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ORIGINAL RESEARCH

Isolation and Characterization of *Bacillus* spp with Potent Proteolytic and Antipathogenic Activities

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ABSTRACT

Objectives: The current study aimed to determine the proteolytic and antipathogenic activity of *Bacillus* spp isolated from soil samples collected across various ecological and land-use categories in multiple locations within the Kathmandu Valley.

Methods: A cross-sectional study was conducted at the Med-Micro Nepal Community Development and Research Centre, Kathmandu. Seventeen different soil samples were collected and processed to isolate *Bacillus* species. The isolates were identified as *Bacillus* spp on the basis of their morphological characteristics, Gram's staining, spore staining and biochemical tests based on Bergey's Manual of Systematics of Archaea and Bacteria. The proteolytic activity was screened and determined by well diffusion method on 1% gelatin agar media for both crude and purified form of enzyme. Similarly, antipathogenic assay was done on Mueller Hinton agar by agar well diffusion method.

Results: All 17 *Bacillus* isolates exhibited proteolytic activity, with enhanced activity observed upon partial purification. Notably, isolate A_M showed the highest increase (25%) in protease activity, while R_S and R_H displayed the largest overall proteolytic zones (32 mm and 31 mm, respectively). Isolates R_H, R_K, F_M, F_N, and F_S demonstrated both strong antipathogenic and proteolytic activities, suggesting a potential correlation between protease production and antimicrobial efficacy.

Conclusion: These findings highlight the therapeutic potential of selected proteolytic *Bacillus* strains, particularly against *Streptococcus* spp and *E. coli*.

Keywords: Protease enzyme, *Bacillus* spp, crude extract, zone of inhibition, antipathogenicity

INTRODUCTION

Bacillus spp are widely recognized for their ecological versatility and biotechnological potential (Contesini et al., 2018; Logan and De Vos 2015; Mokashe et al., 2018). These bacteria thrive in diverse environments including soil, where they contribute to organic matter decomposition and nutrient cycling (Nicholson et al., 2018). Among many attributes, they are prolific producers of extracellular proteases - enzymes that hydrolyze proteins into peptides and amino acids, with applications in industries such as detergents, food processing, and pharmaceuticals (Contesini et al., 2018). The stability of *Bacillus*-derived proteases under extreme conditions, such as high temperatures and varying pH levels, makes them particularly valuable for industrial and therapeutic purposes (Mokashe et al., 2018).

Soil serves as a rich reservoir of diverse microbes, harboring *Bacillus* spp with varying enzymatic capabilities influenced by environmental factors like soil composition and land use (Sharma et al., 2020). The Kathmandu Valley, characterized by diverse ecological niches including residential, agricultural, industrial, and riverine areas, offers a unique opportunity to explore the proteolytic potential of *Bacillus* isolates (Thapa et al., 2019). Recent studies have highlighted the

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ability of soil-derived *Bacillus* spp to have antimicrobial properties, suggesting their potential in combating pathogens such as *Streptococcus* spp and *Escherichia coli* (Saggu & Mishra, 2017; Pant et al., 2021).

Proteases produced from *Bacillus* spp play a critical role in microbial competition by degrading environmental proteins, which may also contribute to antimicrobial activity through the disruption of pathogenic cell structures (Haddar et al., 2021). The agar well diffusion method, a standard technique for assessing enzymatic and antimicrobial activity, has been widely adopted to measure zones of hydrolysis and inhibition (Bauer et al., 2016; Manandhar et al., 2022). These methods enable precise quantification of protease activity and antipathogenic effects, providing insights into the therapeutic potential of *Bacillus* isolates.

This study aims to isolate *Bacillus* spp from soil samples collected across various sites of Kathmandu Valley, characterize their proteolytic activity and evaluate their antipathogenic efficacy against clinical pathogens. By comparing the enzymatic and antimicrobial activities of isolates from different environmental contexts, this research seeks to identify strains with therapeutic potential and investigate potential correlations between protease production and antimicrobial activity. The findings could contribute to the development of novel biotechnological and therapeutic applications byleveraging the natural capabilities of Bacillus spp (Contesini et al., 2018; Pant et al., 2021).

