Enzyme Screening and Antimicrobial Potential of Thermophiles Isolated from Different Environmental Samples

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ABSTRACT

Objectives: This study aimed to examine the growth, enzyme activities, and antimicrobial properties of thermophilic bacteria from compost, soil, and hot springs.

Methods: Six samples each from compost, soil, and hot springs were collected and thermophiles were isolates. These isolates were tested for growth in extreme conditions (high pH, high salt concentrations, temperature up to 65ºC) and identified by morphological and biochemical characteristics. Enzyme production such as amylase, gelatinase, cellulase, pectinase, and lipase were evaluated, and the antimicrobial properties against test organisms were assessed.

Results: From 43 isolates, 17 grew well in high pH, 16 in high salt, and 5 in 65ºC. *Bacillus licheniformis* had the highest amylase and lipase activity. *Bacillus stearothermophilus* and *Thermoactinomyces* had the highest gelatinase activity, *Bacillus polymyxa* had the highest cellulase activity, and *Bacillus macquariensis* and *Bacillus stearothermophilus* had the highest pectinase activity. Antimicrobial activity was observed in 54% of isolates, with 95.7% inhibiting *Staphylococcus aureus* ATCC 25923, 34.8% inhibiting *Escherichia coli, and* 1 isolate inhibiting *Salmonella spp.*

Conclusion: The study showed that compost-derived thermophilic isolates had high enzymatic activity. Furthermore, many isolates were able to inhibit the growth of Staphylococcus aureus ATCC 25923 by producing an antimicrobial substance.

Keywords: Thermophiles, Extreme conditions, Enzyme activity, Antimicrobial substances

INTRODUCTION

Extremophiles are organisms capable of thriving in extreme environments, with thermophiles being a subset that thrives at elevated temperatures ranging from 41 to 122°C (106 to 252°F) (Madigan et al., 2006). Their adaptation mechanisms include protective cell walls, heat-shock proteins, osmoprotectants, specialized enzymes, and DNA repair systems, which enable them to survive and function at high temperatures (Rothschild & Mancinelli, 2001). Thermophiles are categorized into moderate thermophiles (optimal growth at 50–60°C), extreme thermophiles (60–80°C), and hyperthermophiles (80–110°C) (Rampelotto, 2013; Valenzuela et al., 2024). The interest in thermophilic microorganisms has surged since the isolation of the first thermostable enzyme, isoleucyl-t-RNA synthetase, from *Bacillus stearothermophilus* in 1972 (Bertoldo & Antranikian, 2002). These organisms produce thermostable enzymes that are crucial for industrial applications, including cellulases, amylases, chitinases, proteases, lipases, xylanases, esterases, phytases, pectinases, and ananases (Dumorné et al., 2017).

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Thermophilic bacteria such as *Geobacillus*, *Alicyclobacillus*, *Anoxybacillus*, *Pyrococcus*, and *Thermococcus* have shown great potential as biocatalysts due to their ability to grow at high temperatures (Atalah et al., 2019).

Antimicrobial compounds, often produced by microorganisms, inhibit the growth of other microbes. Soil bacteria are major producers of these early antibiotics, but resistance has diminished their effectiveness, creating a pressing need for new antimicrobial sources (Salem et al., 2021). The rise in antibiotic-resistant strains necessitates ongoing exploration for antimicrobial agents, particularly from extremophiles (Mahajan & Balachandran, 2017). Thermophiles have demonstrated the ability to produce antimicrobial agents effective against various microorganisms, including bacteria, fungi, and protozoa (Muhammad et al., 2009).

Nepal's diverse landscapes, including geologically active regions with abundant hot springs, contribute to a rich microbial diversity (Nguyen et al., 2021). Enzymatic studies of thermophilic isolates from Nepalese hot springs have highlighted the significant enzyme-producing capability of the genus *Bacillus*, particularly in amylase production (Acharya et al., 2012; Mahajan & Balachandran, 2017; Yadav, 2018). This study aims to explore the variability in enzyme production among thermophilic isolates from different environmental samples, integrating enzyme screening with antimicrobial production.

METHODS

Study area, design, site, and duration

This descriptive study was conducted in the microbiology laboratory of St. Xavier's College, Maitighar, Kathmandu from October 2023 to April 2023 i.e., 7 months. The environmental samples for the analysis were collected by simple random sampling from various places in Nepal.

Sample Collection and Processing

Compost samples analyzed for the study were collected from gardening sites of Suryabinayak(CS), Palanchowk(CL), Boudha(CP), Buddhanilkantha(CB), Nala(CN) and Kaushaltar(CK). Similarly, soil samples studied in this study were from Rara(SR), Kalinchowk(SK), Mardi Himal(SM), Gosaikunda(SG), Palanchowk(SP) and Korala, Upper Mustang(SL). These samples were taken from a 10 cm deep surface of the soil, and collected in sterile zip-loc bags (Gaete *et al*., 2020). They were air-dried in the laboratory and heat-treated at 50° C to reduce any mesophilic and anaerobic population (Acharya, *et al*., 2012).

