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Isolation and Characterization of *Bacillus thuringiensis* from Crop soil and Evaluation of toxicity on *Bombyx mori*

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Abstract: *Bacillus thuringiensis* (Bt) is a widespread bacterium known for producing multiple proteins with toxicity against various invertebrates, including insects, nematodes, mites, and certain protozoans. The potential of Bt to be used as an insecticide was recognized in the early twentieth century and since that time many Bt-based biopesticides have been commercialized. Our research focused on soil samples from various agricultural fields in Erode, covering crops such as paddy, mango, maize, groundnut, sweet potato, turmeric, banana, chilly, green leaf, and sugar cane.The samples were inoculated on T3 agar plates, resulting in the formation of colonies. T3 agar, functioning as a selective medium for Bt, facilitated the observation of colony morphology, which exhibited characteristics such as circular, white, flat, undulate, or entire. The research revealed that all organisms under study demonstrated the production of spherical-shaped spores and crystals also. Bt, identified as a Gram-positive, rod-shaped bacterium, was found to be prevalent in soil. The strain of Bt was isolated from soil, efficacy was tested against silkworms, *Bombyx mori*. The mortality of Bt against silkworms after 3 days was 70%, respectively. Insecticidal crystal protein profile of Bt produced 97, 68, 43, 29, 20 and 14 kDa bands. The results clearly established the potent insecticidal properties of the Bt strain, suggesting its potential application in disease control through the targeting of insect vectors.

Keywords: **Crop soil, *Bacillus thuringiensis*, Characterization, Insecticidal activity, Silkworm**

1. Introduction

Bacillus thuringiensis, the insecticidal bacterium, stands as one of the most effectively commercialized biocontrol agents and is presently employed in numerous countries (Allwin et al., 2007). *B. thuringiensis* is a versatile pathogen capable of infecting protozoa, nematodes, flatworms, mites and insects that are either plant pests or human and animal health hazards (Feitelson, 1993). *B. thuringiensis* has been sourced from various environments such as soil, the phyllosphere, diseased insects, stored products, dumping pits, and the excreta of vegetarian animals. According to studies by Martin and Travers (1989) and Boucias and Pendland (1998), approximately 30-100% of spore formers in the phyllosphere were identified as B. thuringiensis. An analysis of 27,000 isolates collected from, 100 soil samples all over the world demonstrated that *B. thuringiensis* could be isolated everywhere, including desert, beach and tundra habitats (Atathom et al., 1995). *B. thuringiensis* occasionally exhibits toxicity exclusively towards specific orders of insect species, and even within a particular order, such as Lepidoptera, significant variations in sensitivity are observed among species. For instance, strains HD1 of Bt-based bioinsecticides prove challenging in controlling certain species like the beet and fall armyworm (Spodoptera sp.), while they are effective against others such as the tobacco budworm (*Heliothis virescens Fab*) and the diamond black moth (*Plutella xylostella L.*) (Schnepf et al., 1998).

The efficacy of *B. thuringiensis* varies among different insect species. Beegle and Yamamoto (1992) observed that *B. thuringiensis* is not highly efficient against *S. frugiperda*. In contrast, it demonstrates effectiveness against Trichoplusia ni (Hueb.) and *H. virescens*. Aronson et al. (1991) conducted a study emphasizing the importance of crystal solubility as a crucial factor influencing the insecticidal efficiency of *B. thuringiensis*. They suggested that the low susceptibility of *S. frugiperda* to *B. thuringiensis* may be attributed to this factor. *B. thuringiensis* is the most popular mosquitocidal bacteria, which has high larvicidal activity and is used for decades against mosquito larvae as one of the most powerful biocides (Roy et al., 2021).

