



Extracellular protease-producing ability of *Bacillus* spp. isolated from chicken waste dumping sites and poultry farms in Thrissur district

N. Sonali ^{1*}, Deepa Jolly¹, B. Sunil¹, C. Sethulekshmi¹ and T. Sathu²

¹Department of Veterinary Public health, ²Department of Livestock Products Technology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur-680 651, Kerala Veterinary and Animal Sciences University, Kerala, India.

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Abstract

The present study aimed to investigate the presence of *Bacillus* spp. in chicken waste dumping sites and poultry farms in the Thrissur district to produce extracellular protease. Thirty soil samples were collected from chicken waste disposal sites, as well as from poultry farms and analyzed for physicochemical parameters, soil type, and the presence of *Bacillus* spp. Out of the 68 isolates from 30 heat-treated soil samples, 26 *Bacillus* isolates were identified using standard microbiological methods and confirmed by polymerase chain reaction targeting 16S rRNA. All 26 *Bacillus* isolates were screened for extracellular protease production on skim milk agar plates, identified by the clear zone of hydrolysis around the colony. Among the 26 isolates, 18 *Bacillus* spp. were found to produce protease, accounting for a 69.2 per cent occurrence of protease enzyme-positive *Bacillus* spp. obtained from a total of 30 soil samples. Hence, the study suggests that skim milk agar was effective for screening extracellular protease-producing bacteria, and the aforementioned *Bacillus* spp. isolates demonstrated potential for protease enzyme production.

Keywords: *Bacillus* spp., soil, protease producer, skim milk agar, plate assay

Globally, billions of chicken are slaughtered annually for human consumption, resulting in the production of millions of tons of feather waste as a byproduct. Feathers, inherently durable, take approximately five to seven years to decompose naturally. This resilience is attributed to their complex composition, which consists of 91 per cent keratin protein. The keratin found in chicken feathers comprises both α -helical and some β -sheet conformations, with the central rachis primarily composed of β -sheet structures. The higher cysteine content and the presence of disulfide bonds render β -sheets tougher compared to α -helices (Tamreihao et al., 2019).

Studies have revealed that numerous bacteria, fungi, and actinomycetes thrive in environments rich in keratin, capable of decomposing the keratin present in feathers to sustain themselves through the production of extracellular protease enzymes. This sets the stage for the biodegradation of feather waste. A variety of microorganisms, such as *Bacillus licheniformis*, *B. subtilis*, *B. cereus*, *B. pumilis*, *Microbacterium* (Gram-positive organisms), alongside Gram-negative bacteria like *Vibrio*, *Xanthomonas*, *Chryseobacterium*, *Fervidobacterium*, and *Pseudomonas*, have been identified as producers of protease. This expands the possibilities for microbial involvement in the management of feather

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^{*}Corresponding author: sonalineelakandan@gmail.com Ph. 9944241735

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waste (Sahoo et al., 2017).

Furthermore, within the bacterial domain, *Bacillus* spp. stand out as specific producers of extracellular protease and can be found across various soil types due to their ability to thrive in diverse conditions, including temperate, acidic, alkaline, and neutral soils. Hence, the present study was carried out to isolate and identify *Bacillus* spp. with the potential to produce extracellular protease enzymes.

Materials and methods

Collection of soil samples

Thirty soil samples were collected from both chicken waste dumping sites and poultry farm premises within the Thrissur district. Each sample, weighing approximately 100 g, were collected from depths ranging between five to ten centimeters beneath the soil surface, utilizing sterile polythene bags. The samples were collected from various locations viz., Mannuthy, Vadakancheri, Pattikkad, Thumboor, Kombodinjamakkal, Karoor, Alur, and Chalakudy, between July, 2023 and November, 2023.

Isolation of *Bacillus* spp.

