, and processed.

2) Media selection and isolation of bacteria

Nutrient Broth/Agar (Himedia, India) was chosen among the several media that were available and utilized to isolate extremophilic microorganisms from the stone soil sample. After preparing a 10% w/v soil suspension in sterile distilled water (Milli-Q grade water) and filtering it through sterile 0.22µm membrane filters (Ranbaxy, India), the mixture was centrifuged for ten minutes at 3000 rpm (Remi, India). The isolated supernatant was serially diluted in a sterile environment. To perform the spread plate technique, the sample was serially diluted up to 10^{-7} using sterile water. A nutrient medium (10 ml) was filled with each diluted soil sample and cultured for one week at 35°C. The growth was measured with a spectrophotometer set to 600 nm. Onto nutrient agar plates, broth cultures were then streaked. Isolated colonies were continuously sub-cultured in order to purify them. Colonies that had undergone additional purification and differentiation were streaked onto the appropriate medium and kept at 15% glycerol (v/v).

3) Physicochemical characteristics of mine stone

Using a mortar and pestle, the gathered stone samples were aseptically crushed and ground into a powder. To determine the quality of stone soil, a standard protocol was used to analyse the following physico-chemical parameters: pH (Kicińska et al. 2022), carbon content (Walkley and Black, 1934), nitrogen content (Subbiah and Asija, 1956), presence of heavy metals (Yaqub et al. 2020), and electric conductivity (Richards, 1954) of the sample.

4) Phenotypic Characterization

Staining, morphology, and cultural characteristics

The features of the bacterial isolates that had been purified were assessed. The morphological features of the 24-hour-old bacterial cultures grown on nutrient agar (NA) plates were ascertained. Form, texture, colour, elevation, border, surface opacity, and pigmentation were among these characteristics (Akmar et al. 2011). Gram staining and common biochemical tests, such as the indole test (MacFaddin et al. 2000), IMViC, catalase and glucose fermentation test (Hemraj et al. 2013), methyl red and Voges-Proskauer test (MRVP), starch hydrolysis (observed at 24-48 incubation) (Luang-In et al. 2019), citrate, oxidase and triple sugar iron test(Cappuccino and Sherman 2014)) were used to assess the isolates biochemical activities.

5) Enzyme production study

Selected bacterial strains were isolated and identified from the wide variety of stone samples in order to carry out enzyme production assays: -

6) Amylase production

The chosen isolates were grown on nutrient agar supplemented with 1.5% starch. Gram's iodine solution was added to the petridish after it had been properly incubated at the designated length of time and temperature. This revealed a distinct area that indicated starch hydrolysis (Luang-In et al. 2019).

Protease production

The isolates were cultivated on skimmed milk agar plates and observed the colonies after 24-48 hours were surrounded by zones of skim milk hydrolysis during incubation to show proteolytic activity (Olajuyigbe and Ajele, 2005).

Cellulase production

The pure culture of isolated bacteria was spread on screening medium for the cellulase enzyme production assay. The cultures were incubated for 24-48 hours and stained with 1% Congo red solution. The emergence of a clear area upon destaining indicated cellulose hydrolysis (Teather and Wood, 1982).

Laccase production

A 10 ml pre-culture broth of isolated bacteria was made, with 0.1 ml of inoculums, 1% sodium chloride, and yeast extraction. It was shaken at 120 rpm for 24 hours at 37°C in an incubator. Under the same circumstances, the lone Nutrient broth culture was also incubated for the entire night at 37° C. Both test tubes were filled with 100 mgL^{-1} of Congo red dye, which was then left for ten days of observation and incubation. The ability of bacteria to degrade dyes in comparison to a control is indicated by the decolourization of red colour (Sharma et al. 2022).

7) Physiological Characterizations

Selective bacterial strains growth patterns were examined at various temperature (5°C, 37°C, and 60°C), saline (0%, 5%, 10%, and 20%), and pH (3, 7, 10, and 12) conditions. 200 ml of freshly autoclaved nutrient broth with the appropriate pH and saline concentration were inoculated with 1 ml of the 24-hour-cultured broth of selected strains. Each inoculated flask was then placed in a shaker set at 150 rpm at 5°C, 37°C, and 60°C for 48 hours, with the blank containing only nutrient media correspondingly. After extracting 200µl of the broth culture once per 24 hours, the optical density was determined at 600 nm using a UV-VIS spectrophotometer (Thermo Scientific Multiscan Go). Each bacterial strain's comparative growth pattern was examined by charting an absorbance versus pH and saline concentration at three different temperatures: 5°C, 37°C, and 60°C. All data were obtained from triplicates and were expressed as mean ±S.E.M. In all cases, a difference was considered significant when the p-value was less than 0.05.

