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Exploration of Proteolytic and Amylolytic bacteria from poultry litter soil in Sikkim

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The significant usage of microbial amylases and proteases in various bioindustries has led to their dominance of the global enzyme market among the numerous industrial enzymes. Considering the efficacy and applicability of microbial protease and amylase for sustainable manufacturing and production in commercial industries, the goal of the present study was to explore microorganisms from a poultry litter disposal site at Sikkim that can produce protease and amylase. A total of seven bacterial strains have been isolated and was further examined for proteolytic and amylolytic activities; the isolated strain PLB3 indicated proteolytic and amylolytic potential and had been selected for further characterization and identification. The isolate was identified as a Bacillus species and 16S rRNA sequencing confirms its taxonomic classification as a Bacillus toyonensi.

Keywords : Amylase, *Bacillus toyonensi*, microbial enzymes, protease, Sikkim

INTRODUCTION

Enzymes are bio-catalysts that accelerate the rate of biological processes in live cells by reducing the activation energy (Ea) of the reaction (Nigam, 2013). They are considered environmentally friendly and have the potential to completely reduce or replace the use of hazardous chemical catalysts in industrial processes, thereby, ensuring sustainable manufacturing and production (Singh and Bajaj, 2017). Protease and amylase are two of the most important enzymes, making up over 60% and 25% of the global enzyme market, respectively (Hamza and Woldesenbet, 2017). Generally, plants, animals, and microbes are the natural sources of these enzymes. Amidst the threat to biodiversity loss, plant and animal enzymes are inadequate to fulfill the present global demand for enzymes; therefore, microorganisms are the main source of commercial enzyme synthesis (Ravi *et al*. 2015).

There are many advantages to microbial enzymes, which include lower manufacturing costs, large-scale production, diverse physical and chemical features, and ease in genetic manipulation. The microbial enzymes are more stable and active than their plant and animal equivalent, which makes them ideal for a variety of industrial applications (Dutta *et al*. 2016). Proteases have applications in a variety of industries, which include the processing of leather, the manufacturing of detergents, the processing of food, bioremediation, the production of medicines, the processing of textiles, the processing of waste products, etc. (Sevinc and Demirkan, 2011). Amylases are crucial enzymes; they are mainly used in the starch processing industry, but they also have numerous applications in the food, textile, paper, pharmaceutical, and other industries (Sundarram, 2014). Since bacteria can grow and produce more quickly and can tolerate a wider range of substrates than other microorganisms, they are often preferred. Additionally, bacterial enzymes, particularly those from *Bacillus* spp., have stronger thermal stability than their fungal counterparts (Hyseni *et al.* 2020).

Keratin is the main insoluble structural protein found in feathers of birds. It is known for its recalcitrance and the cross-linking of polypeptide chains by a number of disulfide bonds, which is the reason for mechanical resistance and hinders its breakdown by typical proteolytic enzymes (Brandelli, 2008). Nevertheless, certain microbial enzymes have the ability to hydrolyze the insoluble feather keratins, enabling their

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transformation into feeds, fertilisers, and films (Kornillowicz and Bohacz, 2011). Enzymatic processing may also be helpful for turning protein-rich waste from the poultry industry into worthwhile goods, minimising waste, and preserving the environment (Gupta and Ramnani, 2006). Numerous bacteria that produce amylase and protease have been identified so far from various parts of the world, but no investigation on bacteria that produce these enzymes from poultry dumping sites soil in Sikkim has been reported. Considering these factors, the present investigation was carried out with the intention of isolating, screening, and identifying bacteria that produce enzymes from the poultry dumping site in Sikkim.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from a poultry waste dumping site at Temphyak-Mendu, Rumtek, Sikkim (27.312694° N 88.565254° E) in sterile plastic bags and brought to the laboratory for further analysis.

Isolation of bacterial strains

A series of dilutions, up to 10^{-5}, were applied to the soil sample. The spread plate technique was used to inoculate 0.1 mL of dilutions 10^{-3}, 10^{-4} and 10^{-5} onto nutritional agar medium (NAM) plates, which were then incubated at 37°C ±1 for 2-3 days (Sharma *et al.* 2015).

Proteolytic and Amylolytic activity Screening

By inoculating bacteria isolates on Skimmed Milk Agar plates, the bacteria were screened for proteolytic activity. The SMA plates were incubated for 24-48 hours at 37°C (Olajuyigbe and Ajele, 2005). The development of a clear zone surrounding the bacterial colony demonstrated the isolates' proteolytic activity. Isolates of the bacteria were streaked on Starch Agar plates and incubated at 37°C ±1 for 2–3 days for amylolytic activity activities. Starch agar plates were flooded with Gram's Iodine solution after three days of incubation. Any colour change or halo zone development showed the isolates' amylolytic activity (Sharma *et al.* 2015).