Hot spring water samples were collected from different hot springs of Nepal like Tatopani(HM), Paudwar(HP), Jagat(HJ), Sirchaur(HS), Kodari(HK), and Syabrubesi(HR) where, temperatures of these samples were 72ºC, 71ºC, 75ºC, 52ºC, 60ºC, and 72º C respectively when measured by a thermometer. They were collected in a sterilized thermos flask. The samples were insulated until it was brought to the laboratory, and heat treated at 50°C until further analysis (Soy, *et al*., 2023).

Isolation of thermophiles

Serial dilutions of samples were performed with sterile distilled water in 1:9 ratio, and were cultured on nutrient agar plates using the spread plate technique (Acharya, *et al*., 2012; Indriati & Megahati, 2018; Pawar & Borkar, 2018). The media was incubated at 50°C for 24 hours. Isolated colonies were subcultured for pure culture and stored if necessary for further analysis. Colony morphology of the resultant colony was noted (Masi, *et al.,* 2023). Isolated culture was subcultured in nutrient agar to obtain a pure culture, and stored in nutrient broth with 16% glycerol if necessary for further analysis.

Determination of growth characteristics

Growth temperature range was assessed by cultivating isolates at 37°C to 70°C. Salt tolerance was evaluated at 50°C using NaCl concentrations of 6.5% and 13%. pH tolerance was determined at pH values of 4.0, 7.0, and 9.0 adjusted by 1M NaOH and 1M HCl. All growth were examined on nutrient agar (Lee, *et al.,* 2022).

Biochemical and metabolic characterization of isolated organisms

Each thermophilic isolates examined by gram staining and endospore staining were identified based on the biochemical tests according to Bergey's manual of systematic bacteriology. These tests include starch hydrolysis, growth at 55º C, growth at 6.5% NaCl concentration, acid production in mannitol, arabinose and glucose enriched broth, motility test for *Bacillus.* Actinomycetes were characterized based on colony morphology on substrate and aerial mycelium (Dangol, *et al.,* 2018; Kuo & Hartman, 1966).

Qualitative screening for industrially important enzymes

Amylase: Qualitative screening for amylolytic activity was performed by inoculating the sample culture on 1% starch-enriched nutrient agar and incubated at 50° C for 24 hours. Clear zones around colonies after iodine flooding indicated amylase activity. (Bayoumi*, et al.,* 2007).

Gelatinase: Qualitative screening of gelatinase activity was performed by inoculating the sample culture at 1% gelatin-enriched nutrient agar media at 50° C for 48 hours. Gelatinase activity was determined by the formation of a white cloud precipitate around gelatinase-positive colonies when 15% $HgCl_2$ solution was flooded and left for 5 minutes (Rana Chhetri, *et al.,* 2022).

Cellulase: Qualitative screening of cellulolytic activity was performed by inoculating the sample culture on Carboxymethylcellulose agar (0.2% $NaNO_3$, 0.2% K_2HPO_4. 0.05% $MgSO_4$, 0.05% KCl (Hijam, *et al.*, 2020), 0.2% carboxymethylcellulose sodium salt, 0.02% peptone, and 1.7% agar) with pH adjusted to 7 and incubated at 50° C for 48 hours. Cellulase activity was determined by the use of Gram's Iodine on the colonies that formed on this media after 5 minutes.

Pectinase: Qualitative screening for pectinase activity was performed by point inoculation of sample culture on pectin agar media (0.1% $NaNO_3$, 0.1% KCL, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% yeast extract, 1% pectin, and 2% agar) with pH adjusted to 7. Pectinase activity was determined by the use of gram's Iodine in the colonies after incubation at 50° C for 48 hours and left for 5 minutes (Mohandas, *et al*., 2018).

Lipase: Qualitative screening for lipolytic activity was performed by point inoculation of sample culture on a medium consisting of 0.5% peptone, 0.3% yeast extract, 0.1% $CaCl_2$, 1.5% Agar on which 0.1% Olive oil and 0.1% phenol red indicator (1 mg/ ml) were added after autoclaving and cooling to 60° C. Lipase activity would be determined by yellow coloration around the colony on incubation at 50° C after 24 hours (Tanasupawat *et al*., 2015).

Primary screening of antimicrobial substance

Primary screening was performed by streaking thermophilic isolates as a vertical line on nutrient agar with 2% agar to prevent the spreading growth of *Bacillus* spp. and incubated at 50° C for 24 hours. After incubation, test organisms *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella* spp., and carbapenem-resistant *Klebsiella oxytoca* (with comparison to 0.5 McFarland solution were streaked perpendicular to the line of growth of isolate. Overnight incubation of these plates at 37° C resulted in the inhibition of test organisms if the isolated could produce antimicrobial compounds (Thapa, *et al*., 2021).