8) Molecular characterization of selected bacterial strains

The 16S rRNA gene was sequenced in order to characterize the bacterial strains E1B-LT, E2A-LT, E1D-HT, and E2A-HT molecularly. Using a conventional approach, the genomic DNA of the bacteria was extracted and then utilized as a template for PCR amplification (Tillett and Neilan, 2000). The amplified product was sequenced by Applied Biosystem's commercial service located in Mumbai, India. The 16S rRNA gene sequence was amplified using bacterial universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R

(GGTTACCTTGTTACGACTT). The 16S rDNA sequences obtained by sequencing were analyzed using software tools-GeneTool and BioEdit. After being scrutinized and adjusted, the sequences were tested for homology with other sequence databases-GenBank-accessible reference and type sequences, by using the BLAST (Basic Local Alignment Search Tool). In order to get accession numbers, the corrected 16S rRNA gene sequence was uploaded to GenBank using the Submission Portal. For phylogenetic analysis, all 16S rDNA sequences of type strains that had the highest degree of similarity to the sequence of the test isolate were taken from the NCBI database. For alignment, matching hits with e-values closest to 0.0 were selected. Then, all of the acquired sequences were create consensus sequence by using BioEdit version 5.0.9 program and sequences showing a relevant degree of similarity were imported into the CLUSTAL-W program (Thompson et al 1994). Ultimately, the MEGA 11 version 11.0.13 maximum likelihood algorithm approach was used to generate the phylogenetic tree (Marcheggiani et al. 2008).

Table 1: Samples of sandstone were taken from the Keroo mine No. 47 in Jodhpur, Rajasthan, India. Site and depth of sampling were shown in the table. The alphabetic names of these extremophilic bacterial isolates were followed by numerical designations that indicated depth and temperature sustainability

Site	Depth	S. No	Isolates denotation	Type	Media
Mine No.47 (Serial No.20) Keroo, Jodhpur, Rajasthan, India (latitude (26°33'81.26" N; 72°90'54.87" E),	10 feet	1	E1A - HT	Thermophile	NA
		2	E1A - LT	Psychrophile	NA
		3	E1B - HT	Thermophile	NA
		4	E1B- LT	Psychrophile	NA
		5	E1D - HT	Thermophile	NA
		6	W1A - HT	Thermophile	NA
		7	W1B - HT	Thermophile	NA
		8	N1A - HT	Thermophile	NA
		9	N1C - HT	Thermophile	NA
		10	S1A - HT	Thermophile	NA
		11	S1A - LT	Psychrophile	NA
		12	S1B - HT	Thermophile	NA
		13	S1C - HT	Thermophile	NA
		14	S1D - HT	Thermophile	NA
	20 feet	15	E2A - HT	Thermophile	NA
		16	E2A - LT	Psychrophile	NA
		17	E2B - LT	Psychrophile	NA
		18	E2C - LT	Psychrophile	NA
		19	W2A - HT	Thermophile	NA
		20	N2A - HT	Thermophile	NA
		21	N2B - HT	Thermophile	NA
		22	N2C - HT	Thermophile	NA
		23	S2A - HT	Thermophile	NA
		24	S2B - HT	Thermophile	NA
		25	S2C - HT	Thermophile	NA
		26	S2D - HT	Thermophile	NA
	30 feet	27	E3A - HT	Thermophile	NA
		28	E3A - LT	Psychrophile	NA
		29	E3B - LT	Psychrophile	NA
		30	E3E - HT	Thermophile	NA
		31	E3G - HT	Thermophile	NA
		32	E3H - HT	Thermophile	NA
		33	W3A - HT	Thermophile	NA
		34	W3B - HT	Thermophile	NA
		35	W3C - HT	Thermophile	NA
		36	W3D - HT	Thermophile	NA
		37	N3A - HT	Thermophile	NA
		38	N3B - HT	Thermophile	NA
		39	N3C - HT	Thermophile	NA
		40	S3A - HT	Thermophile	NA
		41	S3B - HT	Thermophile	NA

3. Results

The sandstone samples used in this investigation were taken from the Keroo mine stone in Jodhpur, Rajasthan at various depths (up to 10, 20, and 30 feet) (Figure 1a-c). Figure 1d shows the isolated bacterial strains on NA culture media at different temperatures from the crushed stone sample. From the east location of the sandstone, a total of 41 different types of pure bacterial cultures were recovered for further study (Table 1). These were kept at 20°C in 15% glycerol. To facilitate identification, Table 1 names these microorganisms.

3.1 Physicochemical parameters of mine stone

It has been noted that the physicochemical characteristics of the Keroo mine stones vary with depth. It was found that the pH of mine stone soil ranges from 9.7 to 8.6, making it alkaline to mildly alkaline (Table 2). It was discovered that the differences were significant (at $p < 0.005$). In September 2021, stone samples were taken at a depth of 10 feet revealed a higher pH than samples taken at a depth of 20 feet. The overall findings demonstrated the alkaline pH seen at low depths of stone collection.

