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ORIGINAL RESEARCH

Exploring *Bacillus* spp Protease Activity in Slaughterhouse Soils

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ABSTRACT

Objective: To correlate protease-producing *Bacillus* spp with physicochemical parameters from slaughterhouse soils.

Methods: In this study, eight soil samples were collected from three slaughterhouses located in the Kathmandu Valley and designated as $K_{1,2}$, $L_{1\text{-}3}$, and $B_{1\text{-}3}$. Sterile methods were employed to collect the samples, and their physicochemical properties, including temperature, pH, moisture, electrical conductivity, alkalinity, and nutrients, were analyzed. This was followed by enrichment and isolation of protease-producing *Bacillus* spp.

Results: The collected soils were pale to neutral grey and had alkaline pH. The soils contained moderate water, up to 14%. Even with low organic matter, it had high nitrogen levels. This study isolated *Bacillus* spp that produce proteases and measured the specific protease activity.

Conclusion: Protease-producing *Bacillus* spp found in soils are directly proportional to the nutrients, especially in soils with higher nitrogen levels, which tend to have a greater abundance of protease bacteria. This indicates that soil fertility influences bacterial protease functions.

Keywords: Chemical properties, Nitrogen-rich environments, Physical properties, Protease-producing *Bacillus* spp, Slaughterhouse soil.

INTRODUCTION

Physicochemical properties, including temperature, pH, moisture, nutrient availability, and trace elements, significantly influence bacterial growth, metabolism, and community structure, as these parameters determine enzyme kinetics, membrane stability, and the solubility of nutrients. Optimal temperatures enhance metabolic rates, while extremes will denature enzymes or slow activity. Similarly, pH, electrical conductivity, moisture content, and alkalinity all influence enzyme function and the bioavailability of nutrients and metals (Lu et al., 2025, Yang et al., 2024).

Slaughterhouse soils represent a specialized habitat due to high inputs of animal-derived organic matter (including tissues, blood, and bones) and frequent fresh substrate additions. These soils are often enriched in trace elements, such as copper (Cu^{2+}), iron (Fe^{3+}), and zinc (Zn^{2+}), which act as cofactors for many enzymes; however, these metals could also be inhibitory at high concentrations. The continuous supply of diverse organic substrates supports a wide range of proteolytic, lipolytic, anaerobic, and facultative organisms. It encourages the evolution or enrichment of metabolic pathways for degrading complex animal-derived compounds (Yazdankhah et al., 2014). Microbial interactions in this environment form complex ecological networks that differ from those in typical agricultural soils, producing distinct community structures and functions (Kiprotich et al., 2025, Kracmarova-Farren et al., 2024). Although slaughterhouse soils are rich in microbial diversity, isolating and characterizing these microbes presents

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several research challenges (Alidoosti et al., 2024, Stefanis et al., 2013). The most common bacteria in slaughterhouse soil belong to proteolytic and lipolytic species, which thrive on protein- and fat-rich substrates, such as *Bacillus subtilis*, *Pseudomonas aeruginosa,* and *Clostridium* spp. Secondly, anaerobic and facultative anaerobes such as *Bacteroides* spp, *Enterobacter* spp, and *Methanobacterium* spp. Thirdly, *Actinomycete* spp and soil-dwelling decomposers contribute to organic matter (OM) cleavage and antibiotic production, including *Streptomyces* spp and *Nocardia* spp. Finally, slaughterhouse soils may harbor zoonotic or opportunistic pathogens such as *Listeria monocytogenes, Salmonella* spp, and fecally contaminated *Escherichia coli* (Khan & Rao, 2019).

Extensive research has been conducted on bacterial contamination, patterns of antibacterial resistance, and the associated risks to public health in slaughterhouse soils and meat-handling areas (Bantawa et al., 2018, Singh, 2022). However, exploring isolates that produce proteases with broad industrial applications is an exciting opportunity for further discovery. Therefore, this study aims to isolate protease-producing *Bacillus* spp that can be applied to industries, as slaughterhouse soil is a rich source of nutrients for bacterial growth.