METHODS

Collection of soil samples

Seventeen different soil samples were collected from seventeen different sites of Kathmandu valley. The sites were grouped under six categories viz. residential area- 5, river site- 2, agricultural farm- 3, dumping site- 2, forest area- 3 and industrial area- 2. The five residential samples were taken from Suryabinayak, Mitrapark, Mulpani, Anamnagar, and Koteshwor. Similarly, Bagmati and Dhobikhola were the two river sites. The other three samples were taken from agricultural farms of Suryabinayak, Mulpani and Tarakeshwor. Moreover, two samples were taken from the two dumping sites; Teku and Ratopul. The soil samples from forest areas where taken from three different corners of Kathmandu valley- Suryabinayak, Nagarjun and Mulpani. Finally, two soil samples were obtained from industrial areas of Bhaktapur and Balaju.

The soil samples were collected in sterile aluminum foils. The surface soil was removed and approximately 10-15 grams of soil from depth of 3- 4 inches were collected (Rebecca et al., 2014) with the help of sterilized spatula, kept in a sterile aluminum foil and placed in a zip- lock plastic bags. The soil samples were appropriately labelled mentioning sample code and date and time of collection. The soil samples were brought to laboratory.

Isolation and identification of *Bacillus* species

Heat treatment method was employed to screen heat resistant endospore forming *Bacillus* species as suggested by Logan & De Vos (2015). Five grams of soil sample was added to 45 ml of distilled water and stirred properly. This mixture of soil was heated at 80°C for 15 minutes. The isolation of bacteria was carried out by quadrant streaking method (Lageiro et al., 2025). A loop of processed sample was taken and streaked on a nutrient agar plate. The plate was incubated for 24 hours at 37°C (Lakshmi et al., 2014).

The isolated colonies were identified based on cellular morphology, growth condition, gram staining, endospore staining, growth conditions and biochemical tests according to the guidelines on Bergey's Manual of Systematics of Archaea and Bacteria (Logan & De Vos, 2015). The common biochemical tests to be performed are oxidase test, catalase test and oxidative / fermentative (O/F) test. Schaeffer-Fulton method was used for staining endospores and sub cultured on NA media. The plate was incubated at 37°C for 24 hrs. This was obtained as pure isolated culture of *Bacillus* spp The pure culture was further preserved in a refrigerator at -4°C as master cultures (Pant et al., 2015).

Screening of *Bacillus* isolates for enzyme production

The isolates were screened for protease production by spot assay. The isolated bacteria were point inoculated on 1% gelatin agar plate and incubated for 24 hrs at 30°C. The zone of hydrolysis was checked by flooding the plates with 3% mercuric chloride solution (Rebecca et al., 2014). This is based on gelatin hydrolysis test. The strains producing clear zones were sub cultured on NA and reported as potent isolates.

Production of protease enzyme

The potent isolates from freshly sub cultured NA plates were inoculated in 50mL of gelatin broth. It was fermented in shaking incubator at 30°C for 48hrs.

Enzyme extraction was done by centrifugation process.

At the end of each fermentation period the whole broth culture was centrifuged at 3500 rpm for ten minutes to remove debris. The clear supernatant was recovered by sedimentation and used for further experiments. This is the crude extract of enzyme (Rebecca et al., 2014).

For purification, 5mL of crude extract of enzyme was added with double volume of chilled acetone and refrigerated overnight in a conical flask. The content was then centrifuged at 5000 rpm for five minutes. The supernatant obtained was discarded and the precipitated enzyme pellet was dissolved in small amount of phosphate buffer at pH 7 to obtain partially purified enzyme (Lageiro et al., 2025).

Measurement of enzymatic activity

The enzyme activity was estimated by enzyme assay method based on agar well diffusion procedure (Manandhar & Sharma, 2013). The well was made on 1% gelatin agar plates with the help of 6mm cork borer. 50µl of crude extract of enzyme, 50µl of purified protease and 50µl of autoclaved water were loaded on three different wells bored on a GA media plate and allowed to diffuse at room temperatures for 15 minutes. The plates were incubated at 37°C for 24 hrs. After incubation the plates were flooded by mercury chloride and the zone of hydrolysis was measured (Pant et al., 2014).