The physical and chemical characteristics of the soil combine to produce the soil electrical conductivity (EC) value in millisiemen per meter (mS/m). The EC values are used to quantify the salinity of the soil and show the conductivity of the samples, indicating the presence of dissolved ions. Elevated EC readings could suggest increased mineral content or salinity/alkalinity. The E1 sample had the highest EC value, 43.5 mS/m, supporting a high pH in the same.

The nitrogen found in rock is derived from two sources: either organically bound nitrogen paired with sediment, or nitrogen mixed from meteoric, sedimentary, and mantle sources in thermal fluids. Another sizable potential pool consists of nitrate deposits that have formed in arid and semi-arid regions. They are relatively consistent across the samples. Stone samples E-2 and E-3 had the highest accessible nitrogen concentration (value of 0.5) compared to the sandstone sample E-1, which had a value of 0.4.

Heavy metals' toxicity, resistance to degradation, and propensity for bioaccumulation make them extremely important ecologically. Zn, Cu, Fe, Ni, and Mn were among the specific heavy metals that were found to be present. The amounts of heavy metals in each sample differ significantly. The largest quantities of metals were found in Fe, while

those of Ni and Mn were frequently below detection limits (BDL). Only E-1 sample (52.86 mg/kg) has Cu, although all samples have Fe metal, and E-1 and E-2 samples include Zn.

Table 2: Physico-chemical assessment of Jodhpur's Keroo Mine no. 47 sandstone- east site (September, 2021)

Sample		E-1	E-2	E-3
pH		9.7 ± 0.115	8.6 ± 0.333	8.8 ± 0.333
EC (ms)		43.5 ± 0.866	0.26 ± 0.011	0.358 ± 0.000
OC (*100)		32 ± 0.577	35 ± 0.333	58 ± 1.154
AN		0.4 ± 0.033	0.5 ± 0.033	0.5 ± 0.057
Heavy Metal	Zn (mg/kg)	56.66 ± 0.202	26.48 ± 0.360	BDL
	Cu (mg/kg)	52.86 ± 0.029	BDL	BDL
	Fe (mg/kg)	234 ± 2.309	194 ± 3. 464	268 ± 1.732
	Ni (mg/kg)	BDL	BDL	BDL
	Mn (mg/kg)	BDL	BDL	BDL

*BDL= Below Detection limit

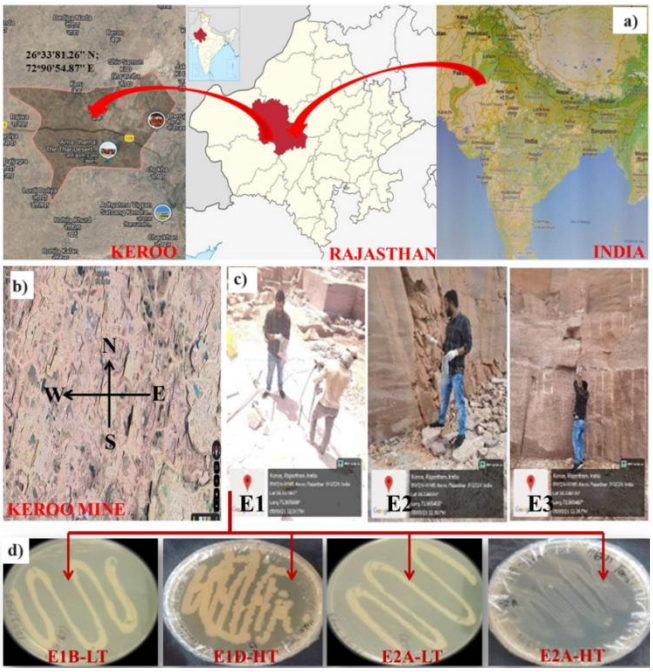


Figure 1: Selecting a location for stone gathering in order to isolate extremophiles. a) A Google map of the mine area; b) The coordinates (26º33'81.26" N; 72º90'54.87" E) of the selected area, Keroo, Jodhpur; c) An approximate 10, 20, and 30 feet depth site from sandstone isolated; d) The selective isolated and pure culture of bacterial strains - E1B -LT; E1D- HT; E2A-LT and E2A-HT at 10º C and 60º C.

3.2 Morphological characteristics of selected strains

Three bacterial strains- E1B-LT, E2A-LT, and E2A-HT exhibited gram positive reactions in the current investigation, while the remaining E1D-HT strain displayed gram negative reaction. The majority of the colonies were yellow in colour, having been chosen visually based on distinctions visible to the unaided eye (Figure 1d). However, over a prolonged incubation period, strains E1B-LT and E2A-HT also showed signs of yellowish colouration whereas E1D-HT showed light orange pigmentation. Table 3 illustrated how the four examined bacterial strains differed significantly in terms of their colony features, including surface, height, texture, and margin. Out of four strains, two had a rod-like morphology and the other two were cocci.