Production of crude extract

Colonies suspected to produce antibiotics were cultured in Nutrient broth for 3 days until proper pellets or surface pellicles were observed. The contents were transferred into sterilized falcon tubes for centrifugation at 6000 rpm for 20 minutes. Equal volumes of ethyl acetate were added to the supernatant, followed by another round of centrifugation at 6000 rpm for 20 minutes. The upper layer was collected and transferred to vials to be labeled as crude extract (Thapa *et al*., 2021).

Secondary screening for antimicrobial substance

The secondary screening was performed for the quantitative determination of antimicrobial compounds by the isolates against the test organisms. The crude extract was tested for antimicrobial activity by agar well diffusion using Mueller Hinton Agar plates. These test organisms were standardized to 0.5 McFarland solution, and the carpet culture was created with the help of a cotton swab. After 10 minutes, 6 mm diameter wells were made in the agar using a cork borer where dimethyl sulphoxide was added to the negative control at the center, and 50 µL of crude extract was pipetted into each well outwards to the center.

The plates were incubated at 37° C for 24 hours and observed for the zone of inhibition and measured in mm (Thapa *et al*., 2021)

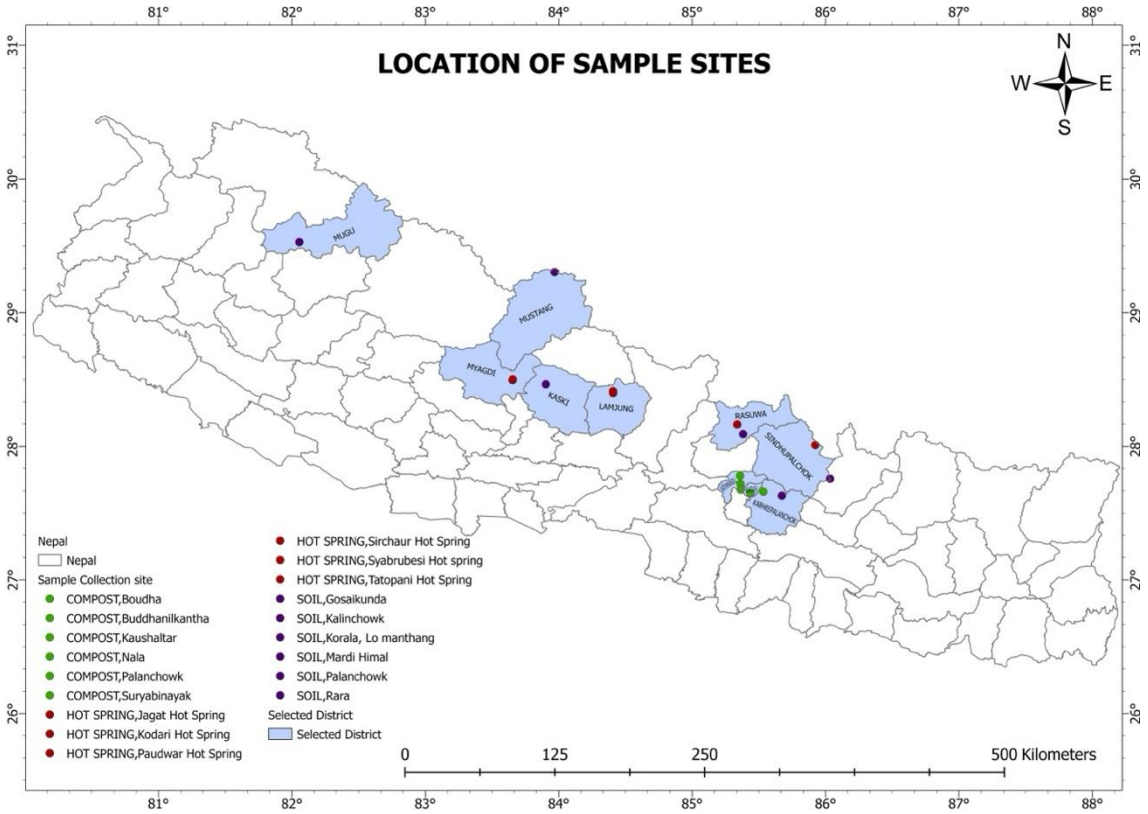


Figure 1: Nepal map showing the sampling sites

RESULTS

In total, 43 thermophilic isolates were processed from 18 environmental samples. This includes 15 isolates from different compost samples, 16 from various soil samples, and 12 from hot spring samples

Growth characteristics of isolated thermophiles

When the isolated thermophiles were exposed to extreme conditions of pH, NaCl concentration, and temperature,16(37.2%) isolates demonstrated resistance to high salt concentrations (13% NaCl), followed by 17 isolates (39.5%) tolerating alkaline environments. 5 isolates managed to grow at 65°. Notably, *Bacillus licheniformis* (SK1B, SM1A, SM2, SP1, HM1A, HR2B, CP2) and *Bacillus stearothermophilus* (CK2, CL2, SL1, HM2A, HJ2B) showed exceptional resilience at 13% NaCl concentration (Table 1).