METHODS

Materials

The laboratory experiments used distilled water and analytical-grade chemicals. All chemicals used in this study were purchased from HiMedia Laboratories Pvt. Ltd., India.

Sampling Sites and Slaughterhouse Soil Samples Collection

Eight soil samples ($K_{1,2}$, $L_{1\text{-}3}$, and $B_{1\text{-}3}$) were collected from three slaughterhouses in the Kathmandu Valley (27±0.2 °C) using sterile techniques at a depth of 15 cm. The samples were transported to the Environmental Engineering laboratory (Pulchowk Engineering Campus), dried, and passed through 2.5 mm and 2 mm sieves for physical, chemical, and biological analysis (Felde et al., 2020).

Physicochemical Analysis of Soil Samples

Different physicochemical parameters, such as colour and moisture content (Little et al., 1998), were detected. Additionally, chemical analyses were conducted, including pH (Al-Busaidi et al., 2005), electric conductance (EC) (dS/m) (Qi et al., 2020), and total alkalinity (Dhoke, 2023). Similarly, total organic matter (%) (Dewis & Freitas, 1970, WRD, 2009) and total nitrogen (TN) (%) (Bremner & Mulvaney, 1982) were also estimated.

Enhancement and Selection of Isolates Producing Extracellular Protease

A modified protocol of Laba et al., (2018) was carried out for screening, in which 1 g sample was mixed with 10 mL of sterilized distilled water and spread on skim milk agar plates (7.5% skim milk powder, 1.5% agar, 0.5% peptone, 0.25% yeast extract, and 0.1% glucose). The plates were incubated (32 °C) until visible growth was observed. Single colony with the surrounding highest clear halo zone was selected (Hyseni et al., 2020). The isolated colonies were inoculated on sterile gelatin plates (1% gelatin, 1% peptone, 1% yeast extract, 0.5% sodium chloride, and 1.5% agar) and incubated for 24 h. After flooding with saturated ammonium sulfate, clear zones were detected around the gelatin (Medina & Baresi, 2007). The morphological and cultural characteristics of the subcultured organisms were used for phenotypic identification of colonies (Hyseni et al., 2020), followed by microscopic examination and biochemical tests (Shen et al., 2022).

Specific Activity of Protease

The highest protease-producing isolates were analyzed using a protease-specific assay (Sigma's non-specific protease activity assay) with 0.65% casein as the substrate in potassium phosphate buffer (pH 7.5) (Cupp-Enyard, 2008). The assay conditions were incubated for 10 min at 37 °C, and were terminated with 10% trichloroacetic acid. Tyrosine was measured using Folin and Ciocalteu's phenol reagent (Hyseni et al., 2020). The total protein content was assessed using the Bradford method (Bradford, 1976).

Statistical Analysis of Data

Results were obtained from three independent experiments and expressed as the mean±standard error (SEM). The data were analyzed statistically in GraphPad Prism 8.0.2, and graphs were plotted in Origin 2019 and GraphPad Prism 8.0.2. The ordinary one-way ANOVA ($p<0.05$) was applied to determine a significant difference among intervals in the same groups. Furthermore, Pearson's correlation (r) was used to investigate the relationships between the physicochemical and/or nutritional parameters with protease-producing *Bacillus* spp.

RESULTS

Physicochemical and Nutritional Diversity of Slaughterhouse Soils across the Valley

Soil samples collected from slaughterhouse areas in Kathmandu (K_{1-2}), Lalitpur (L_{1-3}), and Bhaktapur (B_{1-3}) revealed a broad scope of physical and chemical characteristics. These samples showed variation in physical properties such as colour, ranging from pale to neutral grey; alkaline pH levels, as shown in Figure 1a, indicating that the soils tend to favour alkaline conditions; and moisture content (up to 14%), detailed in Figure 1b, which influences microbial activity and soil stability. The study analyzed the chemical parameters, including EC (Figure 2a) and alkalinity (Figure 2b).