All the collected samples were analyzed for the presence of *Bacillus* spp. The organism was isolated by the method described by Subugadeet *al.* (2017) with certain modifications. Ten grams (10g) of soil sample was suspended in 90mL of 0.85 per cent sodium chloride solution. The soil suspension was subjected to heat shock at 80°C for 10 min in a water-bath to kill non-spore-forming organisms (Manzum & Mamun, 2018). The heat-treated soil suspension was then plated onto nutrient agar plates and was aerobically incubated at 37°C for 24 h and examined for colony formation. Morphologically distinct colonies were selected and subjected to Gram staining. Only Gram-positive colonies were streaked onto HiCrome *Bacillus* agar and incubated at 37°C for 24 h to obtain pure cultures. Isolated colonies from pure culture plates were streaked onto nutrient agar, incubated at 37°C for 24 h, and stored for subsequent identification.

Table 1. Biochemical tests for *Bacillus* spp.

Primary tests	Secondary tests	
Gram reaction	Utilisation of citrate	
Spore staining	Nitrate reduction test	
Motility	Starch hydrolysis	
Catalase	Lecithinase test	
Oxidase	Carbohydrate fermentation test	Mannitol
		Sucrose
		Lactose
		Xylose

Identification of *Bacillus* spp.

Bacillus spp. isolates were identified based on the cultural, morphological and biochemical characteristics. *Bacillus* spp. exhibited a wide range of colony morphologies viz., round, fimbriate edges, mucoid, dry, mucoid beading at the surface, spreading growth, creamy-grey to off-white colonies. The bacterial isolates were characterized by primary and secondary biochemical tests as described by Barrow and Feltham (1993) as shown in Table 1.

Molecular confirmation of *Bacillus* spp.

The genomic DNA extraction was performed using

Table 2. Protease activity of *Bacillus* spp. isolated from soil samples

Sample number	<i>Bacillus</i>	Isolate	Protease activity in Skim milk agar (SMA)
1	Present	B1	Absent
		B2	Absent
		B3	Present
2	Absent	-	-
3	Present	B4	Absent
		B5	Present
4	Absent	-	-
5	Present	B6	Absent
6	Present	B7	Absent
7	Present	B8	Present
		B9	Present
8	Present	B10	Present
9	Present	B11	Present
10	Present	B12	Absent
		B13	Present
11	Absent	-	-
12	Present	B14	Present
13	Present	B15	Present
14	Absent	-	-
15	Present	B16	Present
16	Present	B17	Present
17	Present	B18	Present
18	Present	B19	Present
19	Absent	-	-
20	Present	B20	Absent
21	Present	B21	Present
22	Present	B22	Present
23	Present	B23	Present
24	Absent	-	-
25	Absent	-	-
26	Present	B24	Present
27	Absent	-	-
28	Absent	-	-
29	Present	B25	Absent
30	Present	B26	Present

the snap-chill or boiling technique as described by Singh et al. (2022). Molecular confirmation of *Bacillus* spp. was done by amplifying 16S *rRNA* gene in an automated thermal cycler (BioRad T100 Thermal Cycler) using the primers 16S *rRNA* F (5' AGAGTTTGATCCTGGCTCAG 3') and 16S *rRNA* R (5' TACGGCTACCTTGTACGACTT 3') with an amplicon size of 1516 bp as described by Almahasheer et al. (2022). The cyclic conditions for 25 µL PCR were initial denaturation at 95°C for five minutes followed by 30 cycles of denaturation at 95°C for one minute, annealing at 56°C for one minute, and extension at 72°C for one minute and 30 sec. And then, final extension at 72°C for 10 min. The PCR products were detected by electrophoresis in 1.5 per cent agarose gel and were visualized and documented using a gel documentation system (Syngene, USA).

Screening for protease activity in skim milk agar

The protease activity test was conducted using skim milk agar following the protocol outlined by Subugade et al. (2017). The isolates were point-inoculated onto the skim milk agar plates and then incubated at 37°C for 24 h. After the incubation period, the agar plates were examined for the presence of a clear zone of hydrolysis around the colony.

Results and discussion

Isolation and identification of *Bacillus* spp.