Identification of thermophilic isolates from different samples

Based on the identification of morphological and biochemical characteristics, based on Logan and Allan (2008), the following species were identified: *Bacillus schlegelii*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus badius*, *Bacillus stearothermophilus*, *Bacillus macquariensis*, *Bacillus polymyxa*, and *Thermoactinomyces*. *Bacillus stearothermophilus* was the most abundant of all with 18 isolates isolated (Figure 3).

Qualitative analysis of enzymes in each environmental sample

Thermophilic isolates from compost showed the highest enzyme activity for most enzymes except pectinase. Soil isolates had significant enzyme activity, comparable to or exceeding compost in gelatinase (100%), pectinase (94%), and lipase (88%) (Figure 4). Hot spring isolates generally had lower enzyme activity, though pectinase levels were similar to soil. Notably, gelatinase activity was high, with some hot spring isolates lacking it.

Enzyme activity profile of bacterial isolates

Out of 43 isolates tested for enzyme activity, 39 (90.6%) produced amylase, with SM2 (*Bacillus licheniformis*) showing the highest activity (19 mm hydrolysis).

Gelatinase activity was observed in 41 isolates (95.3%), with CB2A (*Bacillus stearothermophilus*) and CLW (*Thermoactinomyces*) exhibiting the largest zone of hydrolysis (21 mm). Cellulase was produced by 32 isolates (74.4%), with CK1 (*Bacillus polymyxa*) showing the highest activity (21 mm hydrolysis). The pectinolytic activity was present in 33 isolates (76.7%), with CP1 (*Bacillus macquariensis*) and SM1B (*Bacillus stearothermophilus*) demonstrating the highest activity (19 mm). The same number of isolates showed lipase activity, with HR2B (*Bacillus licheniformis*) and HM2A (*Bacillus stearothermophilus*) achieving the largest hydrolysis zone (21 mm). Isolates CK2 (*B. stearothermophilus*) and CK1 (*B. polymyxa*) showed broad enzyme production capability with a great yield for them too (Table 2).