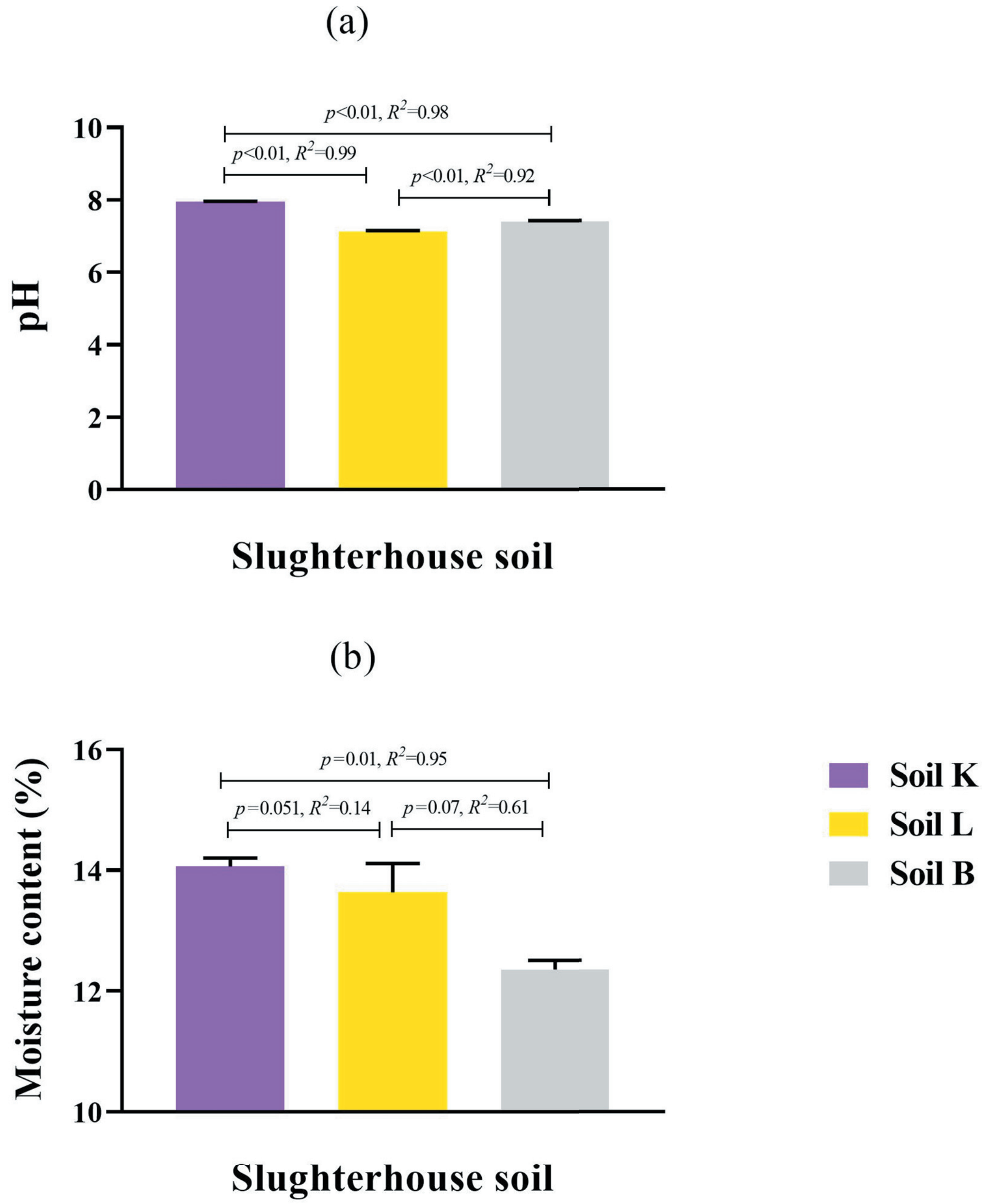
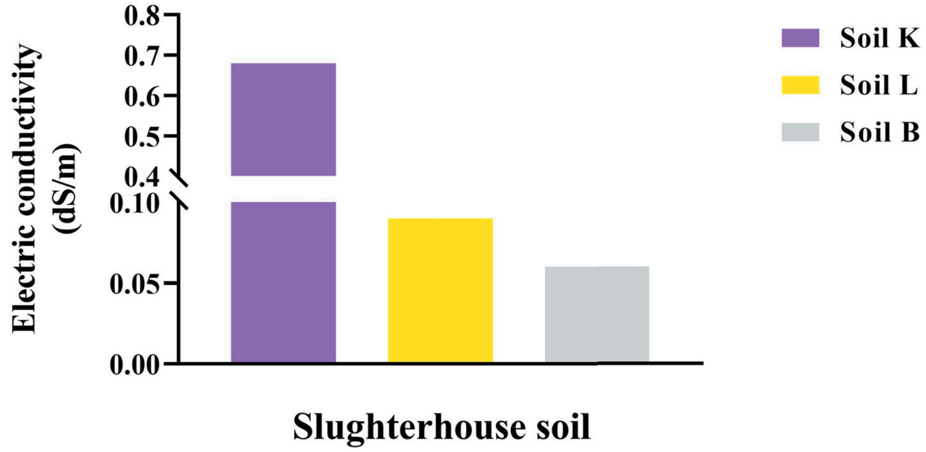