B. thuringiensis is a Gram-positive, aerobic, facultative anaerobic, endospore-forming bacterium and is distinguished from other Bacillus spp. by its ability to produce indigenous crystals during sporulation. Crystal proteins from many *B. thuringiensis* strains are toxic to lepidopteran pests (Zhong et al. 2000). Broadly, Bt strains exhibit specificity towards particular species or hosts, often characterized by a narrow host range. Crystal proteins such as Cry4A, Cry4B, Cry4D, Cry10A, and Cry11A in *B. thuringiensis* are known for their insecticidal activity primarily against mosquitoes and blackflies. Additionally, a distinct dipteran toxin, CytA (27

kDa), interacts with membrane lipids, binding synergistically with Cry toxin to amplify its toxicity (Poopathi and Tyagi, 2006; Federici, 2007; Becker et al., 2010).

The raising of the domesticated silkworm, *Bombyx mori L.* is a popular agroforestry option in India. Sericulture can be practiced even in the most modest circumstances to provide a higher-value silk fibre with a ready world market, as also other useful by-products, such as high-protein animal feed and industrial oils. Recent trend of using insect pathogens particularly *B. thuringiensis* one of the entomobacterial pathogens, for the control of almost all lepidopterous crop pests is encouraging but fraught with risk to silkworm (Jayanthi and Padmavatham, 1997).

This current investigation aimed to isolate and identify *B. thuringiensis* strains from diverse crop soils, including paddy, mango, maize, groundnut, sweet potato, turmeric, banana, chilli, green vegetable, and sugarcane. Samples were collected from various locations in Erode, Tamil Nadu, India. The focus was on characterizing polymorphic crystal-producing strains, with the potential for future exploitation in the biological control of a broad spectrum of insect pests and disease vectors.

2. Materials and Methods

2.1. Sample Collection

Agricultural crop soil was collected from Erode in ten different fields like Paddy, Mango, Maize, Groundnut, Sweet Potato, Turmeric, banana, chilli, green leafy vegetables and sugar cane by random selection method. From each field, 10g of soil was collected from five spots. Samples were mixed thoroughly and put in polythene packets.

2.2. Isolation of *Bacillus thuriengiensis* from soil sample

Soil samples (0.5gm) were incubated in a water bath shaker for 4 hours at 30° C along with 10 ml of luria broth in 0.25M sodium acetate. For 100 ml preparation, 50 ml of LB2x, 12.5ml sodium acetate (2M) and 37.5ml of distilled water were taken. To that 2M concentration of sodium acetate can be added. From the samples, 2ml solution was taken and heat shocked in a water bath 60°C for an hour. The luria broth contains tryptone-1gm; sodium chloride-1gm; yeast extract-0.5gm; distilled water-100ml. The sodium acetate was prepared by dissolving 1.951g in 50ml of distilled water.Then the samples were diluted with phosphate buffer saline and spreaded on Is agar plates. Plates were incubated at 26°C for 2 days and the colony morphology was observed by using colony counters. Selective Medium used (Traver's et al., 1987) Is Agar Medium (Tryptone -3gm, Tryptose -2gm, Yeast extract-1.5gm, Sodium phosphate -0.05gm, Manganese chloride-0.005gm, Agar-15gm, Distilled Water-1000ml, pH-6.8). Crystal producing colonies were isolated confirming presence of crystal inclusions under objective of a phase contrast microscope. Isolated bacteria were purified and confirmed for spore and crystal formation under the microscope, slants and stabs of pure cultures were prepared and incubated for 72h at 30 ± 1◦C temperature. Each bacterial isolate was grown on a rotary shaker for 72h to attain about O.D. at 620nm i.e., about culture and concentrated up to 10×102 spores of each isolate for bioassay.

2.3. Colonial characterization

The colony shape, size, colour and opacity were recorded with reference to (Lacey et al., 1997). Antibiotics viz. Erythromycin, Ampicillin, Penicillin, Vancomycin and Bacitracin sensitivity of the organisms were also recorded. Diameter of the inhibition zone of sensitive bacteria was measured with an antibiotic zone scale.

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2.4. Insecticidal activity

Silkworms were obtained from silkworm rearing centres. For analysis of toxicity assay 10 numbers of silkworms were used for a single isolate. Three mi of old sporulated cultures contain 10×102 spores/ml of each isolate which was sprayed on mulberry leaves. The larvae were fed on this contaminated leaf. The test was done in duplicate and mortality was examined daily for 3 days at 25°C. The isolates that are killed >80% of insect tested were assigned to a toxic strain.