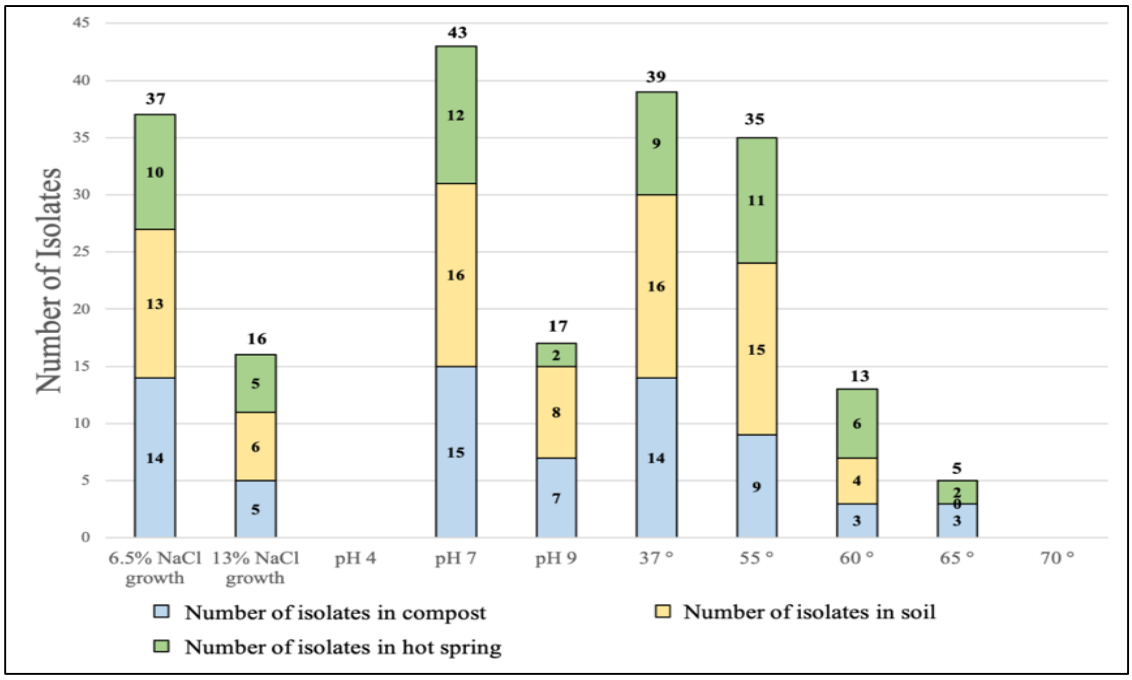


Figure 2: Growth characteristics of isolated thermophiles

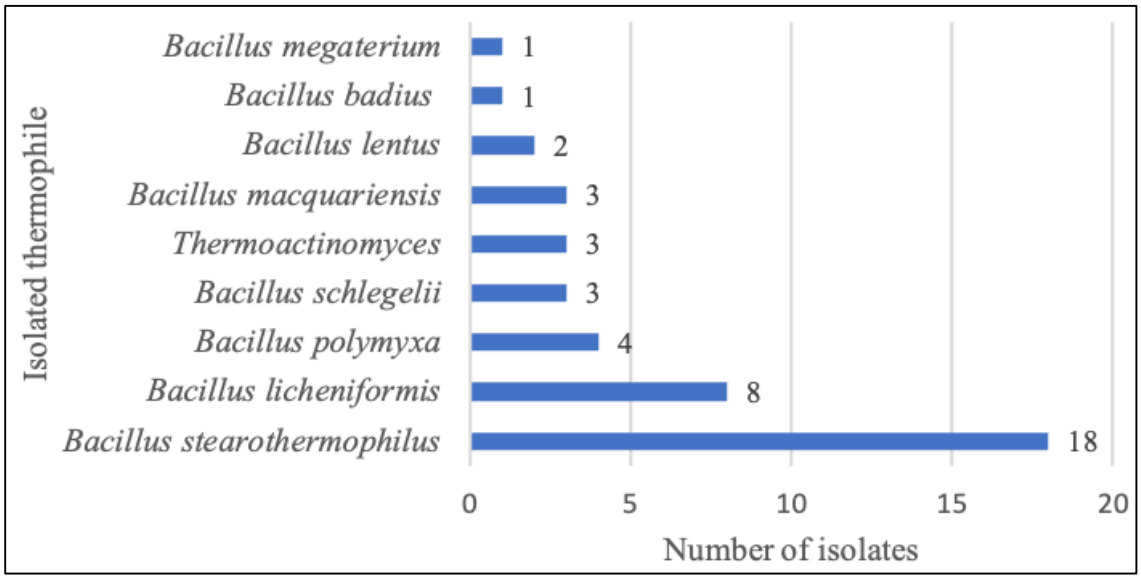


Figure 3: Number of identified thermophilic isolates

Table 1: Thermophilic isolates able to resist different extreme conditions

Extreme conditions used in the study		
13% NaCl	**pH 9**	**65°**
CN1 (*B. macquariensis)*	CN1*(B. macquariensis)*	CB2 *(B. stearothermophilus)*
CN3 *(B. lentus)*	CN3 *(B. badius)*	CK1 *(B. polymyxa)*
CK2 *(B. stearothermophilus*	CN4*(B. macquariensis)*	CP2 *(B. licheniformis*)
CL1 *(B. lentus)*	CS2 *(B. polymyxa)*	HJ2A *(B. schlegelii)*
CL2 *(B. stearothermophilus)*	CL1*(B. lentus)*	HR2A *(Thermoactinomyces)*
SR1A *(B. lentus)*	CLW *(Thermoactinomyces)*	
SK1B *(B. licheniformis)*	SK1B *(B. licheniformis)*	
SM1A *(B. licheniformis)*	SR1B *(B. schegelii)*	
SM2 *(B. licheniformis)*	SR2B *(B. stearothermophilus)*	
SP1 *(B. licheniformis)*	SK1A *(B. stearothermophilus)*	
SL1 *(B. stearothermophilus)*	SK1B (*B. licheniformis)*	
HM1A *(B. licheniformis)*	SK2A *(B. stearothermophilus)*	
HM2A *(B. stearothermophilus)*	SG3 *(B. stearothermophilus)*	
HP1 *(B. stearothermophilus)*	SM1B *(B. stearothermophilus)*	
HR2B *(B. licheniformis)*	SL1 *(B. stearothermophilus)*	
HJ2B *(B. stearothermophilus)*	HJ2B *(B. stearothermophilus)*	
	HP2 (*B. megaterium)*	