All soil samples (n=30) collected from chicken waste disposal sites and poultry farm premises in Thrissur district were subjected to isolation and identification of *Bacillus* spp. Out of the 68 isolates obtained from the heat-treated soil samples, 26 were confirmed to be *Bacillus* spp. through biochemical and molecular analysis by PCR with an amplicon size of 1516 bp (Fig. 1). This represented an occurrence rate of 38.24 per cent for *Bacillus* spp. in the soil samples (Fig. 2). Similar results were also reported by Herzog et al. (2015), who reported 42 per cent occurrence of *Bacillus* spp. in Canada. However, Ibrahim and Zaki (2019) documented a higher occurrence, with 38 out of 58 isolates (73.1%) identified as *Bacillus* spp. from 40 soil

samples collected from different regions in Baghdad city in Iraq. In a study, Manzum and Mamum (2018) reported that 54 *Bacillus* isolates were obtained from 24 soil samples in Dhaka. The difference between the present study and that of Manzum and Mamum (2018) is that in the latter study, all Gram-positive rods isolated from heat-treated soil samples were presumptively considered *Bacillus* spp. without biochemical and molecular confirmation.

In this study, it was observed that, without heat treatment, all other Gram-positive and Gram-negative non-spore-forming bacteria had outgrown *Bacillus* spp. Hence, all soil samples were subjected to heat treatment at 80°C for 10 minutes. This process aimed to eliminate non-spore-forming vegetative bacterial cells and promote the germination of spores. The comparatively lower occurrence of *Bacillus* spp. observed in our study, in contrast to some previous research, could be attributed to variations in the ecosystems of sample collection and differences in the methodologies employed across studies.

Screening for protease activity in skim milk agar

All 26 *Bacillus* isolates (designated as B1 to B26) were screened on skim milk agar to identify producers of protease enzyme. Among these isolates, 18 exhibited extracellular protease activity on skim milk agar plates (Table 2), as evidenced by clear hydrolysis zones surrounding the colonies (Fig. 4). Consequently, the occurrence of extracellular protease-producing *Bacillus* spp. was determined to be 69.2 percent, as illustrated in Figure 3. Barman et al. (2017) reported a similar finding, with 61 percent of protease producers isolated from soil samples collected from poultry farms and the Hazaribagh tannery industry in Dhaka. In contrast to the present study, Vidyalakshmi et al. (2020) reported a lower occurrence of 38 percent of protease-positive isolates from the Hoskote region of Bangalore.

Proteases, which are enzymes responsible for breaking down proteins, constitute a class of degradative enzymes. In the present study, eight *Bacillus* isolates did not exhibit protease activity when tested on skim