Table 3: Morphological and colony traits which distinct colonies isolated from mine samples.

Sample	E1B-LT	E1D-HT	E2A- HT	E2A- LT
Colony form	Irregular	Irregular	Regular	Regular
Transparency/ opacity	Opaque	Opaque	Transparent	Opaque
Colony elevation	Convex	Convex	Flat	Convex
Colony margin	Undulate	Lobulated	Entire	Undulate
Pigmentation	Yellowish	Light Orange	Yellowish	Whitish
Cell shape	Cocci	Rod	Rod	Cocci
Gram staining	Gram Positive	Gram Negative	Gram Positive	Gram Positive

3.3 Biochemical characteristics of selected strains

According to the variance in morphological traits, Table 4 indicates a significant divergence in the biochemical activity of all investigated strains. Different testing revealed different features for the bacterial strains that were the subject of the inquiry. Tests for oxidase, IMViC, glucose fermentation, and triple sugar iron showed favorable results for strains E2A-HT (Figure 2). All strain has a positive catalase test result. In the Triple Sugar Iron profile (A/A) test, strain E1D-HT was found to be positive for glucose fermentation.

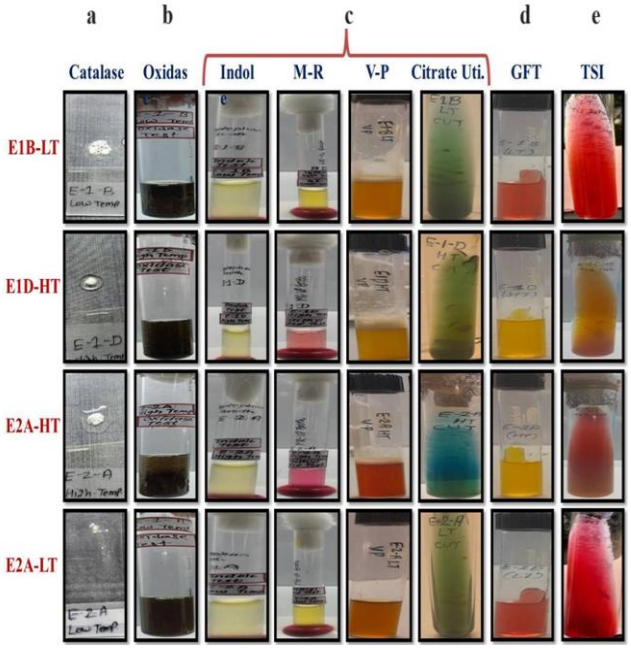


Figure 2: Isolates E1B-LT, E1D-HT, E2A-LT, and E2A-HT shown biochemical activity **a)** Catalase activity; **b)** Oxidase test **c)** IMViC test; **d)** Glucose fermentation test; and e**)** Triple sugar iron test.

Table 4: Biochemical characteristics of extremophilic bacterial strains obtained from the sandstone mines of Keroo, Jodhpur, Rajasthan, India.

Strain ID		E1B-LT	E1D-HT	E2A-HT	E2A-LT
Test					
IMVic Test	Indole Test	-	-	-	-
	Methyl Red	-	+	+	-
	Vogues Proskauer Test	-	-	+	-
	Citrate Test	-	-	+	-
Glucose fermentation test		-	+	+	-
Catalase Test		+	+	+	+
Oxidase Test		-	-	-	-
Triple	Slant/	K/K	A/A	K/A	K/K

Sugar Iron Test	Butt				
	Gas	-	+	-	-
	H2S	-	-	+	-

'+' denote the activity and'-'denotes no activity.
K denotes alkaline slant/butt; A denotes alkaline slant/butt
K/A - alkaline/acid (red slant/yellow butt) reaction: It is indicative of dextrose fermentation only.
A/A - acid/acid (yellow slant/yellow butt) reaction: It indicates the fermentation of dextrose, lactose and/or sucrose.
K/K alkaline/alkaline (red slant, red butt) reaction: Absence of carbohydrate fermentation results.
Blackening of the medium: Occurs in the presence of H_2
Gas production: Bubbles or cracks in the agar indicate the production of gas (formation of $CO_2$and H_2)

3.4 Enzyme production assay

Employing culture medium, the ability of each extremophile to produce the extracellular hydrolytic enzymes amylase, protease, cellulase, and laccase was investigated (Table 5). The isolates demonstrated enzymatic activity for one or more enzymes, according to preliminary assays conducted on media loaded with the substrate for each enzyme. While E2A-HT had significant amylolytic, proteolytic, cellulolytic, and dye degradation activities at high temperature, E1D-HT strain of thermophilic bacterial isolates possessed high amylolytic and dye degradation activities (Figure 3). At low temperatures, both the psychrophilic bacterial isolates E1B-LTand E2A-LT demonstrated amylolytic, proteolytic and cellulolytic capabilities. There were differences in the enzyme activity based on the formation of zone on culture plate, indicating that some strains were more active than others (Figure 3).