2.4. SDS Page

One gram of each *B. thuringiensis* isolate from freshly prepared slants were extracted with one millilitre of 0. 1M sodium phosphate buffer (pH 7.0) at 4°C. The homogenates were centrifuged for 20 minutes at 10,000 pm, and the supernatant was used for the SDS-PAGE. The protein content of the sample was determined (Bradford,1976).100 microgram of protein from different treatments was taken and mixed with 10 microlitre of sample buffer in a microfuge tube, boiled for 4 minutes and incubated at 4°C for 30 min. The samples containing equal amounts of proteins were loaded into the wells of polyacrylamide gels. The medium range molecular weight markers were used, and electrophoresis was carried out at constant voltage of 75 volts for 2 hours. The gels were stained with 0.2% Coomassie brilliant blue solution.

2.5. Statistical analysis

The analyses were conducted using SPSS software version 16.0. Probit analysis, as outlined by Finney in 1971, was employed to scrutinize insecticidal tests and the associated data.

3. Results and Discussion

3.1. Isolation, Morphology and biochemical properties of *Bacillus thuringiensis*

Soil samples from various agricultural fields, including paddy, mango, maize, groundnut, sweet potato, turmeric, banana, chili, leafy greens, and sugarcane, were gathered in Erode. Subsequently, each soil sample was cultivated on both nutrient agar and T3 agar, as depicted in Figures 1 and 2. The sample 1 (paddy) which produces 196.5×10-2 on nutrient agar and 29.5×10 2 cfu/gm in T3 agar. The sample 2 (mango) which produces 241.5×10-2 on nutrient agar and 42.0 ×102 cfu/gm in T3 agar. The sample 3 (maize) produces 177.0×10-2 on nutrient agar and 37.5×102 CFU / gm in T3 agar. The sample 4 (groundnut) produces 220.0×10-2 on nutrient agar and 44.0×102 CFU /gm in Is agar. The sample 5 (sweet potato) produces 189.0×10-2 on nutrient agar and 39.5×102 CFU / gm in T3 agar. The sample 6 (turmeric) produces 238.0×10-2 on nutrient agar and 34.5×102cfu/gm in Ts agar. The sample 7 (Banana) produces 250.0×10-2 on nutrient agar and 80.034.5×102 CFU / gm in T3 agar. The sample8 (chilly) produces 252.5×10-2 on nutrient agar and 40.0×102 CFU / gm in T3 agar. The sample 9 (Green leaf) produces 228.5x10-2 on nutrient agar and 35.0×102 CFU/gm in T3 agar. The sample l0 (Sugarcane) produces 249.0× 102 on nutrient agar and 63.5×102 CFU / gm in T3 agar. Figure 3 displays the colony morphology observed on T3 Agar.

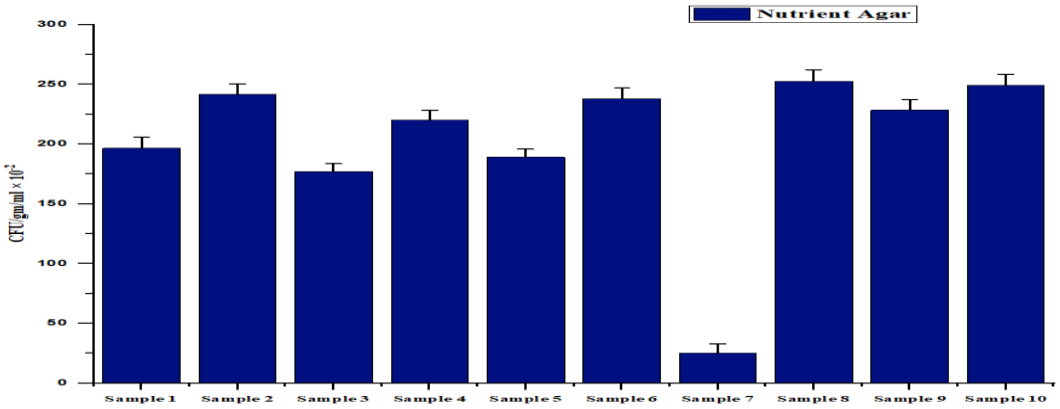

Figure 1: Numbers of colonies formed on Nutrient agar.

