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ORIGINAL ARTICLE

Isolation, Characterization, and Optimization of Protease-Producing Bacterium *Bacillus thuringiensis* from Paddy Field Soil

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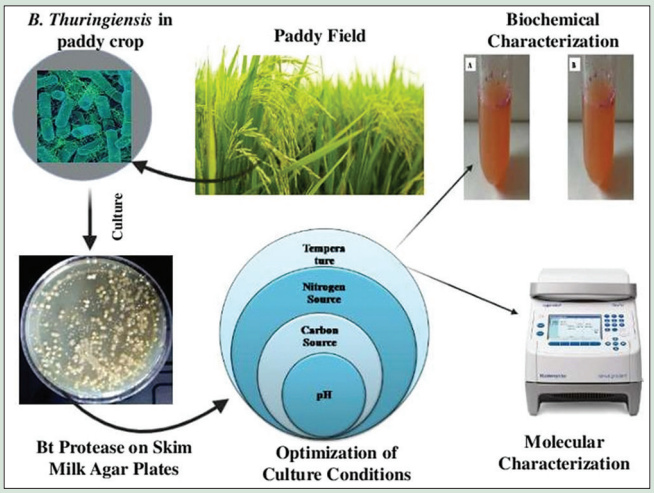
ABSTRACT

Background: The ubiquitous proteases that are commonly found in all living organisms play an important role in cell growth and cell differentiation. The bacterium *Bacillus thuringiensis* (*Bt*) produces delta-endotoxins that exhibit toxic properties against various insecticides and has demonstrated its potency and safety as a biopesticide agent for decades. The *Bt* protein includes vegetative, insecticidal, and crystal proteins that exhibit highly toxicants against immature insects (larvae). **Objectives:** The aim of this research was to use *Bt* as an alternative to chemical insecticides, and the source of *Bt* genes aids in the development of a resistant transgenic plant that improves not only productivity but also shift life. **Materials and Methods:** In the present study, bacterium *Bt* was isolated from various paddy files around the Hunsur region, Karnataka. The isolated bacteria show a potent protease activity on skim agar plates. Morphology, colony assay, and biochemical characterization were performed for the characteristic properties of bacteria. Further, 16S ribosomal RNA partial sequencing was carried out to identify the specific species of *Bacillus*. **Results:** Among nine samples from different paddy soils, three *Bacillus* isolates SAL-P1, SAL-P2, and SAL-P3 are the major dominant colonies which were streaked onto the fresh skim milk agar plates, out of which SAL-PI shows an abundant growth and production of an enzyme at pH 7.0, 37°C, and 48 h, respectively. The study also shows the optimum condition of temperature, carbon, nitrogen source, pH for growth, as well as for biomass production. **Conclusion:** The results of this study confirm the significance of continuous exploration of new *Bt* strains from different ecological regions that could be more useful for *Bt*-based bioformulations and the generation of transgenic plants. Furthermore, the growth and biomass production of *Bt*g (isolated from paddy soil) and *Bt*i (reference strain) were found to be identical.

Key words: 16S ribosomal RNA, *Bacillus thuringiensis*, biochemical identification, isolation, optimization, paddy soil

SUMMARY

- Screening of samples from various habitats may be useful to obtain *Bacillus thuringiensis (Bt)* strains with wide host ranges and novel crystal proteins
- Morphology, colony assay, and biochemical characterization were performed for the selected bacterial strains to determine the characteristic properties of the bacteria
- Further, 16S ribosomal RNA partial sequencing was carried out to identify the specific species of *Bt* bacteria
- In this study, the optimal pH, temperature, and incubation time for growth and biomass production were also specified.



Abbreviations Used: *Bt: Bacillus thuringiensis*; 16SrRNA: 16S ribosomal RNA; pH: Potential of hydrogen; B.P: Base pair: DNA: Deoxyribonucleic acid; GIS: Geographic information system; $HgCl_2$: Mercury (II) chloride; kb: Kilo base; Mb: Mega base; MR: Methyl red; PCR: Polymerase chain reaction; sp.: Species; VP; Voges-Proskauer.