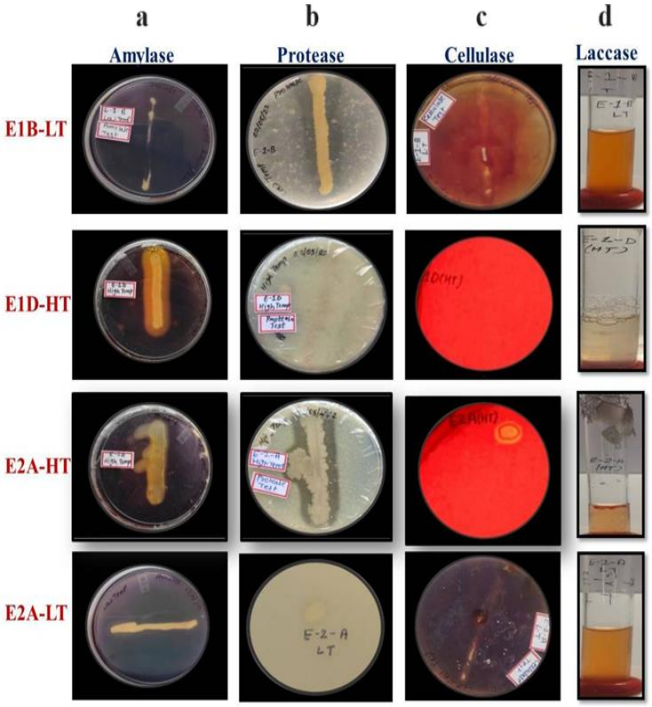


Figure 3: Assay for the production of enzymes using isolates E1B-LT, E1D-HT, E2A-LT, and E2A-HT respectively **a)** Amylolytic; b**)** Proteolytic; **c)** Cellulolytic and d**)** Congo Red dye degradation activities.

Table 5: The production of enzymes by the isolated, selected strains.

Strain ID	Enzyme Production			
	Amylase	Protease	Cellulase	Laccase
E1B- LT	+	+	+	-
E1D-HT	+	-	-	++
E2A- HT	+	+	+	+
E2A- LT	+	+	+	-

3.5 Physiological characterization of selected strains

3.5.1 Growth at different pH at 35^{O}C temperature

Among all the isolates tested, strain E1D-HT exhibits the highest growth capacity in an alkaline environment with a pH of 12, which indicates that the sample has a high alkalophilic character due to the alkali nature of sand stone in the physicochemical parameter. Others exhibit their best growth at pH 7.

3.5.2 Growth at different saline concentration at 10^{o}C, 35^{o}C and 60^{o}C temperature

The most rapid growth of the selective strain E2A-HT occurs in saline environments. E2A-HT demonstrates growth at temperature of 10^{o}C and 20% saline concentrations as well as 35^{o}C and 10% saline, respectively. At 35^o C, E2A-LT likewise exhibited increase growth of 5% saline concentrations. E1D-HT shows their optimum growth at 60^0 C.