Bio-chemical characterization

Catalase test

The bacterial culture was taken and placed in a microscopic slide. With the help of a dropper, one drop of 3% H_2O_2 was dropped on the slide and immediate bubble formation was observed (Reiner, 2010).

Citrate test

Freshly grown bacterial culture was streaked in the plates containing Simmons' citrate agar and incubated at 35°C±2°C for 18-48 hrs. The utilisation of citrate by the organism was indicated by both observable growth and a change in the colour of the medium (Macwilliams, 2009).

Protease test

The isolate was plated on Skimmed Milk Agar (SMA media) and cultured for 24 to 48 hours at 37°C. The clean zone around the bacterial growth was checked on the plate.

Lipase test

Tributyrin Agar plate was prepared and inoculated with fresh bacteria. The plate was incubated at 37°C ±1 for 24 to 48 hours. The agar plate was examined for clearing around the bacterial growth. The formation of a clear zone indicated the lipolytic activity of the bacteria (Smith and Hussey, 2005).

Gelatin hydrolysis test

Gelatin Agar Medium plate was prepared and inoculated with fresh bacteria and incubated at 37°C ±1 for 24 to 48 hours. After incubation period the plate was flooded with 1% mercuric solutions and observed for color change/halo zone (Smith and Hussey, 2005).

Indole test

4 mL of tryptone broth was transferred to a test tube. The fresh bacterial culture was added to the broth and incubated at 35°C±2 for 24 to 48 hours. Add 5 drops of Kovac2 s reagent to the incubated broth, the immediate formation of a pink

to red colour on top of the medium indicated the production of Indole (Mac Williams, 2009).

Detection of ammonia production

A freshly developed culture was inoculated in 10 mL of peptone water and incubated at 28 oC for 48–72 hours. Nessler's reagent 0.5 mL was added to the broth following the incubation time. The development of a yellow-brown precipitate indicates the presence of ammonia (Smith and Hussey,2005).

The 9th edition of Bergey's Manual of Determinative Bacteriology was then used to compare the findings (Bergey and Holt, 1994).

Morphological and molecular characterization

The identification of the isolate was based on cultural, morphological, biochemical and molecular characterization (16S rRNA gene). The morphology of bacteria was observed using two staining techniques: Gram Staining and Endospore staining (Hussey and Zayaitz, 2007; Cappucina and Sherman, 1992). For molecular identification, the standard phenol/chloroform extraction technique was used for genomic DNA extraction (Sambrook *et al.* 1989), and the 16S rRNA gene was then amplified via PCR utilising universal primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1492 (5'-TAC GGY TAC CTT ACG ACT T-3'). The amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer as per manufacturer's instructions. Identification was carried out using the Lasergene software and the EzBioCloud database (Yoon *et al.,* 2017), using the sequencing facilities at the National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune.

Table 1: Cultural, Morphological and Biochemical characteristics of the isolate PLB3

Cultural characteristics in Nutrient Agar Medium (NA plates)	
Color	Off-White
Form	Round
Margin	Slightly wavy
Elevation	Flat/ Slightly raised
Opacity	Opaque
Morphological characteristics	
Form	Rod- shaped
Gram stain	Positive
Spore	Endospore
Biochemical characteristics	
Catalase	Positive
Citrate	Positive
Protease	Positive
Amylase	Positive
Lipase	Positive
Gelatinase	Positive
Indole	Negative
Ammonia	Positive

RESULTS AND DISCUSSION

Protease enzymes catalyse the breaking of peptide bonds in proteins, releasing short sequences of amino acids known as peptides (Barrett *et al*. 2002). The understanding that proteolytic enzymes not only play a crucial part in cellular metabolic processes but have also attracted a lot of attention from industrial sectors has led to resurgence in interest in the study of these enzymes (Kirk *et al.* 2002). Amylases are extracellular enzymes that catalyse the breakdown of internal 1,4-glycosidic bonds in starch to produce dextrin and other small