Sample 1 - paddy field, Sample 2 - mango field, Sample 3 - Maize, Sample 4 - groundnut, Sample 5 - sweet potato, Sample 6 - turmeric, Sample 7- banana, Sample 8 - chilly, Sample 9- greeny leaf and Sample 10- Sugarcane.

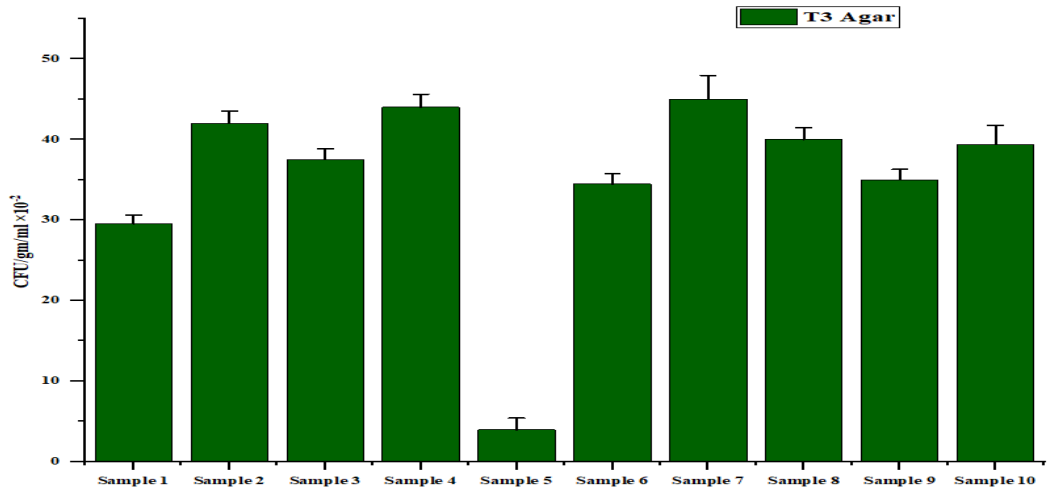

Figure 2: Numbers of colonies formed on T3 agar.

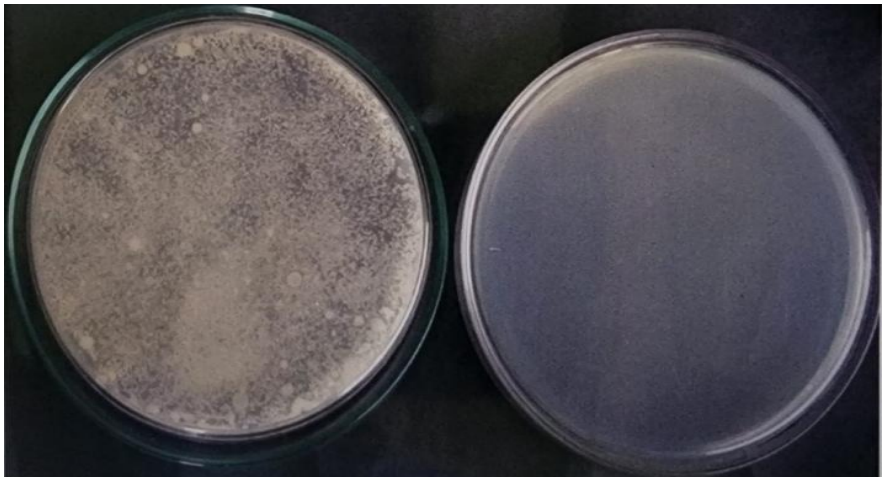

Figure 3: Colonies of *Bacillus thuringiensis* on T3 agar

Soil contains vastly diverse microbial communities. Generally, the genus Bacillus is recognized by being rod shaped. Gram-positive bacteria typically exhibit catalase production and can function as either aerobic or facultatively anaerobic organisms. They are commonly saprophytic and widely distributed, particularly in soil (Helgason et al., 2000). The observed colony characteristics include circular, undulate, or entire, as detailed in Table 2.