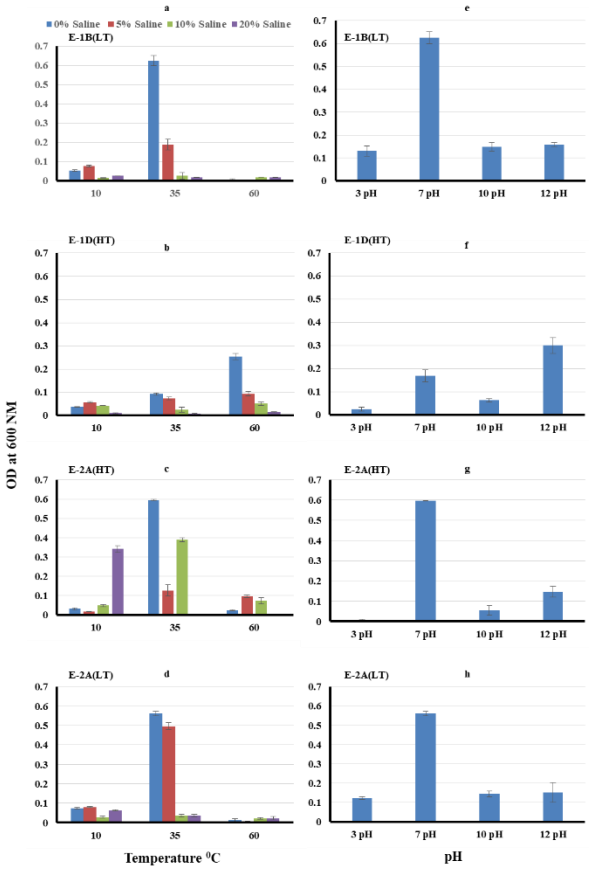


Figure 4: The kinetics of the growth patterns of the isolated extremophiles were depicted:-Growth curve of isolated strains on different saline conditions 0%, 5%, 10%, 20% at different temperatures 10^{o}C, 35^{o}C, 60^{o}C each **a)** E1B-LT; **b)** E1D-HT; **c)** E2A-HT and **d)** E2A-LT as well as at different pH 3, 7, 10 and 12 respectively at 35^{o}C temperature **e)** E1B-LT ; **f)** E1D-HT; **g)** E2A-HT and **h)** E2A-LT.

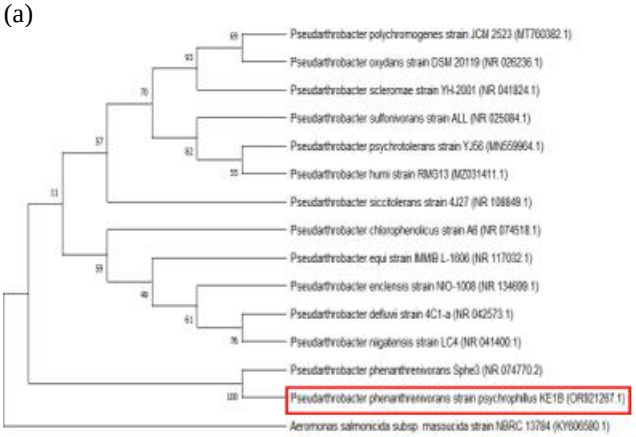
3.6 Molecular characterization

Based on morphological, biochemical, and physiological criteria, four bacterial strains were chosen among 41 isolates from the sandstone of Keroo, Jodhpur, for 16S ribosomal RNA gene sequencing. Following the successful submission of the 16S rRNA gene sequences for E1B-LT, E1D-HT, E2A-LT, and E2A-HT, the accession number (Table 6) was obtained from NCBI GenBank. The 16S rRNA gene sequence phylogeny indicates that the recently isolated psychrophilic bacterial strains E1B-LT and E2A-LT belong to *Pseudarthrobacter phenanthrenivorans Sphe3* (Accession No.-NR074770.2) which was isolated from a creosote-contaminated soil in Greece and exhibit 100% and 99.77% sequence identity with the type strain (Kallimanis et al. 2009)(Figure 5). The other two isolated thermophilic bacterial strains, E1D-HT and E2A-HTexhibited 99.56% and 99.62% sequence identity with the type strains of *Geobacillus sp.*G4-1 and *Bacillus sp.* NG4-2, respectively. An endophytic bacterium called *Bacillus sp. NG4-2* was isolated from the Chinese desert plant *Lepidium perfoliatum* L whereas *Geobacillus sp.*strains G4-1 was isolated from China.

Table 6: Name of isolated strain and their corresponding accession number obtained from the sandstone mines of Keroo, Jodhpur, Rajasthan, India

Isolated strain designated as	Accession Number of isolated strain
Pseudarthrobacter phenanthrenivorans strain psychrophillus KE1B	OR921267.1
Geobacillus sp. KE1D	PP250152.1
Bacillus sp. KE2A	PP250151.1
Pseudarthrobacter phenanthrenivorans psychrophillus KE2A	PP237444.1

(a)



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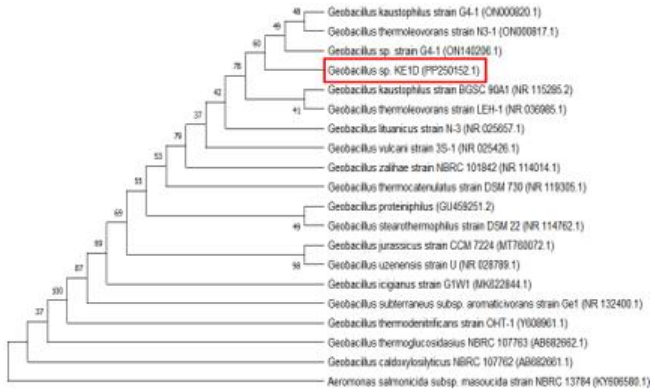