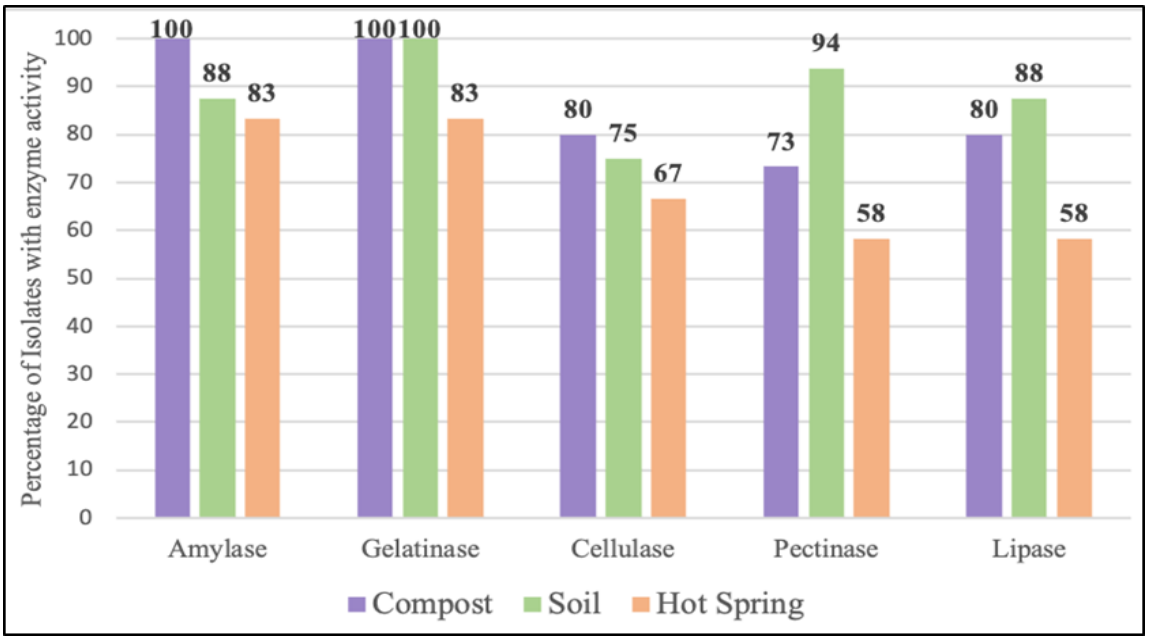


Figure 4: Enzyme activity of thermophiles observed in each environmental sample

Table 2: Production of enzymes in thermophilic isolates

Isolate	Enzyme activity				
	Amylase	Gelatinase	Cellulase	Pectinase	Lipase
CN1 *(B. macquariensis)*	++	+	+	+	+
CB1 *(B. stearothermophilus)*	+	+	+++	+	+
CB2A *(B. stearothermophilus)*	+	++++	+	-	++
CK1 *(B. polymyxa)*	+	+	++++	+++	+
CK2 *(B. stearothermophilus)*	++	+++	+++	+	+
CLW *(Thermoactinomyces)*	+	++++	+	+	+
CP1 *(B. macquariensis)*	+	++	+	+++	+
SK1B *(B. licheniformis)*	++	+	+	+	+++
SK2A *(B. stearothermophilus)*	-	++	+	++	++
SM1A *(B. licheniformis)*	+	+	+++	+	+
SM1B (*B. stearothermophilus)*	-	+	+	+++	+
SM2 *(B. licheniformis)*	+++	+	+	+	+
SP2 *(B. licheniformis)*	+	+	+	++	+
HM2A *(B. stearothermophilus)*	+	+	+	+	++++
HP1 *(B. stearothermophilus)*	++	++	-	-	+++
HP2 *(B. megaterium)*	++	++	+	-	-
HR2B *(B. licheniformis)*	+	++	+	+	++++
HK2 *(B. polymyxa)*	+	++	+++	+	+

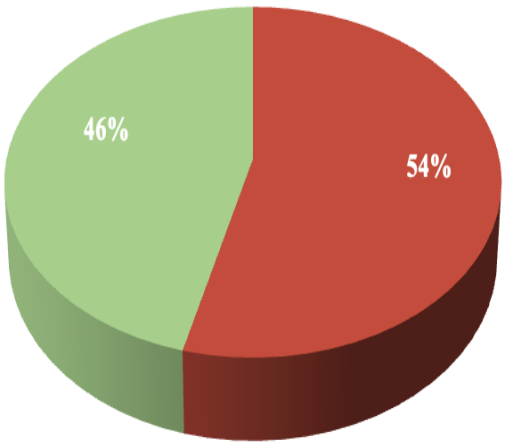
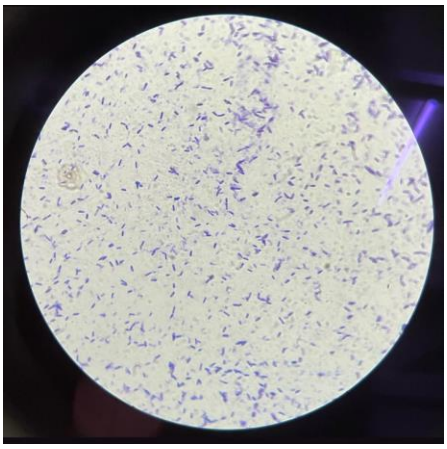
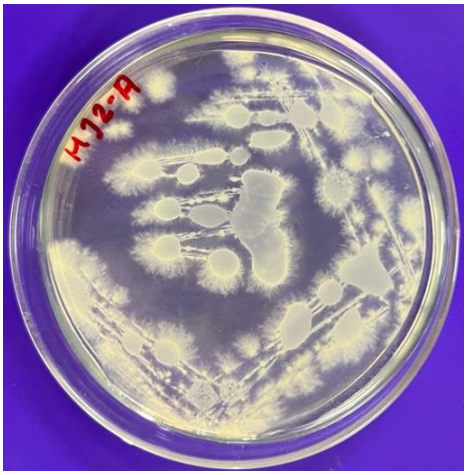