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INTRODUCTION

Plant pests and diseases affect 20%–40% of food production globally.[1] Inadequate use of chemical pesticides to control pests has increased selection pressure, resulting in insect resistance and affecting soil fertility.[2] To resolve these issues, a new form of pest control is desperately needed. Most of the micro-organisms are capable of surviving in almost all sorts of environments.[3] In the present scenario, development of diseases, resistance varieties, and chemical pesticide resistance are a few major hurdles for insect pest management. Since most of the microbes are capable of developing potential insecticidal properties that act differently

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from known chemicals, this leads insects to develop the least chances of resistance.[4] Over the past two decades, biopesticides provide 1% of total plant protection worldwide; about 175 biopesticide compounds and 700 biopesticide products have already existed in the market globally.[5] With various bioproducts, *Bacillus thuringiensis* (*Bt*) has been used as an important plant protectant. The uniqueness of this *Bt* is that they produce epizootic which has the capability of controlling the natural phenomenon of some insect pests.[6,7] This *Bt* is a rod-shaped, motile, Gram-positive, facultative aerobic bacterium with endospores.[8] The size of the *Bt* also varies from 1 μm width to 5 μm in length. However, the genome size ranges from 2.4 to 5.7 base pair.[9] These bacteria can produce a variety of insecticidal toxic parasporal proteins, but not all strains are effective against insect pests. These toxicants primarily affect Lepidoptera (pigeon pea, cotton bollworm, rice yellow stem borer, etc.), Diptera (*Drosophila melanogaster*), *Coleoptera* (bruchids of various pulse grains), and other organisms.[10] About 50,000 *Bt* strains have been isolated from different environments. It has been reported that these *Bt* strains are found in various habitats such as grains, olive tree-related habitats, soil, various plants, aquatic environments, and stored product dust and also in agricultural soil.[11] The diversity of various *Bt* and their genes from different locality across India has been studied earlier. Various researches have been carried out on the identification and characterization of local *Bt* strains from soil to develop a novel toxicant against agriculture pests.[12] *Bt* cotton, *Bt* corn, and *Bt* soya been are few *Bt* transgenic crops which are already available in the market.[13] Based on these reports, novel *Bt* genes were identified by various techniques. The studies suggest that the *Bacillus cereus* species have similar features with *Bt* species due to their biochemical and genomic compositions.[14] In this study, we isolated *Bt* bacterium from a local paddy field in the Hunsur region, Karnataka. After isolation, various strategies were followed to identify novel *Bt* strains which include partial sequencing, use of degenerate primers, polymerase chain reaction (PCR) amplification, development of DNA library, biochemical characterization, and optimization techniques used to develop even environmental factors such as pH, sunlight, and rainfall; the temperature may also affect the activity of *Bt* and biomass yield.[15] Out of these factors, temperature and pH play an important role in a successful fermentation reaction. In the current study, we isolated a local *Bacillus* strain from paddy field soils and examine novel *Bt* gene (s) used PCR amplification and biochemical morphological approaches.[16] However, PCR amplification and sequencing of the amplicon are the most common and also widely used techniques to identify novel *Bt* genes.[17]

MATERIALS AND METHODS

Sampling and site selection

Soil samples were collected from nine different paddy fields around Hunsur Taluk, Mysore district, Karnataka. About 25 g of surface soil (after removed 2 cm of soil surface) was collected in a sterile cup. The obtained samples were then shifted to the laboratory and were processed within 2 h of collection. The sources of collected samples were not sprayed with any of the *Bt* biopesticides before. The geographic information system of different sampling locations is presented in Table 1. About 1 g of collected soil samples was dissolved in 10 ml of sterile distilled water. To remove unwanted vegetative cells, the samples were heat treated with 80°C for about 10 min.[18] For serial dilution, each sample was carried out separately and the aliquots of each dilution were spread on skim milk agar medium and this colony is shown in Figure 1. The inoculated media plates were incubated at 37°C for about 48 h for the production of enzymes. After 48 h, 5 ml of 10% $HgCl_2$ was poured into the Petri plates and the colonies with the highest clearance indicated protease activity and were subjected to Gram staining.[16]