Figure 1: Variation in physical properties of slaughterhouse soil samples. (a) pH and (b) Moisture content. Note: Data represent the mean±standard error (n=3). Statistical significance was determined by ordinary one-way ANOVA (*$p<0.05$*).

(a)



(b)

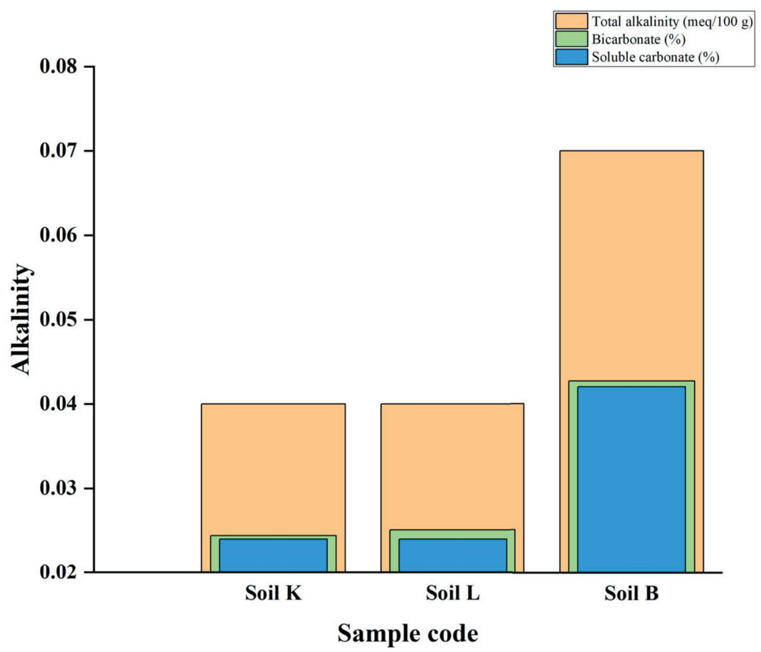


Figure 2: Variation in chemical properties of slaughterhouse soil samples. (a) Electric conductivity and (b) Alkalinity. Note: Data represent the mean±standard error (n=3). Statistical significance was determined by ordinary one-way ANOVA (*p<0.05*).

These variations in physicochemical parameters contributed to the total carbon (TC) (%) and total nitrogen (TN) (%) content, as shown in Figure 3.