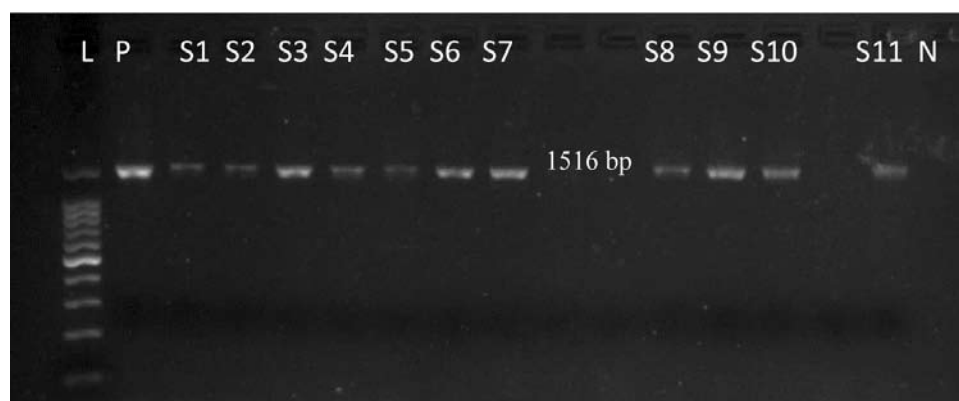


Fig. 1. PCR profile of *Bacillus* spp. using 16S *rRNA* gene

L – 100bp Ladder
P – Positive control
N – Negative control
S (1-11) - Samples

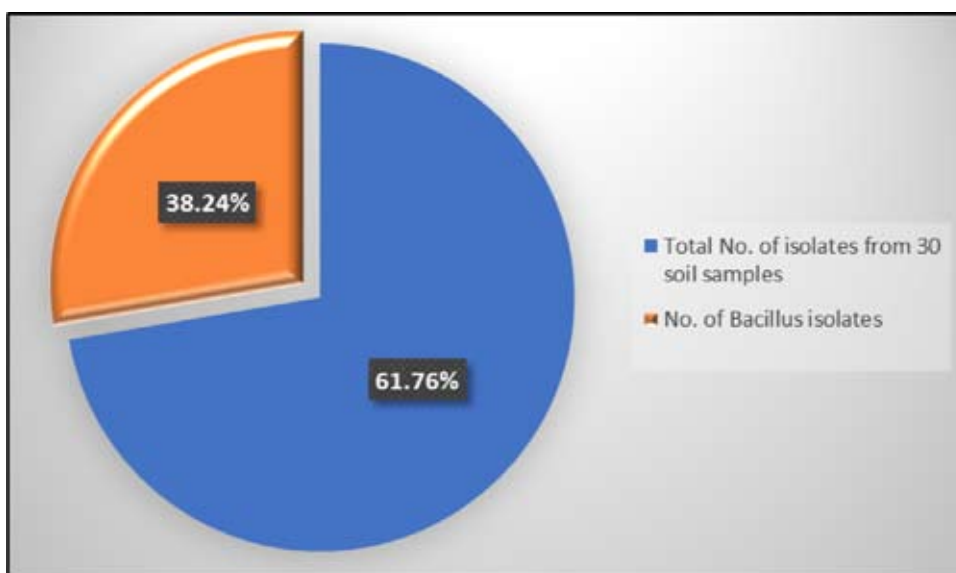


Fig. 2. Occurrence of *Bacillus* spp. in soil samples

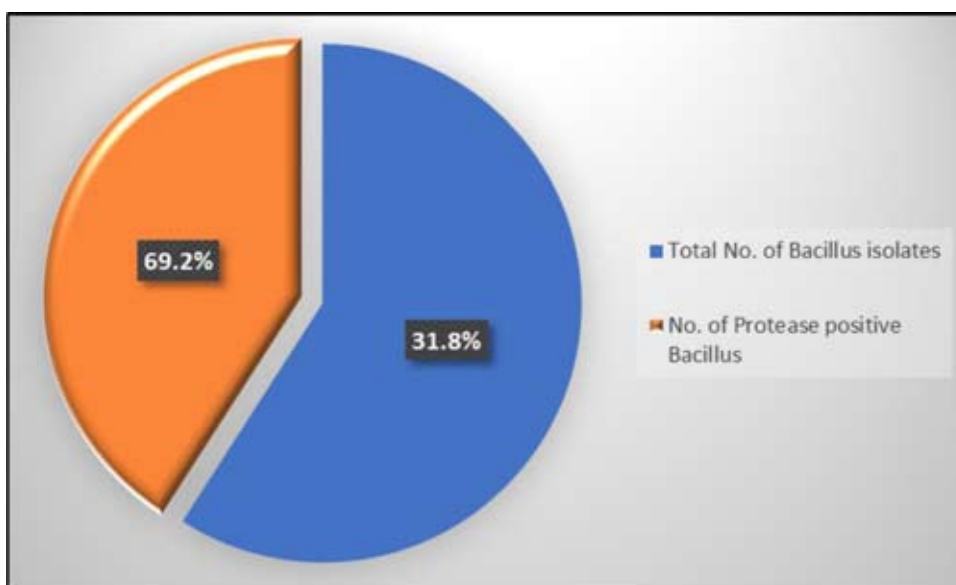


Fig. 3. Occurrence of Protease positive *Bacillus* spp.

milk agar. This lack of activity could be linked to various factors affecting an organism's protease activity, including temperature, pH, incubation duration, genetic diversity (which may involve the absence of genes encoding for protease production), and the strain's specificity towards substrates for protease activity. Consequently, under the experimental conditions utilized in this study, 18 isolates were identified as producers of extracellular protease enzymes.

Conclusion

The results of the present study demonstrate that heat treatment of soil samples is effective for isolating *Bacillus* spp. from soil. *Bacillus* isolates which had shown extracellular protease activity have greater potential for



Fig. 4. Clearance zone around colony of isolate B22

feather waste management and processing. Therefore, these isolates can be used for further investigation of feather degradation.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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