Isolation identification

Gram staining

The smears of the isolated were made on a clean glass slide. The smear was air-dried and heat-fixed. The slide was held using a slide rack with the smear on it and was covered with crystal violet stain for about 30 s. The slide was then washed with distilled water under slow flowing tap water, then treated with Gram's iodine for 1 minute before being washed with distilled water. To decolorize smear, 95% ethanol was added and incubated for about 20 s and was washed with distilled water.[19] Finally, safranin was added and incubated for about 30 s, and the slide was rinsed with distilled water and observed under ×100 high and oil immersion objectives.

Spore staining

From the SLP-1 sample, the smear was prepared as described previously.[20] The fixed smear slides were stained with Malachite Green. Then, the slide was steamed for about 5 min and washed with sterile water. The rinsed slide was then counterstained with safranin for about 30 s. After incubation, the slides were rinsed and observed under high and oil immersion objectives and the slide was shown in Figure 2.

Culture characterization

Three dominant colonies named SAL-P1, SAL-P2, and SAL-P3 colonies were taken and single streaked onto the fresh skim milk agar plates to screen for the most dominant protease producer.[21] Among those, the selected SPL-1 isolate was plated onto the surface of *Bt* chromogenic agar and incubated for about 24 h at 30°C is shown in Figure 3. Then, the plates were subjected for growth, and colony morphology, size, color, and texture were shown in Table 2.

Biochemical identification

Indole production test

1% tryptone broth was prepared and 10 ml of it was dispensed into two test tubes, and both the tubes were sterilized in an autoclave at 12°C for about 15 min. After sterilization, one of the tubes was inoculated with isolated culture and other tubes were used as a control; both the tubes were then incubated at 37°C for about 48 h. After incubation, 1 ml of Kovac's reagent was directly added to both the tubes. Then, the tubes were gently shaken for about 15 min. The tubes were allowed to stand until a cherry-red color develops at the surface of the tube which gives a positive test for indole production [Figure 4 and Table 3].[22]

Methyl red and Voges-Proskauer test

About 5 ml of methyl red and Voges-Proskauer (MR-VP) broth was dispensed into clean five test tubes. The tubes were autoclaved at 121°C for about 15 min. After sterilization, four tubes were inoculated with the isolated culture and one test tube was used as control. The tubes were then incubated at 37°C for about 48 h after incubation MR indicator (5 drops) was added to each tube. The change in color of MR for the MR test was observed and noted. For the VP test, VP reagent I (12 drops) and VP reagent II (2–3 drops) were added to each tube as described previously.[23] The tubes were then gently shaken for about 30 s. The reaction was allowed for about 15 min, and the test tube was observed for change in color of the VP test [Figure 5 and Table 3].

Citrate utilization test

Using sterile technique, Simmons citrate agar medium was prepared and was poured into two tubes and they were autoclaved. One tube was inoculated with the isolated culture, while the other tube was used as control. The tubes were incubated at 37°C for about 48 h. Then, slant was

Table 1: Geographic information system locations of the collected soil samples from different locations of paddy fields

Soil numbers	Location	GIS
1	Aspathre kaval	
2	Bannikuppe	
3	Dasanapura	
4	Gowdikere	
5	Hanchya	
6	Heggandhuru	
7	Harave	
8	Manuganahalli	
9	Yavagumba	

GIS: Geographic information system

observed for the presence or absence of growth, and coloration of the medium was recorded [Figure 6 and Table 3].[24]

Starch hydrolysis test

The starch agar medium was prepared and was autoclaved at 121°C for about 15 min. After sterilization, the medium was poured into the sterile Petri plates under sterile conditions and allowed to solidify. The isolate culture was inoculated onto the Petri plates and was incubated at 37°C for about 24–48 h. After incubation, for about 30 min, the surface of the agar was flooded with Gram's iodine solution. The iodine solution was poured off, and the zone of clearance was observed [Figure 7 and Table 3].[25]