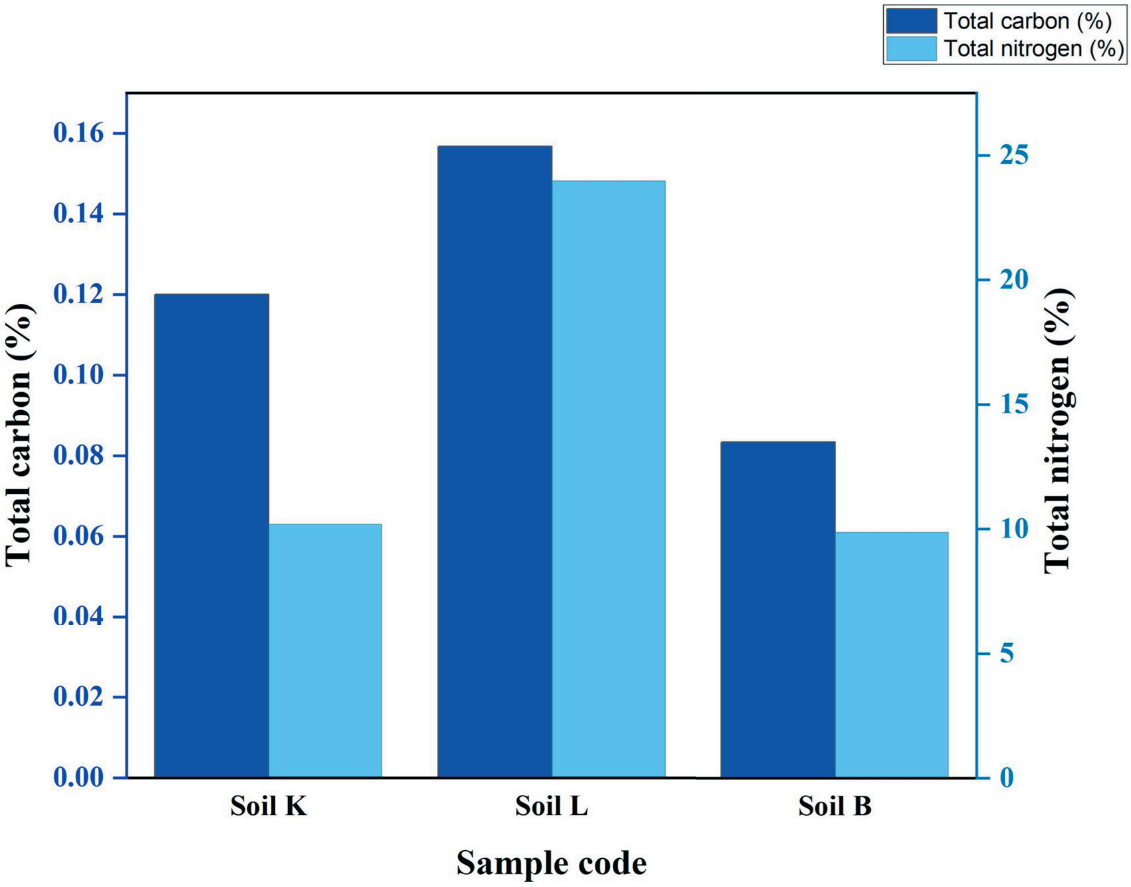


Figure 3: Variation in nutrient contents of slaughterhouse soil samples. Note: Data represent the mean±standard error (n=3). Statistical significance was determined by ordinary one-way ANOVA (*p<0.05*).

Distribution of Gram-Positive Bacterial Isolates Exhibiting Protease Activity

After subculture, 25 bacterial isolates were observed to be Gram-positive and rod-shaped (Figure 4), exhibiting biochemical characteristics consistent with those of *Bacillus* spp. The study included 28% isolates from Kathmandu, 40% from Lalitpur, and 32% from Bhaktapur, as depicted in Figure 5a. The isolates exhibited protease activity (0.25-482.20 U/mL, y = 4000x-182.67, $R^2 = 1$) (Figure 5b) and were subsequently stored in glycerol stock.