Table 2. Colony characters of *Bacillus thuringiensis* isolates on T3 agar plates

Samples	Form	Color	Elevation	Margin
Sample 1	Circular	White	Flat	Undulate
Sample 2	Circular	White	Flat	Entire
Sample 3	Circular	White	Flat	Undulate
Sample 4	Circular	White	Flat	Entire
Sample 5	Circular	White	Flat	Entire
Sample 6	Circular	White	Flat	Entire
Sample 7	Circular	White	Flat	Entire
Sample 8	Circular	White	Flat	Entire
Sample 9	Circular	White	Flat	Entire
Sample 10	Circular	White	Flat	Undulate

Soil sample size -100/ crop

The bacteria were cultured on nutrient agar at 27°C for a period of 3-4 days. Cultures that exhibited sporulation were examined under a microscope to observe morphological features such as spores and parasporal inclusions. The isolates displayed spherical crystal formations in terms of morphology. Microscopic observations of Gram staining and crystal staining, as depicted in Figure 4, revealed that these isolates were gram-positive rods with rounded ends and were non-motile. Table 3 presents the characterization of *B. thuringiensis* bacteria.

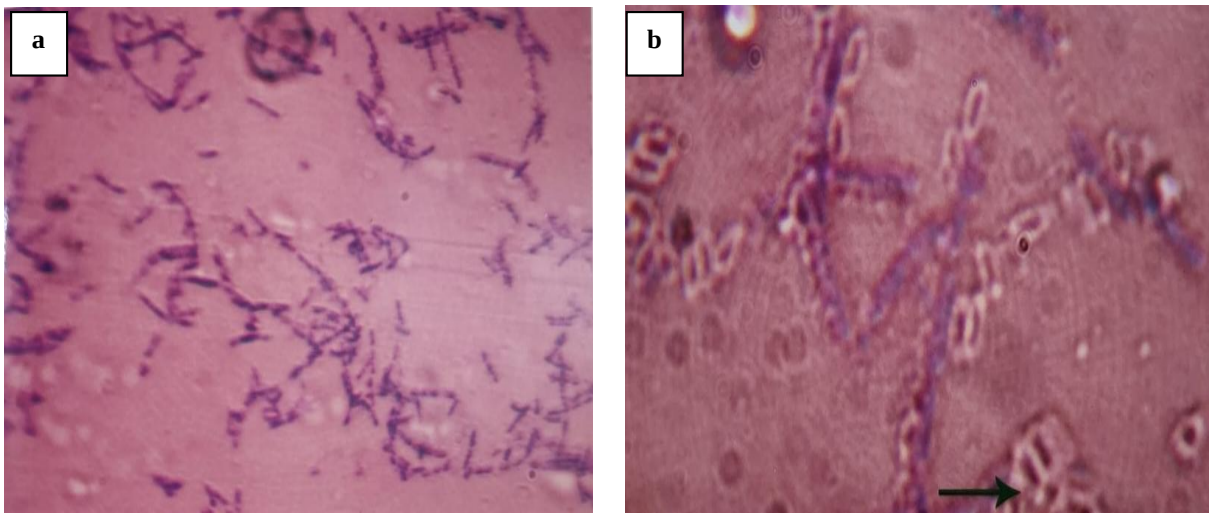

Figure 4: *Bacillus thuringiensi* (a) Gram staining and (b) Crystal staining

Table 3: Characterization of the *Bacillus thuringiensi* bacteria

***Bacillus thuringiensis* isolate**	**Gram Staining**	**Shape**	**Motility**
Sample 1	+	Rods	-
Sample 2	+	Rods	-
Sample 3	+	Rods	-
Sample 4	+	Rods	-
Sample 5	+	Rods	-
Sample 6	+	Rods	-
Sample 7	+	Rods	-
Sample 8	+	Rods	-
Sample 9	+	Rods	-
Sample 10	+	Rods	-

+ = Positive: - = Negative

The sensitivity assay was conducted, revealing that all isolates exhibited resistance to antibiotics Erythromycin, Ampicillin, Penicillin, and Vancomycin. Among the isolates, three showed intermediate sensitivity to Bacitracin, as detailed in Table 4. The resistance profile of *B. thuringiensis* (Bt) to Erythromycin, Ampicillin, Penicillin, and Vancomycin aligns with that of similar counterparts. This observation supports the general resistance of Bt to group of antibiotics (Roy *et al*., 2021).