Catalase test

The nutrient agar medium was prepared and poured into culture tubes; the tubes were autoclaved at 121°C for 15 min. The culture tubes were inoculated with isolate culture and were incubated at 37°C for about 48 h. Inoculums of the culture were taken on a clean glass slide, and 3–4 drops of H_2O_2 was added. The slides were observed for the appearance of the gas bubbles [Figure 8 and Table 3].[26-27]

Genomic DNA isolation

DNA isolation

Isolation of DNA was performed by Rebecca *et al.* (2011) method.[18] The selected *Bt* strain was cultured and incubated at 37°C for 48 h. From the culture plate, the cells were transferred into 0.2 ml of sterile distilled water and mixed. Then, the samples were frozen at −80°C for about 20 min; after incubation, they were transferred into boiling water for about 10 min. Then, the cell lysate was centrifuged at 10,000 rpm for 10 s and the obtained supernatant (15 µL) was used as a DNA template in PCR analysis.

16S rDNA gene sequencing

The preliminary screening of *Bt* strain by 16S rDNA was done using universal primers which are parallel to the phylogenetically conserved position with 5'–3' ends. Primers, 20F 5'-GAGTTTGATCCTGGCTCAG-3' (position 9-27), and 1500R 5'-GTTACCTTGTTACGACTT-3' (position1509-1492) were used for 16S rDNA gene sequencing. From the study of Haitao *et al.* (2011),[27] the amplification and sequencing study was carried out. By applying the biosystem model of 3130 DNA sequence and ABI prism sequencing kit, the sequencing result obtained was a purified PCR product which resolved by 3'primers (20F, 520F, and 920F). GenBank and BLAST program were used to compare the sequencing report.

Optimization of culture conditions

Optimization of carbon source

Mineral salt medium (MSM) is used to grow the screened isolates (g/L: KH_2PO_4,0.42;K_2HPO_4, 0.375; $(NH_4)_2SO_4$, 0.244; NaCl, 0.015; $CaCl_2.2H_2O$, 0.015; $MgSO_4.7H_2O$, 0.05; and $FeCl_3.6H_2O$, 0.054; pH 7); some are added with carbon sources such as 1% (w/v) lactose, glucose, maltose, and sucrose. Further inoculated tubers are incubated at 37°C for 48 h. After incubation, a protease movement test was completed to decide the most potential carbon substrate that incites the greatest for the production of protease enzyme.[28]

Optimization of nitrogen source

MSM is used to grow the screened isolates (g/L: KH_2PO_4, 0.42; K_2HPO_4, 0.375; (NH_4) $2SO_4$, 0.244; NaCl, 0.015; $CaCl_2.2H_2O$, 0.015; $MgSO_4.7H_2O$, 0.05; and $FeCl_3.6H_2O$, 0.054; pH 7) supplemented with different nitrogen sources such as 1% (w/v)-peptone, beef extract, yeast extract, and sodium nitrate. The tubes were incubated at 37°C for 48 h. The most potential nitrogen substrates were checked for maximum protease enzyme productions, after the incubation of the protease activity.[29]

Optimization of media pH

MSM is used to grow the screened isolates (g/L: KH_2PO_4, 0.42; K_2HPO_4, 0.375; $(NH_4)_2SO_4$, 0.244; NaCl, 0.015; $CaCl_2.2H_2O$, 0.015; $MgSO_4.7H_2O$, 0.05; and $FeCl_3.6H_2O$, 0.054;) with different pH 4, 6, 7, and 9. The pH medium was adjusted using 1N HCl or 1N NaOH. The tubes were incubated at 37for 48 h. After incubation, a protease activity assay was carried out to determine the optimum pH that induces the maximum protease enzyme production.[30]

Optimization of media temperature

MSM is used to grow the screened isolates (g/L: KH_2PO_4, 0.42; K_2HPO_4, 0.375; $(NH_4)_2SO_4$, 0.244; NaCl, 0.015; $CaCl_2.2H_2O$, 0.015; $MgSO_4.7H_2O$, 0.05; and $FeCl_3.6H_2O$, 0.054; pH 7). About the temperatures of 30°C, 37°C, and 45°C, the tubes were incubated up to 48 h. After incubation, the activity of protease is checked to determine the optimum temperature for the maximum protease enzyme production.[31]

RESULTS AND DISCUSSION

Isolation of *Bacillus thuringiensis* from paddy soil

Nine samples from different paddy soils were collected. Sodium acetate selection methods were used for the isolation of *Bt*. Colonies on plates of nutrient agar that had similar colony morphology to *Bt* were selected as references (*Bti*).