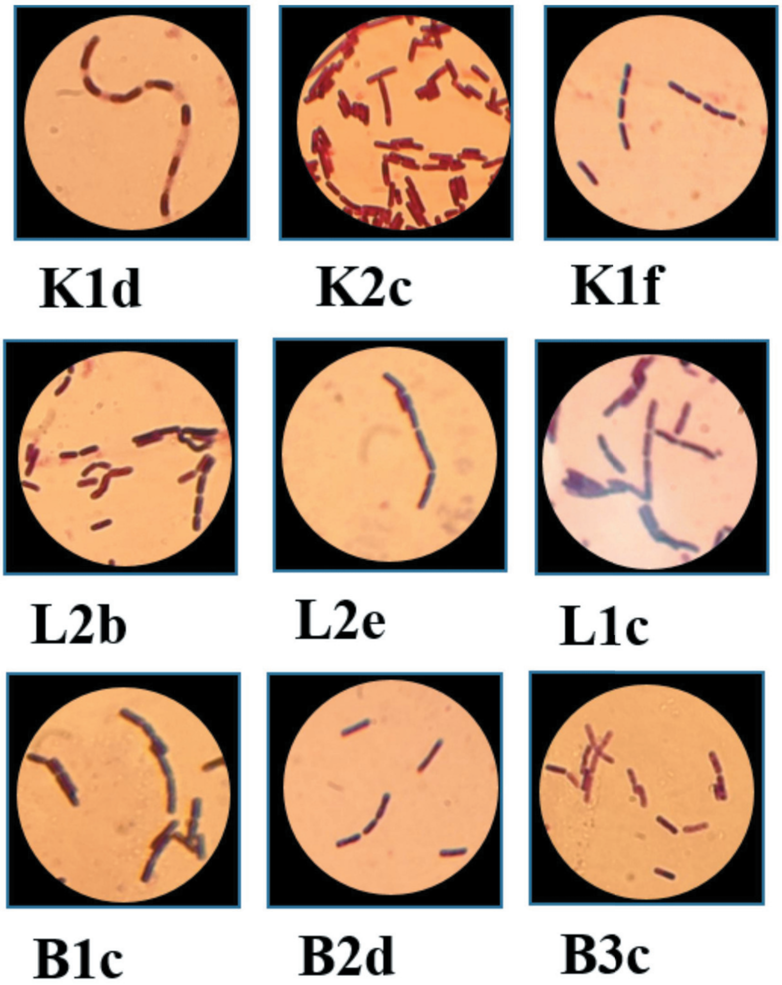


Figure 4: Gram staining of selected protease-producing *Bacillus* spp from slaughterhouse soil samples.