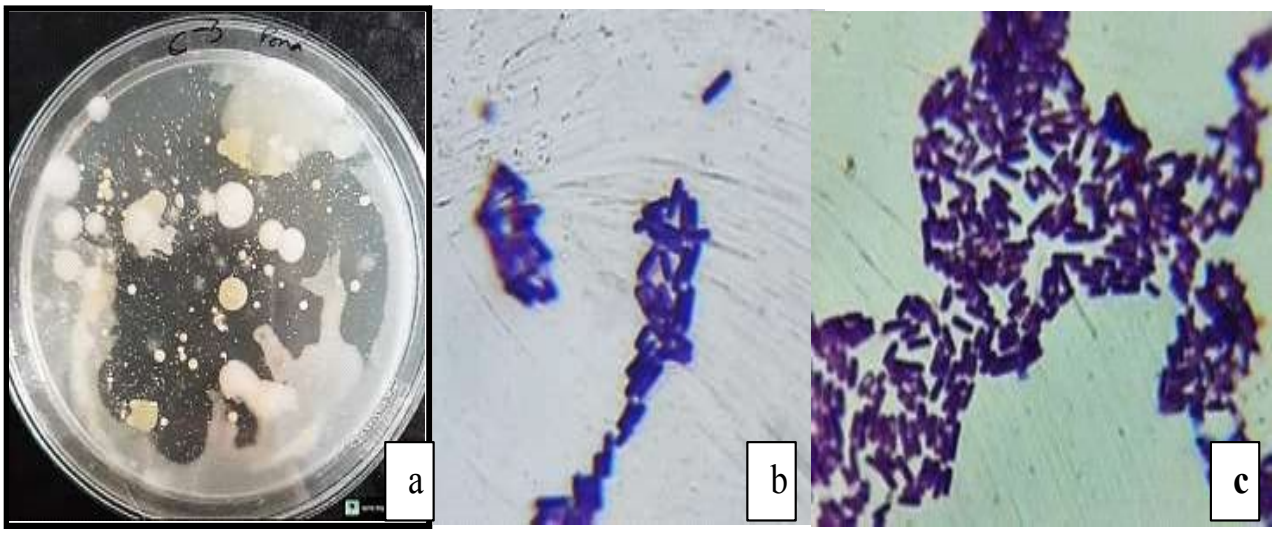

Fig. 1: Bacterial colonies isolated from soil sample (10^{-3} dilution) on (a)NA Medium, ; and (b-c) rod-shaped gram-positive bacteria under X100 microscope