Bacillus thuringiensis site selection

From the skim milk agar plates, three dominant colonies named SAL-P1, SAL-P2, and SAL-P3 colonies were taken and single streaked onto the fresh skim milk agar plates to screen for the most dominant protease producer. After 48 h of incubation at 37°C, there was abundant growth and production of the enzyme in only one plate. After the addition of $HgCl_2$, the zone of clearance was measured in millimeters.

The cultural characteristics of the suspected *Bt* isolates were examined. Generally, colonies were white-to-cream in color, tend to have a large frosted glass appearance, initially, but may become opaque. Some colonies were mucoid, while others were brittle. The isolates are Gram-positive, spore formers, and motile. The spore is found in the center of the cell. The shape of the spores is ellipsoidal. All isolates produced crystal proteins with various forms and sizes are shown in Figure 3.

Various biochemical tests were performed to further characterize the strains.

Partial sequencing of 16S ribosomal RNA

Genomic DNA isolation

The genomic DNA was isolated from the cells of the isolate. It was loaded onto 1% agarose gel and a DNA band was observed. The obtained band is shown in Figure 9.

Polymerase chain reaction amplification using universal 16S rDNA primers

The specific sequence of 16SrDNA was amplified using sequence-specific forward and reverse primers, and the PCR product thus obtained was observed in 1% agarose gel. 1.5 Kb DNA band was separated on the agarose gel as shown in Figure 10. The obtained PCR-amplified results were sequenced and compared with the 16S rRNA gene sequences which were submitted in the GenBank Database using the phylogenetic analysis method. The isolated gene sequence-16S rRNA of SAL-P1 showed 99% similarity with sequences of *Bt* and identified as *Bt* strain [Figure 11].

Optimization of culture conditions

Optimization of carbon source

Among the various carbon sources used, protease production was highest in the medium containing lactose (0.36 U/mL), followed by glucose (0.34 U/mL). The use of maltose and sucrose as the carbon