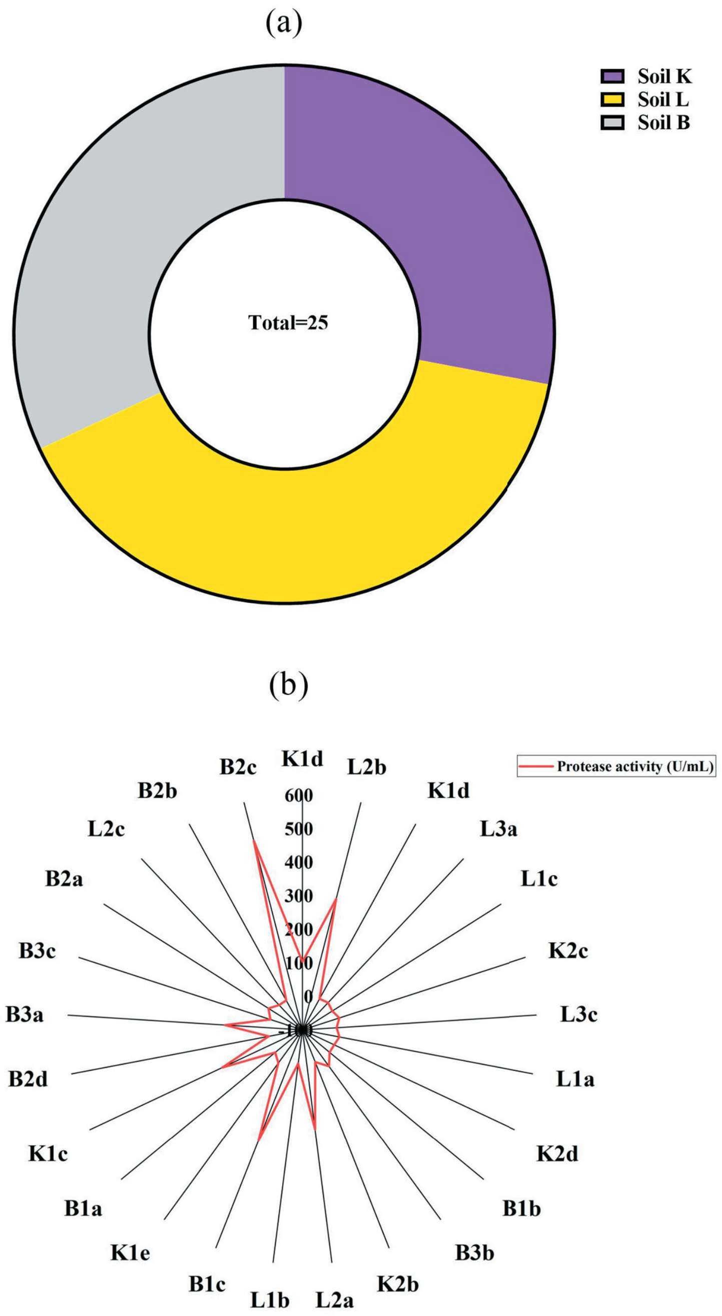


Figure 5: Isolates exhibiting protease activities. (a) Different locations of Kathmandu Valley and (b) Specific protease activity (U/mL).

Association between Slaughterhouse Soil Parameters and Protease-Producing *Bacillus* spp

The study suggested that the individual physical and nutritional parameters have a favourable association; however, the chemical parameters, such as electric conductivity and total alkalinity, have an unfavourable association. The physical and chemical parameters also have a favourable association. Additionally, the moisture content of slaughterhouse soil influences its nutritional content; however, the nutritional content was unaffected by the pH of the slaughterhouse. Electric conductivity exhibits favourable and unfavourable associations with organic carbon and total nitrogen contents. Interestingly, the study observed that all parameters highly influence the protease-producing *Bacillus* spp in the respective soils, as shown in Table 1.

Table 1: Correlation between different parameters of slaughterhouse soil samples

Parameters	pH	MC (%)	EC (dS/m)	TA (meq/ 100 g)	OC (%)	TN (%)	Isolates
pH	1	0.42^{3*}	0.93^{5*}	0.18^{*}	$-0.33^{\#}$	$-0.75^{4\#}$	0.60^{4*}
MC (%)	0.42^{2*}	1	0.73^{4*}	0.97^{5*}	0.72^{4*}	0.29^{2*}	0.98^{5*}
EC (dS/m)	0.93^{5*}	0.73^{4*}	1	$-0.54^{3\#}$	0.04^{*}	$-0.44^{3\#}$	0.86^{5*}
TA (meq/ 100 g)	0.18^{*}	0.97^{5*}	$-0.54^{3\#}$	1	$-0.87^{5\#}$	$-0.52^{3\#}$	0.8^{5*}
OC (%)	$-0.33^{\#}$	0.72^{4*}	0.04^{*}	$-0.87^{5\#}$	1	0.88^{5*}	0.55^{3*}
TN (%)	$-0.75^{4\#}$	0.29^{2*}	$-0.44^{3\#}$	$-0.52^{3\#}$	0.88^{5*}	1	0.89^{5*}
Isolates	0.60^{4*}	0.98^{5*}	0.86^{5*}	0.8^{5*}	0.55^{3*}	0.89^{5*}	1

Note: One-tailed Pearson correlation ($p>0.05$) where '*': Very low but positive association, '2*': Low but positive association, '3*': Moderately and positive association, '4*': Highly and positive association, and '5*': Very highly and positive association, '3#': Moderately but negative association, '4#': Highly but negative association, and '5#': Very highly but negative association.