Table 4: Antibiotic sensitivity of the isolate *Bacillus thuringiensis*

***Bacillus thuringiensis* isolate**	**Antibiotics**									
	Erythromycin		**Ampicillin**		**Penicillin**		**Vancomycin**		**Bacitracin**	
	Re	**Zf (mm)**	**Re**	**Zf (mm)**	**Re**	**Zf (mm)**	**Re**	**Zf (mm)**	**Re**	**Zf (mm)**
Sample 1	R	1.50	R	1.50	R	1.00	R	1.00	I	1.00
Sample 2	R	1.00	R	1.70	R	1.30	R	0.90	I	0.90
Sample 3	R	1.10	R	1.60	R	1.10	R	0.90	I	0.90
Sample 4	R	1.00	R	0.20	R	1.20	R	0.70	R	0.70
Sample 5	R	0.50	R	1.10	R	0.50	R	0.50	R	0.60
Sample 6	R	0.0	R	1.60	R	0.60	R	0.90	R	0.60
Sample 7	R	0.90	R	1.50	R	0.40	R	0.90	R	0.60
Sample 8	R	1.00	R	1.30	R	0.50	R	0.70	R	0.70
Sample 9	R	1.10	R	0.90	R	0.50	R	1.30	R	0.30
Sample 10	R	1.00	R	1.50	R	1.00	R	1.10	R	0.10

Re - Reaction; R - Resistance; S - Sensitive; I - Intermediate; Zf - Clear zone formation (mm)

The bacterial strains exhibited consistent phenotypic and biochemical properties. The presence of spherical, green-colored spores and crystals, as observed under microscopy, confirmed the strains as *B. thuringiensis*. The length and breadth of all isolates were determined through micrometry, as presented in Table 5. The identification of these organisms as *B. thuringiensis* was further confirmed by the production of crystals and spores, along with their growth in T3 medium, in accordance with established references (Sneath, 1986; Smibert and Krieg, 1994; Thiery and Frachon, 1997; Garrity, 2001). Although all isolates belonged to *Bacillus thuringiensis*, variations in reactions to different substrates might be attributed to their specific ecological niches. Nonetheless, the continual selection of newer strains through a genetic pool buildup is essential to effectively combat pests that co-evolve with biocontrol agents (McGaughey *et al*., 1992).

Table 5. Characters of spores and crystals of *Bacillus thuringiensis* isolates

Samples	Shape	Spores		Crystals		Spore and Crystal stain
		Length (μm)	Breath (μm	Length (μm)	Length (μm	
Sample 1	Spherical	1.0±0.03	0.25±0.01	2.00±0.14	1.50±0.08	Positive
Sample 2	Spherical	0.75±2.08	0.52±0.01	0.75±2.08	0.52±0.10	Positive
Sample 3	Spherical	3.20±0.03	2.60±0.25	2.20±0.17	1.67±0.03	Positive
Sample 4	Spherical	1.00±0.05	0.75±0.02	2.50±0.23	2.00±0.08	Positive
Sample 5	Spherical	2.03±0.14	2.00±0.03	2.90±0.31	1.50±0.03	Positive
Sample 6	Spherical	2.00±0.43	1.50±0.08	2.50±0.23	0.92±0.53	Positive
Sample 7	Spherical	3.48±0.31	1.46±0.07	4.00±0.59	3.80±0.07	Positive
Sample 8	Spherical	2.90±0.44	1.50±0.08	3.40±0.44	1.46±0.09	Positive
Sample 9	Spherical	3.48±0.37	1.46±0.77	2.00±0.14	1.62±0.02	Positive
Sample 10	Spherical	2.50±0.23	2.00±0.03	1.00±0.03	0.75±0.01	Positive