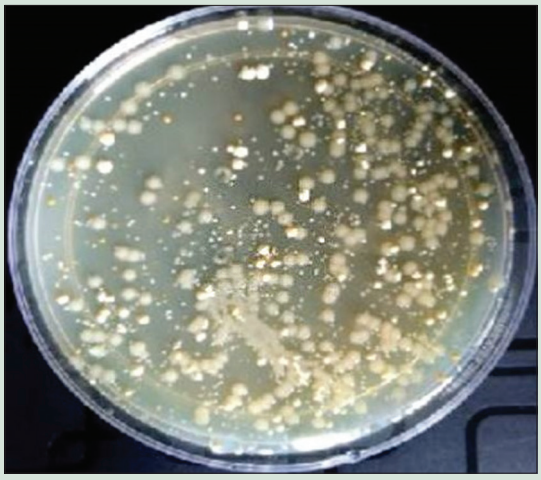


Figure 1: Protease-producing bacteria isolated on skim milk agar plates

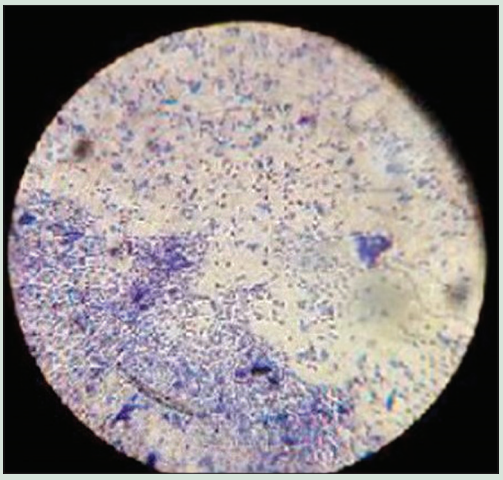


Figure 2: Gram-positive rod-shaped bacteria

Figure 3: SAL-P1 isolate shows zone of clearance

Figure 4: Negative test for indole production

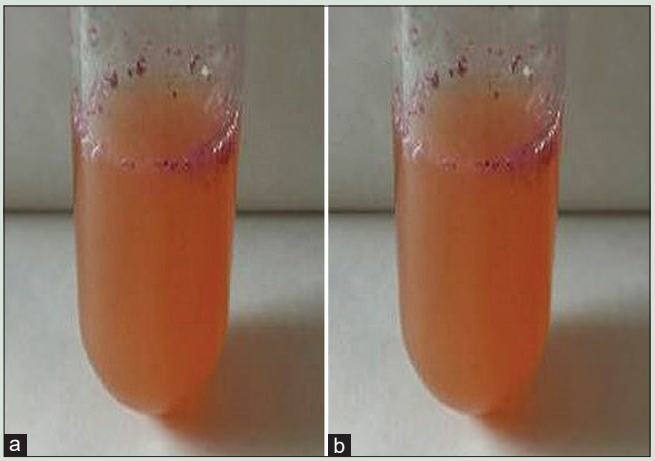


Figure 5: Bacterial isolate showed a positive result for methyl red test (a) while negative results for Voges-Proskauer test (b)

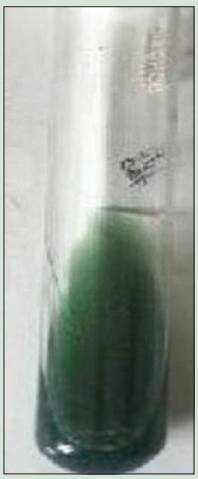


Figure 6: Negative citrate utilization test for *Bacillus thuriengeinsis* isolate

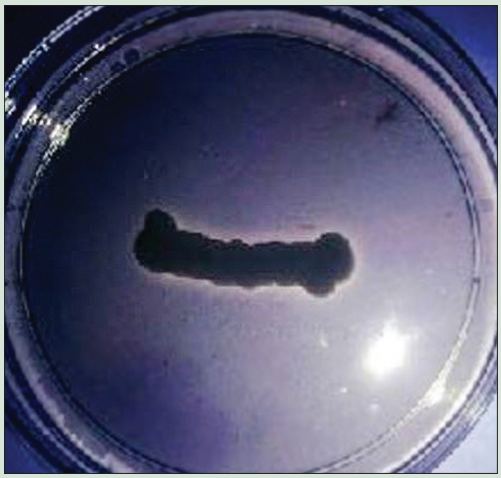


Figure 7: Positive for starch hydrolysis test

Figure 8: Positive result for catalase test

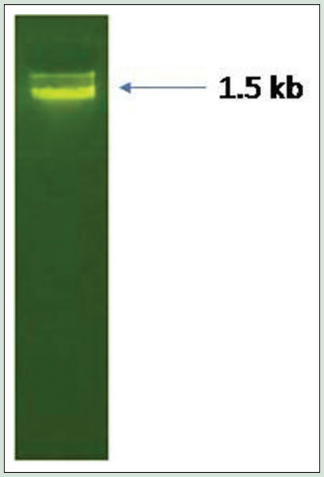


Figure 9: Genomic DNA Loaded on 1% agarose gel electrophoresis

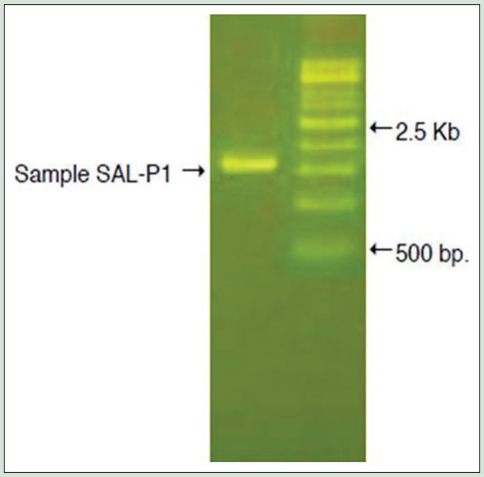


Figure 10: PCR product load on 1% agarose gel

sources yielded almost similar protease production, 0.27 and 0.26 U/mL, respectively. Lactose, glucose, maltose, and sucrose were found to be stimulatory carbon sources, with lactose showing the best results, as seen in Graph 1.