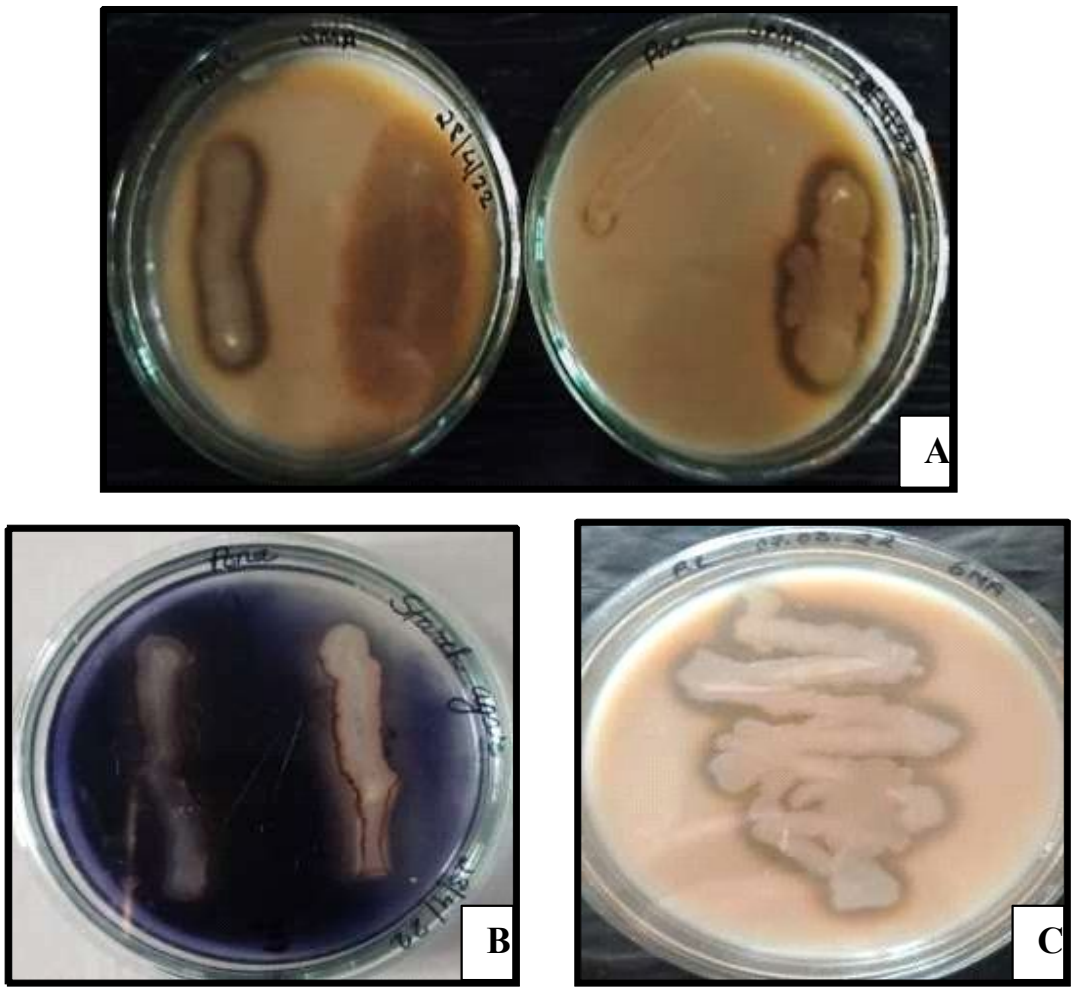

Fig. 2: (A-C) Screening of bacterial isolates:(A) Qualitative screening of isolates for proteolytic activity on Skimmed Milk Agar plates, (B) Qualitative screening of the isolates for amylolytic activity on Starch Agar plates, (C) Formation of clear zone around the bacterial colony on Skimmed Milk Agar.

glucose-containing molecules (Gupta *et al*. 2003). *Bacillus* sp. is the best source of amylase producers among terrestrial bacteria (Dash *et al*. 2015). In the present study, an effort has been made to isolate bacteria from unique soil samples that can produce both protease and amylase. A dilution of the soil sample by 10^{-3} led to the isolation of a total of 7 bacterial colonies. The isolates were given codes: PLB1, PLB2, PLB3, PLB4, PLB5, PLB6, and PLB7. Screening for protease and amylase activities was performed in Skimmed Milk Agar and Starch Agar, respectively. Out of seven isolated bacteria from poultry waste soil, the isolate PLB3 was selected for further characterization and identification because it exhibited both amylolytic and proteolytic activities. The isolate formed off-white, smooth colonies in NAM plates. Microscopic examination of the isolate revealed gram-positive rod-shaped cells that developed endospores (Fig. 1 a,b,c).

The results of the isolated strain PLB3's morphological, cultural, and biochemical properties were summarized in Table 1 and Figure 2 (A-C)**.** The results of morphological and cultural characterization indicated that the isolate PLB3 belonged to the genus *Bacillus sp.* Among the biochemical tests performed, the isolate tested positive for the hydrolysis of casein, starch, gelatin, and lipid, presence of enzyme catalase, and utilisation of citrate while it was negative for the indole test. The isolate also tested positive for the production of ammonia. Ammonia in poultry litter is generated from uric acid degradation by microorganisms and its production depends mainly on the enzyme uricase (Kin and Patterson, 2003). Based on the results of the aforementioned biochemical tests, it was determined that the isolate in the present study confirms the characteristics to the genus *Bacillus* (Table 1). The genus level identification of this isolate was based on colony and cell morphology, several biochemical characteristics, and 16S rRNA sequencing confirms the taxonomic designation of the isolate PLB3 as *Bacillus toyonensis*. The identification of isolates was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. The NCBI gene bank's nucleotide BLAST algorithm revealed a 99.93% similarity to *Bacillus toyonensis* BCT-7112(T). The bacterial culture was deposited at NCCS, Pune with accession number CP006863.

Numerous investigations have shown that *Bacillus* sp. is the most effective source of microbial amylase and protease with a significant amount of enzyme activity (Hussey and Zayaitz, 2007; Ravi *et al.,* 2015; Luang *et al.* 2019). Considering the efficacy and applicability of the protease and amylase produced by *Bacillus sp.*, as reported by numerous researchers, it is hypothesized that the *Bacillus toyonensis* identified in the present investigation has comparable properties. Quantitative assays, activity analysis of the aforementioned enzymes, and the optimization of different growth parameters for the synthesis of enzyme (temperature, pH, nutrient source, etc.) from *Bacillus toyonensis* can give a clear picture of potential of this species.

CONCLUSION

In this study, bacteria were successfully isolated from unique soil samples (poultry waste dumping site), with an emphasis on identifying strains capable of producing both protease and amylase enzymes. A total of seven bacterial colonies were isolated using a dilution technique, among which the strain PLB3 demonstrated both amylolytic and proteolytic activities. The isolate PLB3 exhibited morphological and biochemical characteristics typical of the *Bacillus* genus, such as gram-positive rod-shaped cells with endospore formation. Through various biochemical tests, PLB3 was found to hydrolyze casein, starch, gelatin, and lipid, while also producing catalase, ammonia, and utilizing citrate, but testing negative for indole production. The isolate was further identified at the genus level as *Bacillus*, and 16S rRNA sequencing confirmed its taxonomic designation as *Bacillus toyonensis*. This dual enzyme-producing strain holds potential for biotechnological applications in industries requiring both protease and amylase enzymes, such as in food processing, pharmaceuticals, and bio-waste management.

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DECLARATION

Conflict of interest. Authors declare no conflict of interest to declare.

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