Anti-pathogenic Assay

Each of five different clinical isolates - Methicillin Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus* spp were swabbed on different Mueller Hinton Agar (MHA) plates. Six well were made with 6 mm borer in each plate, where *Bacillus* isolates were filled. The zone of inhibition were measured after incubation for 24 hours.

Data Analysis

The zone of hydrolysis was recorded and analyzed by using Microsoft word and MS- excel. The bar charts and tables were prepared. Similarly, mean of the samples was also calculated as statistical analysis.

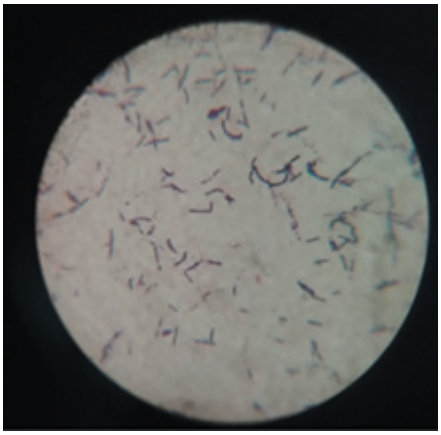

Figure 1: Gram staining of *Bacillus* spp: Gram positive (purple colour) bacteria are seen under light microscopy at 100x magnification

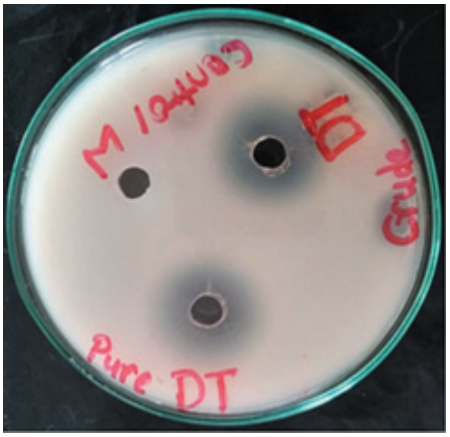

Figure 2: Proteolytic activity of protease extract on gelatin agar: Halo zone around the well shows the zone of hydrolysis of crude (crude DT) and partially purified (pure DT) protease extract from the isolates of dumping sites from Teku. No halo zone was seen around the control (distilled water).

RESULTS

Only the most thermotolerant *Bacillus* spp from each sampling site was taken for isolation and characterization. The description of soil samples is presented in table 1.

Table 1: Description of all soil samples

Soil samples	Location	Code name
Residential area	Suryavinayak	R_S
	Umakunda (Mitrapark)	R_{Mu}
	Mulpani	R_M
	Hanumanthan (Anamnagar)	R_H
	Koteshwor	R_K
River site	Bagmati	Ri_B
	Dhobikhola	Ri_D
Agricultural farm	Suryabinayak	A_S
	Mulpani	A_M
	Tarakeshwor	A_T
Dumping site	Teku	D_T
	Ratopul	D_R
Forest area	Suryabinayak	F_S
	Mulpani	F_M
	Nagarjun	F_N
Industrial area	Bhaktapur	I_{Bkt}
	Balaju	I_B

A total of 17 isolates were identified as *Bacillus* spp on the basis of microscopic characteristics and biochemical tests. The characteristics of the isolates is detailed in table 2. All the Gram positive, catalase positive, motile and spore forming isolates were positive in their proteolytic activity.