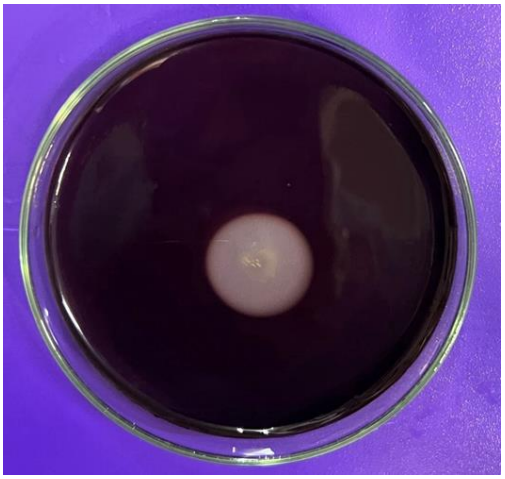
Figure 5: Proportion of antimicrobial producer (Right) and non-producer (Left) by thermophilic isolates



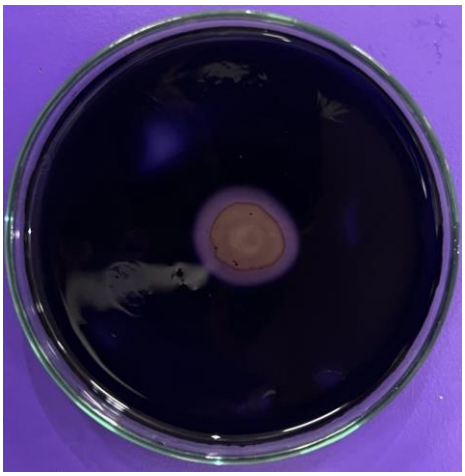
Photograph 1: Gram staining of *Bacillus stearothermophilus*



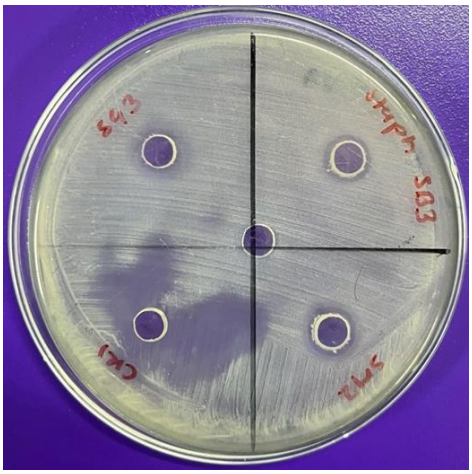
Photograph 2: Colony morphology of *Bacillus stearothermophilus*



Photograph 5: Zone of hydrolysis observed in Cellulase activity of SM1A *(Bacillus licheniformis)*



Photograph 3: Zone of hydrolysis observed in Amylase activity of Sample CK2 (*Bacillus stearothermophilus)*



Photograph 6: Zone of Inhibition of CK1 (*Bacillus polymyxa*) observed in secondary screening of antimicrobial substance against *Staphylococcus aureus* ATCC 25923

Photograph 4: Zone of hydrolysis observed in Gelatinase activity of CLW *(Thermoactinomyces)*

Production of antimicrobial substance by thermophilic isolates

Out of 43 thermophilic isolates, 23 (54%) were capable of producing antimicrobial substances, while the remaining 20 (46%) did not exhibit any antimicrobial activity (Figure 5). Among the 23 (54%) isolates, *Bacillus stearothermophilus* was the most prolific, accounting for 47.8% of the antimicrobial production. Other notable antimicrobial producers included *Bacillus licheniformis* and *Bacillus polymyxa*, each contributing 13%, followed by *Bacillus schlegelii* and *Bacillus macquariensis*, both at 8.9%. Additionally, *Bacillus megaterium* and *Bacillus badius* contributed 4.3% and 4.1%, respectively. 22 isolates (95.7%) produced antimicrobial substances that inhibited the growth of the Gram-positive organism *Staphylococcus aureus* ATCC 25923, except *Bacillus stearothermophilus.* Isolate CK1 showed the highest inhibition zone of 18 mm against *Staphylococcus aureus.* Similarly, 8 isolates (34.8%) were able to demonstrate antimicrobial activity against *Escherichia coli* ATCC 25922. Furthermore, only one isolate was able to inhibit the growth of *Salmonella* spp.