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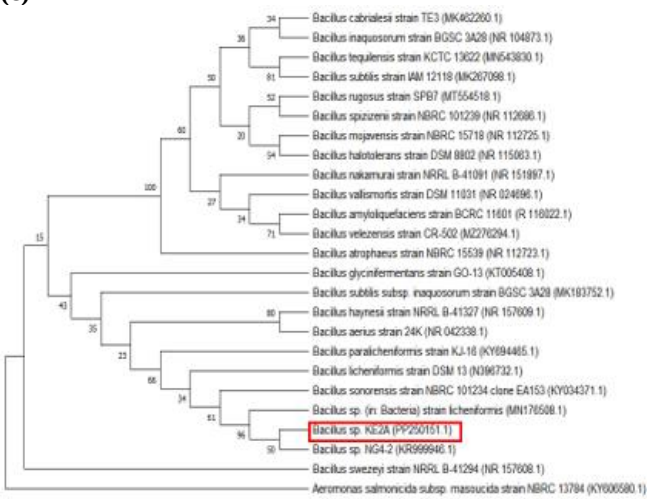

(d)

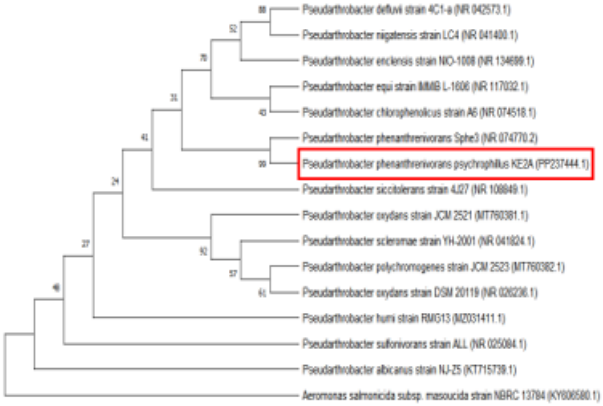

Figure 5: Phylogenetic tree of extremophile 16S rRNA gene sequences obtained from the east site of the Keroo mine in Jodhpur, Rajasthan together with related type strains. **a)** E1B-LT is inscribe as *Pseudarthrobacter phenanthrenivorans strain psychrophillus KE1B* (Accession No.-OR921267.1) is closely related to *Pseudarthrobacter phenanthrenivorans Sphe3* (NR074770.2), **b)**E1D-HT is inscribe as *Geobacillus sp. KE1D* (Accession No.-PP250152.1) is closely related to type strains of *Geobacillus sp. G4-1* (ON140206.1), **c)**E2A-HT is inscribe as *Bacillus sp. KE2A* (Accession No.-PP250151.1) is closely related to *Bacillus sp. NG4-2* (KR999946.1) and **d)** E2A-LT is inscribe as *Pseudarthrobacter phenanthrenivorans psychrophillus KE2A* (Accession No.-PP237444.1) and closely related to type strains of *Pseudarthrobacter phenanthrenivorans Sphe3* (NR074770.2). Neighbour-Joining (NJ) was used to construct the tree, and the bootstrap values for 1000 replications are displayed at the internodes. 1% replacements per site are shown by the scale

bar. GenBank accession numbers are enclosed in parenthesis. *Aeromonas salmonicida subsp. Masoucida strain* NBRC 13784 (KY606580.1) was used as an out group.

4. Discussion

The research on the variety of extremophilic bacteria in the Keroo mining area is being done first time. It provides information about ancient life forms and the persistence of extremophile microbial species over geological eras. The physicochemical parameters showed alkaline pH and high EC value with high nitrogen content supports the existence of halophile as well as saline ecosystem. An accumulation of salts and $CaCO_3$ may be the cause of this alkaline pH level. Because of the temperate temperature in Keroo, Jodhpur, the mineralization process proceeds slowly, accumulating organic matter that breaks down gradually and lowers the pH of the soil at depth by producing an acid equivalent that may have a direct impact on higher soil acidity. Similarly, high EC suggests that salt-sensitive soil is present. The sample E1 exhibits high EC and pH parameters, which may be signs of effects on biological activity where changes in EC or pH may have an impact on specific microbial mediated processes (Delgado-García et al. 2018; Ma et al. 2010). The halophile's presence in these sedimentary rocks is supported by the physico-chemical parameter mentioned above.

In the present investigation, extremeophilic bacteria from the Keroo mines east site sandstone were recovered. These results showed that a variety of habitats is home to thermophilic, acidophilic, alkalophilic, and psychrophilic bacteria. The isolated bacteria showed closed phylogenetic relationships with the genera *Geobacillus* as well as *Bacillus* (phylum = Bacillota), and *Pseudarthrobacter* (phylum: Actinomycetota). Moreover, these ecosystem's extremophile microorganisms have special metabolic traits that allow them to endure harsh circumstances. Similar extremophiles were also reported in Fidusar and Keroo that supports the present findings (Yadav et al. 2022; 2024)
The gram-positive *Pseudarthrobacter* genus includes both mesophilic and psychrophilic microbes that have been isolated from various locations in Epirus, Greece (Kallimanis et al. 2009). Protease and cellulase are produced by the isolated strains E1B-LT and E2A-LT, which can withstand temperatures of up to 10 °C. Both are capable of growing in the 3–12 pH range (Tsagogiannis et al. 2021). These characteristics corroborate the existence of *Pseudarthrobacter phenanthrenivorans Sphe3*, which was primarily detected at the site in Keroo sandstone, and share 99.77% of its molecular similarities (Asimakoula et al. 2023).