Table 2: Characterization of isolates

Isolate No.	Catalase	Oxidase	Spore Staining	Motility	Gelatinase
R_S	+	+	+ (central, oval)	+	+
R_{Mu}	+	+	+ (central, oval)	+	+
R_M	+	+	+ (central, oval)	+	+
R_H	+	-	+ (sub-terminal, oval)	+	+
R_K	+	+	+ (central, oval)	+	+
Ri_B	+	+	+ (sub-terminal, oval)	+	+
Ri_D	+	+	+ (sub-terminal, oval)	+	+
A_S	+	-	+ (sub-terminal, oval)	+	+
A_M	+	-	+ (sub-terminal, oval)	+	+
A_T	+	-	+ (sub-terminal, oval)	+	+
D_T	+	-	+ (sub-terminal, oval)	+	+
D_R	+	-	+ (sub-terminal, oval)	+	+
F_S	+	-	+ (sub-terminal, oval)	+	+
F_M	+	+	+ (central, oval)	+	+
F_N	+	+	+ (central, oval)	+	+
I_{Bkt}	+	-	+ (sub-terminal, oval)	+	+
I_B	+	+	+ (sub-terminal, oval)	+	+

The protease enzyme prepared by fermentation with all primarily screened isolates showed increased activity with partially purified extract than the crude extract indicating that the increase in zone of hydrolysis after partial purification is statistically significant. On average, the partially purified samples exhibited 3.18 mm larger zone of hydrolysis than crude samples. (Table 3).

Table 3: Zone of hydrolysis of crude and partially purified protease extract from all samples (t = -9.8182, df = 16, mean difference = -3.18)

Sample	Crude (mm)	Partially_Purified (mm)	Increase (mm)	% Increase	P-value (t-test)
R_S	28	32	4	14.29%	
R_{Mu}	27	30	3	11.11%	
R_M	24	25	1	4.17%	
R_H	27	31	4	14.81%	
R_K	26	30	4	15.38%	
Ri_B	25	30	5	20.00%	
Ri_D	20	22	2	10.00%	
A_S	23	26	3	13.04%	
A_M	24	30	6	25.00%	3.54×10^{-8}
A_T	23	25	2	8.70%	
D_T	18	20	2	11.11%	
D_R	20	24	4	20.00%	
F_S	18	20	2	11.11%	
F_M	20	22	2	10.00%	
F_N	20	22	2	10.00%	
I_{Bkt}	20	24	4	20.00%	
I_B	20	24	4	20.00%	

Similarly, all the *Bacillus* spp isolated from residential area, agricultural area, river bank, industrial area, forest area and dumping site showed a zone of hydrolysis greater than 19 mm (Figure 1).

Figure 3: Zone of hydrolysis by crude and partially purified protease enzyme across different sites

All seventeen proteolytic *Bacillus* isolates showed notable anti-pathogenic activity against five different clinical isolates - Methicillin Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus* spp (Table 4).

Table 4: Antipathogenic activity of different proteolytic isolates

Isolates	Zone of inhibition (in mm)				
	MRSA	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Streptococcus* spp
R_S	20	23	18	19	24
R_{Mu}	18	20	16	17	21
R_M	19	22	17	18	22
R_H	22	25	19	21	25
R_K	21	24	18	20	24
Ri_B	17	19	15	16	20
Ri_D	16	18	14	15	19
A_S	18	21	17	18	22
A_M	19	22	17	19	23
A_T	17	20	16	17	21
D_T	15	17	14	15	18
D_R	14	16	13	14	17
F_S	20	23	18	19	24
F_M	21	24	19	20	25
F_N	22	25	18	21	25
I_{Bkt}	16	18	15	16	20
I_B	17	19	16	17	21

DISCUSSION

The 17 *Bacillus* isolates were selected based on their thermotolerant properties and were identified as Gram-positive, catalase-positive, motile, and spore-forming bacteria. All of them demonstrated proteolytic activity. These characteristics were consistent with *Bacillus* spp, which are widely recognized for their ability to survive in diverse environments, including those with varying temperatures and stress conditions (Parvez et al., 2020). Notably, all isolates were positive for protease production, aligning with findings from previous studies that highlight the proteolytic capabilities of *Bacillus* species, which are essential for various industrial applications, including food processing, detergents, and leather (Bhalerao et al., 2019).

The morphological features of the isolates varied, with some showing central oval spores (e.g., R_S, R_{Mu}, R_M), while others exhibited sub-terminal oval spores (e.g., R_H, R_K, Ri_B), a feature commonly observed in *Bacillus* species (Logan & De Vos, 2015). This variability may reflect the ecological adaptations of these isolates to different environmental conditions across the sampled sites. For instance, differences in spore location could indicate genetic diversity within *Bacillus* populations, a finding that warrants further genomic exploration to better understand their ecological and functional differentiation (Singh et al., 2024).