DISCUSSION

In this study, the thermophilic bacteria isolates were characterized for their growth and enzyme activities under various environmental conditions. None of the isolates tolerated an acidic pH of 4.0. However, 37.2% of the isolates demonstrated growth at NaCl concentrations up to 13%, surpassing the 9% tolerance reported by Lee et al. (2022). In alkaline conditions at pH 9, 16 isolates grew, with only 2 from hot springs, 8 from soil, and 7 from compost showing resilience. This resilience contrasts with Fierer et al. (2006), which highlighted bacterial diversity in neutral soils and their adaptability to pH levels.

Soil thermophilic isolates showed a broad growth capability, particularly from high-altitude, dry areas in Nepal, supporting Marchant et al. (2008) who linked thermophile occurrence to environmental factors like wind and sunlight exposure. All isolates growing at 50°C were gram-positive, rod-shaped, and endospore-forming. These were tested for biochemical properties, revealing Bacillus stearothermophilus (18 isolates) as the most prevalent, followed by Bacillus licheniformis (8 isolates). Notably, Bacillus schlegelii was identified, previously reported from Antarctic soil by Andrew et al. (1988), and Thermoactinomyces was recognized for its unique thermophilic habitat and branched mycelium (Tendler & Burkholder, 1960).

Enzyme activity varied among the environmental samples. Compost was rich in diverse enzyme producers, while soil also showed significant enzyme activity but less than compost. Hot spring samples had relatively lower enzyme activities. This variation may stem from differences in microbial communities, organic matter, and environmental conditions. Sharp et al. (2014) found similar results, with water samples showing higher enzymatic activity compared to soil.

Enzyme activities were qualitatively determined as '+' for low, '++' for moderate, '+++' for high, and '++++' for very high activity (Joshi et al., 2016). Bacillus stearothermophilus is noted for producing stable enzymes at high temperatures (Kotzekidou, 2014), relevant for processes like milk powder drying and cleaning, which can cause spoilage of low-acid canned foods.

Research on thermostable enzymes supported these findings, with *Bacillus megaterium* producing thermostable amylase optimal at 55°C (Aguloglu, 2022). Bacillus species are also noted for gelatinase production (Balan et al., 2012), and thermophilic actinomycetes produce significant gelatinase quantities under specific conditions (Tendler & Burkholder, 1960). Bacillus licheniformis was efficient in cellulase production in Himalayan soils (Shyaula et al., 2023) and showed thermostability and increased production at high temperatures (Saidan et al., 2024). Pectinolytic activity was highest in Bacillus licheniformis and *Geobacillus stearothermophilus* (Bayoumi et al., 2007).

Antimicrobial production was prevalent among *Bacillus* isolates. Tran et al. (2022) noted Bacillus as a significant antimicrobial producer, with *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus velezensis* being prominent. *Bacillus polymyxa* from this study showed the highest inhibition against *Staphylococcus aureus* ATCC 25923, aligning with Li & Chen (2019) who found antimicrobial activity in *Paenibacillus polymyxa* from hot springs. He et al. (2007) reported *Paenibacillus polymyxa*'s ability to produce antibiotics and lantibiotics inhibiting gram-negative bacteria, which contrasts with this study's findings.

39.1% of isolates inhibited gram-negative organisms, with only one showing activity against *Salmonella* spp. Shleeva et al. (2023) identified *Bacillus licheniformis* producing licheniformin, which targets various pathogens, including multidrug-resistant mycobacteria. However, this research

did not find antimicrobial agents effective against carbapenem-resistant *Klebsiella* spp., as noted by Abdelraouf et al. (2018), who reviewed substances active against *Klebsiella* spp.

Conclusion

The study focused on isolating thermophiles from 18 environmental samples that included 6 each of compost, soil, and hot spring samples. 43 isolates were studied from these environmental samples, and identified by phenotypic and biochemical features. On Identification following Bergey's manual of systematic bacteriology, it can be noted that *Bacillus stearothermophilus* was found in abundance. Characterization of thermophilic bacteria based on ability to grow at growth features at 6.5 % NaCl, 13% NaCl, pH 4, pH 7, pH 9, and temperatures of 37ºC, 50ºC, 55ºC, 60ºC, 65ºC, and 70ºC revealed that isolates were able to withstand these extreme conditions.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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