Both the isolated strains of *Geobacillus* sp. KE1D and *Bacillus* sp. KE2A can withstand temperatures as high as 60º C. Additionally, both strains of the bacterial genera *Geobacillus* sp. and *Bacillus* sp. NG4-2 can survive in a wide pH range (3–12) (Zebrowska et al. 2022). The bacterial strain *Geobacillus* sp. KE1D is a thermo-alkalophile, meaning that it can survive at both temperatures of 60º C and 12 pH. This supports that the isolate can survive in a variety of environmental conditions and is notable for being a poly-extremophile. The ability of *Geobacillus* sp. KE1D to produce amylase and laccase enzymes supports both its capacity to hydrolyze carbohydrates and break down Congo red dye which is mutagenic and teratogenic dye in nature. Because of these characteristics, isolates have significance for bioremediation.

The bacterial strain *Bacillus* sp. KE2A is a thermo-alkalophile that can withstand high salinity concentrations (10-20% salinity at range of 10-35°C temperature). This ability to survive in high salinity conditions, similar to those found in the sea, may provide supportive evidence that the marine environment may be once existed in Rajasthan. Furthermore, under extreme conditions, a large number of extremophiles create proteins and enzymes with remarkable stability and activity, which makes them useful for applications like bioremediation. Pollutants and toxins can adapt to degrade in harsh conditions when traditional cleanup techniques would not work (Martínez-Espinosa, 2020). The ability of the bacterial strains *Geobacillus* sp. KE1D and *Bacillus* sp. KE2A to break down the Congo red dye at 60º C temperature supports the sandstone's cleanliness and its capacity for biodegradation (Gopinath et al. 2009).

Because of their remarkable metabolic properties and ability to withstand harsh environments, isolated extremophilic bacteria are highly valued for biotechnological applications in the industrial sector because they can produce biomolecules and enzymes with remarkable stability and activity. In the present investigation, the bacteria *Geobacillus* sp. KE1D and *Bacillus* sp. KE2A are capable of producing amylase enzyme, and *Pseudarthrobacter phenanthrenivorans* strain *psychrophillus* KE1B, *Bacillus* sp. KE2A, and *Pseudarthrobacter phenanthrenivorans strain psychrophillus KE2A* are capable of producing cellulase and protease enzymes. Extremophile enzymes, such as laccase, amylases, and cellulases, are used in a variety of industrial applications. *Bacillus* species KE2A and *Geobacillus* sp. KE1D have the ability to break down Congo red dye, a sign that laccase enzyme is being produced (Chakravarthi et al. 2021). The discovery of microbial presence in ancient geological formations from the period when the marine existed lends credence to hypotheses like continental drift and plate tectonics presented in this investigation. The discovery of *Geobacillus* sp. has also been documented in the Mariana Trench, the Arctic Deep Sea Hydrothermal Water, and the Andes Mountains of Bolivia (Hussein et al. 2015). According to this study, some extremophiles may have a marine origin, implying that they have evolutionary ties to ancient marine habitats in Rajasthan. The microbiological richness of polyextremophilic bacteria in the sandstone of the Rajasthan Keroo mine is first time highlighted in this research, opening up possibilities for further investigation and use in the synthesis of extremozymes.

5. Conclusion

The research has produced a collection of axenic polyextremophilic strains from 780-million-year-old sandstone in Keroo mines. This pioneering study sheds light

on ancient microbial life and demonstrates the adaptability of extremophiles over geological timescales. The unique sandstone environment has yielded a wealth of genetic resources from extremophilic microorganisms with promising biotechnological potential. Representing genera such as *Pseudarthrobacter*, *Geobacillus*, and *Bacillus*, these microorganisms offer a stable genetic platform for exploring thermally stable extremozymes and industrial applications. Notably, the collection of 41 extremophiles includes strains that produce significant hydrolytic enzymes like laccase, protease, cellulose, and amylase, underscoring their potential in bioremediation. The findings not only enhance our understanding of microbial evolution and biotechnology but also support theories of plate tectonics and continental drift. Additionally, the discovery of *Geobacillus* in diverse locations like the Mariana Trench and the Bolivian Andes suggests possible evolutionary links to ancient marine environments. Overall, this research underscores the importance of extremophiles in advancing our knowledge of marine environments, including those in Rajasthan.

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