Proteolytic activity is a key feature of *Bacillus* spp, enhancing their industrial applications. This study showed that all isolates exhibited notable protein hydrolysis, with partially purified protease extracts demonstrating increased activity compared to crude extracts (Table 3). This suggests that the proteases produced by these strains can be effectively concentrated and refined for enhanced industrial utility (Contesini et al., 2018). The increase in zone of hydrolysis upon partial purification ranged from 4% to 25%, with the highest increase observed in isolates from the agricultural farm (A_M) and river bank (Ri_B), indicating that certain environmental factors may enhance protease activity (Razzaq et al., 2019).

The variation in protease activity among isolates could be linked to specific environmental conditions at the sampling sites. For instance, isolates from residential areas exhibited higher proteolytic activity than those from industrial or forest sites, possibly due to differing nutrient availability and microbial competition (Saggu & Mishra, 2019). Such environmental gradients could influence the metabolic pathways of *Bacillus* spp, leading to differential enzyme production (Liu et al., 2020).

In examining site-specific proteolytic activity (Table 4), isolates from residential areas showed the highest average proteolytic activity (29.6 mm for partially purified extracts), followed by agricultural areas (27.6 mm). River bank and industrial areas had moderate activity, while forest and dumping sites exhibited the lowest proteolytic zones. These differences could be attributed to the organic content and microbial diversity in the soil of each sampling site (Radhakrishnan et

al., 2017). Residential and agricultural areas likely have higher organic matter and microbial abundance, enhancing protease production. Conversely, dumping and industrial areas may present polluted environments with limited nutrient availability, restricting *Bacillus* growth and protease production (Saggu & Mishra, 2019).

The antipathogenic activity of the *Bacillus* isolates was evaluated against pathogenic bacteria, including Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Streptococcus* spp All isolates demonstrated significant antipathogenic activity, with inhibition zones ranging from 14 mm to 25 mm. This is consistent with studies highlighting the antimicrobial potential of *Bacillus* spp, particularly against multi-drug resistant pathogens (Banerjee et al., 2018; Molla et al., 2019). Isolates from residential and agricultural areas, such as R_S, R_H, and F_M, showed the most potent inhibition, possibly due to higher proteolytic activity and the presence of antimicrobial compounds like bacteriocins or lipopeptides (Abriouel et al., 2011).

Interestingly, isolates from industrial areas, which exhibited lower proteolytic activity, also showed reduced antimicrobial activity, suggesting that environmental stressors might impact both proteolytic and antimicrobial potential (LeBlanc et al., 2020). Conversely, isolates from forest areas (F_N, F_S) exhibited strong inhibition against pathogenic bacteria, suggesting production of a broader range of antimicrobial substances (Moldes et al., 2020).

Proteolytic and antimicrobial activities exhibited by the *Bacillus* spp isolated in this study suggest their potential for various biotechnological applications. The enzymes produced could be valuable in industrial processes requiring protein degradation, such as in the production of detergents, leather, or food products (Bhalerao et al., 2019). Moreover, the demonstrated antimicrobial activity, particularly against drug-resistant pathogens like MRSA and *Klebsiella pneumoniae*, highlights the potential of these *Bacillus* strains in developing novel antimicrobial agents (Schillaci et al., 2020). Given the rising concerns about antibiotic resistance, the use of *Bacillus* spp as a source of natural antimicrobial compounds could offer a promising alternative to conventional antibiotics (Gull et al., 2020).

CONCLUSION

This study identified 17 *Bacillus* spp from diverse soils in Kathmandu Valley, demonstrating strong proteolytic and antimicrobial activities. High-performing isolates such as A_M, R_S and R_H showed notable enzyme production and pathogen inhibition. These results highlight the biotechnological potential of soil-derived *Bacillus* spp and warrant further investigation to optimize their use in industrial and antimicrobial applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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