Mean of three observations

3.2. Protein profile of *Bacillus thuringiensis* isolates and Insecticidal activity

The protein profile of *B. thuringiensis* isolates was established through the utilization of eight percent SDS-PAGE. The primary aim was to discern variations in the protein banding patterns among all the isolates. Notably, there was observed diversity in the protein banding patterns, with varying intensities among isolates. Some isolates exhibited high intensity in their protein bands, while others displayed a lower intensity. Figure 5 illustrates the Protein Profile-SDS PAGE, capturing these variations.

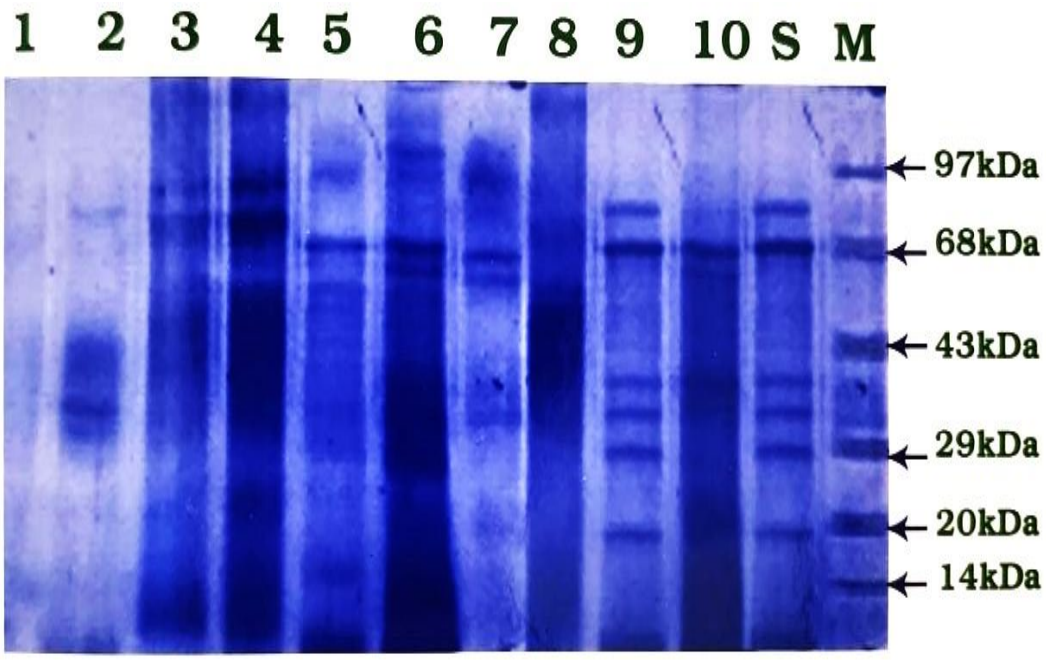

Figure 5 : Protein profile of *Bacillus thuringiensis*
(1 to 10: Samples, S: Standard *Bacillus thuringiensis*, M: Marker protein)

On SDS- PAGE, *B. thuringienis* isolates showed polypeptides, 68-97 kDa, multiple bands at 43, 29 and 23-14kDa. *B. thuringiensis* showed mainly protein bands when tested on a toxicity assay challenging Bombyx mori samples corresponding to the lanes 1, 2 and 3 causes 40% mortality. The heavy protein bands obtained in the lanes 4, 6, 8, 9 and 10 cause 70% mortality. Further investigation is required in which the portion of protein which acts as biopesticides is compared with standard *B. thuringiensis* used and also checked the efficiency of isolated or protein act as biopesticides. It should be emphasised that recent studies noted that only small proportions of *B. thuringiensis*, natural populations exhibited insecticidal activity, when tested on several major insect species (Ohba *et al*., 2000; Lee *et al*., 2003, Yasutake *et al*., 2006; Armengol *et al*., 2007). The observations may provide an ecological niche of this bacterial species in natural environments. The toxicity of these isolates tested against *Bombyx mori* (Figure 6).