DISCUSSION

This study concluded that the slaughterhouse soil has an alkaline pH (Figure 1a), in contrast to the study conducted by Fernández-Calviñoand Baath (2010) that observed the pH range of 4.5 to 7.8 was optimal for bacterial growth in the respective soils. Alter in soil pH by ±1-2 units influence the nitrogen cycling genes, indicating functional consequences beyond bacterial population shifts (Xiong et al., 2024).

Additionally, the moisture content ($r=0.98$, $p>0.05$) (Table 1) also directly relates to the bacterial growth, its diversity, and enzymatic activities (Bogati et al., 2025, Iovieno & Baath, 2008). However, reducing alkalinity through microbial amendments enhances microbial diversity and enzymatic activity, whereas higher pH and alkalinity suppress microbial growth by altering soil structure and limiting nutrient availability (Lopes et al., 2021, Tian et al., 2025).

Similarly, to previous studies, this study also concluded that the chemical property, such as a moderate increase in EC, directly relates to microbial biomass by improving soil nutrient availability (Feng et al., 2025). Whereas, higher EC levels may enhance bacterial growth, however induce bacterial stress and shift community composition due to salinity (Kim et al., 2016, Lee et al., 2011) ($r=0.86$, $p>0.05$) (Figure 2).

This study also observed that the protease-producing *Bacillus* spp were positively correlated with pH, EC, TC, and TN (Table 1). Protease-producing *Bacillus* spp were enhanced in nutrient-rich soils, suggesting that physicochemical properties directly influence the metabolic potential of bacteria. However, a survey by Meena et al., (2020) indicates that bacterial populations positively correlated with pH, EC, TC, and TN, and inversely with EC. This study also observed that TN availability ($r=0.89$, $p>0.05$) had a more substantial impact on microbial communities than pH ($r=0.6$, $p>0.05$) and OC ($r=0.55$, $p>0.05$). A study conducted by Zhang et al., (2024) also showed that nutrient availability had a more substantial impact on microbial communities than pH, and nitrogen addition promoted the growth of *Proteobacteria* spp.

CONCLUSION

Slaughterhouse soils, the natural habitats of the protease-producing *Bacillus* spp, hold promising potential for various industrial applications, and this is a relatively underexplored field. Exploring these isolates can lead to novel discoveries. However, the physicochemical parameters and high nitrogen levels are essential nutrients that support bacterial growth, affecting the inhabitants of these *Bacillus* spp. A broad range of protease-producing *Bacillus* spp was isolated, which have a wide scope in various industries, such as the pharmaceutical sector (development of new drugs and diagnostics), the leather industry for dehairing, detergent manufacturing, and in the food industry as an eco-friendly alternative. However, this study only focused on the broad-spectrum protease-producing *Bacillus* spp, limiting the other enzyme-producing isolates that can be isolated from this environment. Therefore, the future direction should focus on the isolation, detection of different enzymes, and optimization for the maximal enzyme expression and finally extraction for the respective industries. Hence, conducting comprehensive studies to identify, isolate, and better understanding these microbes is critical, which could lead to industrial microbiology and environmental sustainability breakthroughs.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest pertinent to this work.

AUTHOR CONTRIBUTIONS

Designed the study: PB and MA, collected samples: PB, LM, KS, and MA, Laboratory work: PB and LM, data analysis: PB, first draft of manuscript: PB, final version of manuscript: PB, BKD, IPA, and MA.

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