Optimization of nitrogen source

Among the various nitrogen sources used, protease production was highest in the medium containing yeast extract (0.33 U/mL), followed by peptone (0.254 U/mL). Beef extract as a nitrogen source showed a moderate enzyme production (0.17 U/mL). When sodium nitrate was used as a nitrogen source, it produced the least amount of enzyme activity (0.12 U/mL), indicating that inorganic nitrogen sources are inferior to organic nitrogen sources in protease production. The combination of yeast extract, peptone, beef extract, and sodium nitrate among all these yeast extracts showed the best nitrogen source as shown in Graph 2. We also discovered that replacing yeast extract with peptone or casamino acids instead of casein had a positive impact on crystal development.[29]

Table 2: Colony characterization of SAL-P1

Characterization of Bacillus colony isolates from SAL P1	Result
Shape	Round
Elevation	Flat
Margin	Entire
Surface	Smooth
Color	White
Degree of growth	Moderate

Table 3: Biochemical identifications: Various biochemical tests were performed to further characterize the strains

Biochemical test	Result
Indole production test	Negative
Methyl red test	Positive
Voges-Proskauer test	Negative
Citrate utilization test	Negative
Starch utilization test	Positive
Catalase test	Positive

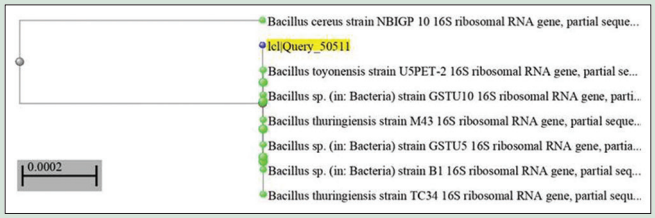
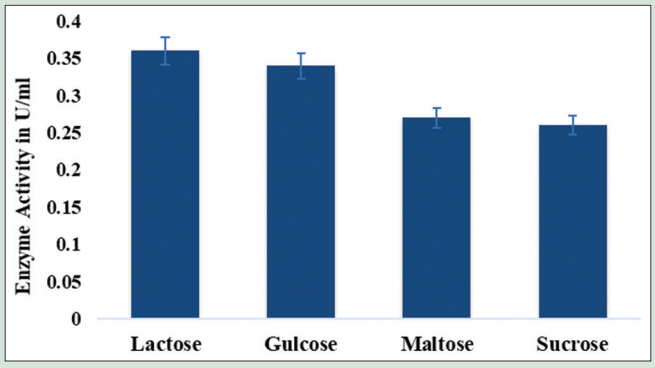


Figure 11: Phylogenetic analysis of SAL-P1 based on 16S ribosomal RNA gene sequence



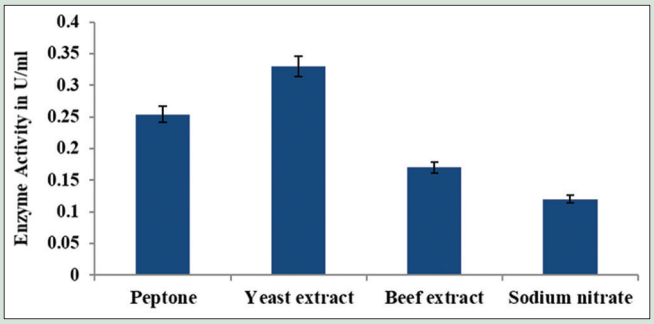
Graph 1: Effect of various carbon source on protease production in *Bacillus thuringiensis*