Each sample, consisting of 1 ml with a spore concentration of 10 x 10^{-2} ml, was applied to mulberry leaves. Daily observations of silkworm mortality were conducted. In sample 1 over a three-day period, the mortality observed was one death on day 1, three deaths on day 2, and four deaths on day 3, resulting in a 40% mortality rate. Similar mortality rates of 40% were recorded for samples 2 and 5. Sample 3 exhibited a 50% mortality rate with two deaths on day 1, three deaths on day 2, and five deaths on day 3. Samples 4, 6, 7, 8, 9, and 10 showed a high mortality rate of 70%, as they caused two or more deaths in the observed period. These samples were classified as toxic strains due to their ability to kill more than 80% of silkworms.

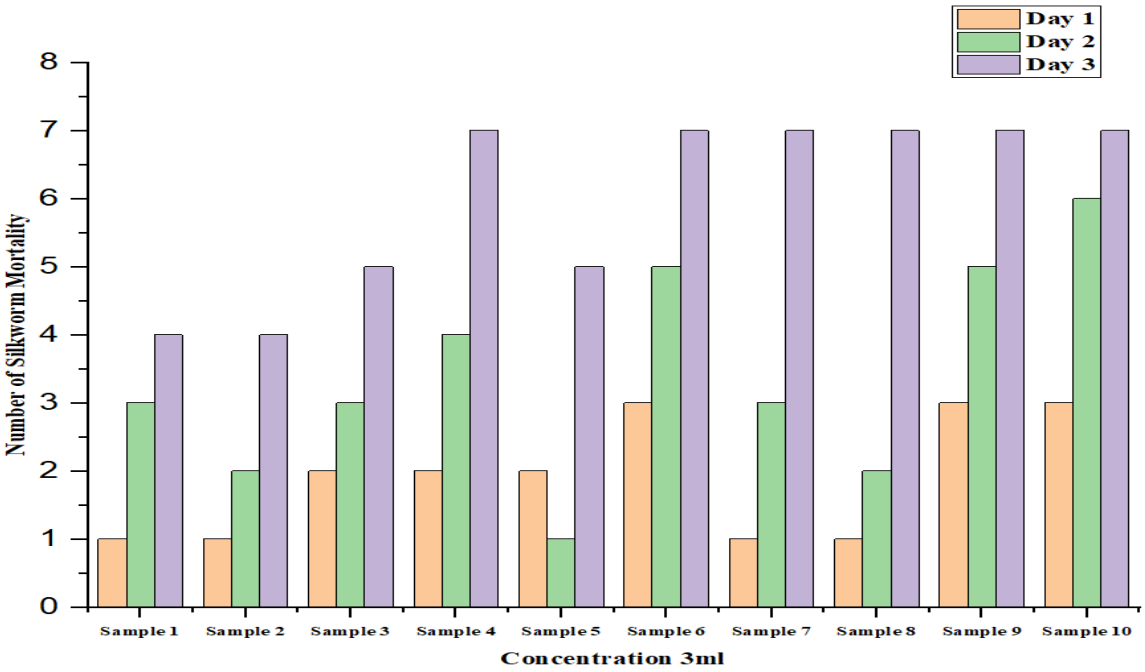

Figure 6: *Bacillus thuringiensis* isolates tested against Silkworm, *Bombyx mori.*

4. Conclusion

The present study highlights the insecticidal efficacy of *B. thuringiensis* strain against silkworms. While bacterial strains from various agricultural fields exhibited similarities in phenotypic and biochemical properties, the presence of crystals and crystal proteins served as confirming markers for the identification of the strain as *B. thuringiensis*. The crystal proteins produced by *B. thuringiensis* demonstrated target specificity and proved highly effective against the larvae of dipterans and lepidopterans. This environmentally friendly and target-specific strain of *B. thuringiensis* holds potential for disease control by targeting insect vectors in Tamil Nadu and other regions of India.

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