Optimization of media pH

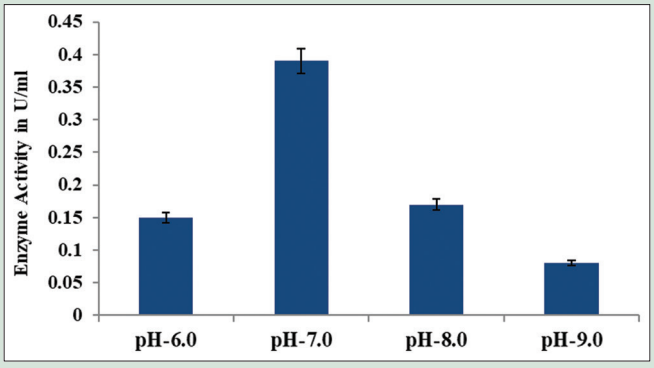
Among the various physical parameters, the pH of the media plays an important role in inducing the production of protease. The bacteria could produce an enzyme at a wide range of pH 6–9. The isolate was tested for enzyme production at pH 6, 7, 8, and 9. It was discovered that at pH 7–0.39 U/ml, the most enzyme was released. From the results obtained, the protease production increased with the increase in pH from acidic to neutral but decreased at alkaline pH. Hence, it can be observed that the optimum media pH for the isolated organism was found to be pH 7. The data observed were in agreement with the supporting literature that *Bt* was a neutral protease and that maximum production at that pH 7.0 is shown in Graph 3. The pH profile also indicates that acid or alkaline protease did not constitute a significant part of the total protease activity.

Optimization of media temperature

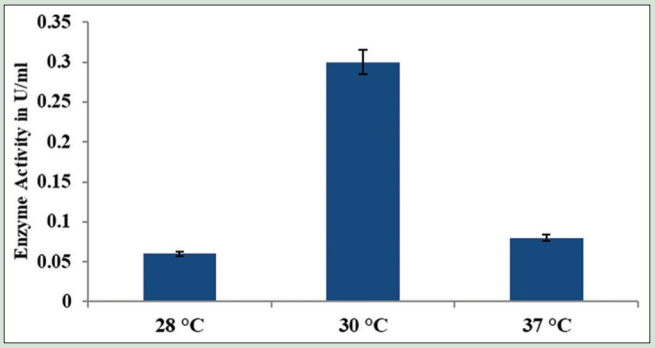
Temperature is the most important factor that governs the enzyme activity. Generally, the enzyme activity increases with an increase in temperature, and at the optimum temperature, the maximum enzyme activity is achieved. Further increase in temperature above the optimum temperature may decrease the enzyme activity because of denaturation of proteins. *Bt* strain was incubated at different temperatures 28°C, 30°C, and 37°C; it was found that the maximum protease production occurred at 30°C showing enzyme production of 0.3U/ml as shown in Graph 4.



Graph 2: Effect of various nitrogen sources on protease production in *Bacillus thuriengeinsis*



Graph 3: Effect of various pH on protease production in *Bacillus thuriengeinsis*



Graph 4: Effect of various temperatures on protease production in *Bacillus thuriengeinsis*

CONCLUSION

Proteases are hydrolytic enzymes that act on proteins and degrade them into peptides or amino acids. Apart from plant and animal sources, micro-organisms such as bacteria, fungi, and some viruses also produce different types of proteases. Among bacterial species, the organisms belonging to the genus *Bacillus* are the most potent producers of proteases. They mainly produce neutral and alkaline proteases, other than acidic, serine, and metalloproteases. In the present study, morphology, colony assay, and biochemical characterization were performed for the selected bacterial strain to determine the characteristic properties of bacteria. The result showed that the isolate belongs to the genus *Bacillus*. Further 16SrRNA partial sequencing was carried out to identify the specific species of *Bacillus*. In conclusion, this is the first study to isolate and classify *Bt* from paddy field soil, with a thorough study of these novel genes, including isolation of the full-length version of these novel genes and efficacy of these genes against insect pests, providing valuable knowledge for those working on bioinsecticides and transgenic crop production using *Bt* proteins, to reach a new insecticidal protein with greater efficacy, specificity, and wider host range that will also be harmless to non-target insects.

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Conflicts of interest

There are no conflicts of interest.

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