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Full Length Research Paper

Occurrence of *Bacillus thuringiensis* in faeces of herbivorous farm animals

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***Bacillus thuringiensis* (Berliner), the insect pathogen has been isolated from a variety of habitat. It is understood that the habitat of *B. thuringiensis* has always been associated with their biological activity. In the present study, *B. thuringiensis* was isolated from faeces of cows and goats. The phenotypic characterization revealed that the *B. thuringiensis* isolates are motile and harbour bipyramidal and spherical crystals, which was confirmed by the electron microscopic studies. The biochemical characterization confirmed the haemolytic nature and nitrate reduction ability of the *B. thuringiensis* isolates. There is a distinct variation in protein profiles among the isolates as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. From this study we hypothesize that application of cattle manure in addition to maintaining the soil health also help augment *B. thuringiensis* population in soil, as a natural biocontrol agent and may play a role in the biological nitrogen cycle.**

Key words: *Bacillus thuringiensis*, isolation, animal faces.

INTRODUCTION

Bacillus thuringiensis (Berliner) (Bt) is a Gram-positive, spore-forming soil bacterium that produces parasporal crystal inclusions which are toxic to the larval forms of a wide variety of insects including nematodes (Bravo et al., 2007). Bt has been isolated from different environments including soil (Martin and Travers, 1989), phylloplanes (Smith and Couche, 1991), stored grains (Meadows et al., 1992), faecal samples of greenhouse workers (Jensen et al., 2002) and sericulture environments (Xavier et al., 2007). Due to the much environmental and health concern, there has been a renewed interest in organic forming. One of the major sources of crop nutrients in organic farming is the application of farmyard manure from cattle. The decomposed form of cattle manures in addition to the supply of plant nutrients, also paly a major role in maintaining soil health.

Generally, cattle feed on the green foliage of grasses and are also fed with commercially available cattle feeds. It is assumed that Bt on the phylloplanes of grasses may enter the animal system, while grazing and eliminated through the faeces. This may not be just a physical process. The ingestion of Bt along with the feed may have a biological significance in the animal system. Earlier studies with the faecal samples of herbivorous animals maintained in a zoological garden in Japan indicated the presence of Bt isolates (Lee et al., 2002). Probably this may be the first study to investigate the occurrence of Bt in the faeces of farm animals. The present study is aimed at isolation and characterisation of Bt from fresh and dried faeces of herbivorous farm animals. It is presumed that the occurrence of Bt in the faeces of farm animals, play a critical role in the environmental distribution and enrichment of Bt in the cropping environment. In this paper, we report the presence of Bt in the faeces of herbivorous farm animals, which reflects the ecological significance of application of organic manures in the form of composted cattle manure.

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Abbreviations: SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **Bt,** *Bacillus thuringiensis*; **FM,** farmyard manure; **CBB,** Coomassie brilliant blue.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bt used in this study was isolated from faecal samples of herbivorous animals such as cows and goats. The bacteria were grown in nutrient broth or nutrient agar for sporulation and crystal production at 30°C.

Animal faeces sample collection

Faecal samples of cows and goats were collected from different cattle farms (wet samples) and grazing lands (dry samples) located in Kuala Ketil, Kedah Darul Aman State in Malaysia. Totally, 50 faecal samples were collected each weighing approximately 50 g in a sterile plastic bag, using a sterile spatula. The samples were collected carefully to avoid soil contamination. The samples were stored in the laboratory at 4°C until use. The animals in the farms were healthy and show no symptoms of illness. The sampling areas had not been previously sprayed with any commercial *B. thuringiensis* based microbial pesticides. The animals were taken out periodically for grazing and also supplemented with commercial animal feed and straw in the farm.

Isolation of *B. thuringiensis*

Isolation of Bt from faecal samples was carried out by a modified version of temperature selection method (Xavier et al., 2007). Each sample was thoroughly ground, mixed and approximately, 15 g of the sample was introduced into a 250 ml conical flask containing sterile distilled water. The sample was homogenized on an orbital shaker at 250 rpm for 5 h. One millilitre (1 ml) aliquot was taken from the sample and subjected to heat shock at 80°C for 15 min. Then the sample was serially diluted and plated onto nutrient agar. The plates were then incubated at 30°C for 2 days. The bacterial isolates were selected based on the colonial morphology resembling *Bacillus* spp. Microbiological and biochemical, electron microscopic studies were carried out to characterize the isolates.

Coomassie Brilliant Blue (CBB) staining and crystal protein morphology

Selected isolates were examined for the vegetative cell morphology, showing a typical rod shaped appearance and also for the presence of spores. All the sporulating isolates were further analysed for the presence of parasporal inclusions. The sporulated cultures of Bt isolates were used for CBB staining to study the crystal morphology. A straight inoculating wire was used to transfer an aliquot of a sporulated culture onto a microscopic slide. The slide was then heat fixed and stained (0.133% CBB stain in 50% acetic acid), rinsed in distilled water, dried and observed under light microscope using a 100x oil immersion objective (Rampersad and Ammons, 2002). The presence of parasporal bodies were clearly observed as dark blue stained objects.

Motility test

Motility test was performed as previously described (Apaydin et al., 2008). Motility plates were prepared using 1% (w/v) tryptone, 0.5% (w/v) NaCl and 0.3% (w/v) agar and the isolates were streak-inoculated onto the middle of the plate from top to bottom by drawing two closely drawn parallel lines with 2 mm width along the diameter of the motility plates. The plates were incubated overnight at 30°C. If an isolate was observed to spread out from the inoculation site, the isolate was scored motile, if not the isolate was considered non-motile.

Haemolytic assay

The haemolytic activity of the isolates was tested against human erythrocytes. Five millilitres (5 ml) of blood was mixed with 100 ml molten, lukewarm blood agar base and mixed thoroughly and poured onto glass Petri plates. After complete solidification of the medium, the isolates were streak inoculated and incubated at 37°C. The haemolysis was visually examined after 24 h.

Nitrate reduction test

The test was performed as directed by the manufacturer (Becton, Dickinson and company, USA). The isolates were grown in nitrate broth for 24 h at 37°C. To this, Nitrate A and B reagents were added in equal proportions. The medium is expected to turn pink or red if the organism is nitrate positive. The reaction indicates the ability of the organism to reduce nitrate (NO_3) to nitrite (NO_2), nitrous oxide (N_2O), nitric oxide (NO) or ammonia (NH_3).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The bacterial isolates were grown on nutrient broth. When 90% of the cells were lysed, the culture was harvested. This spore-crystal mixture was thoroughly washed with 1M NaCl for three times and centrifuge at 12,000 g for 10 min. The pellet was resuspended in distilled water in an eppendorf tube. The total protein concentration was estimated by Bradford (1976) method. The samples were boiled for 10 min in 5X sample buffer and loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel (SDSPAGE). Upon completion of electrophoresis, the gels were stained with CBB R250 [50% (v/v) ethanol, 10% (v/v) acetic acid and 0.1% CBB dye] for 1h for complete staining. The excess stain was removed by destaining with a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) ethanol for 2 to 3 h on a rocker. The molecular mass of proteins was determined by comparison with protein standards.

Transmission electron microscopy

The Bt isolate C4 was grown in nutrient broth until sporulation. A droplet of the culture to be examined was placed on a carbon film coated with 400 mesh copper grid (held with a self locking fine forceps). After 1 to 3 min (during which the cells attach to the film), the droplet was wicked to dryness using pieces of filter paper and the grid was left for 1 min. Then a droplet of the negative stain solution was added to the surface of the grid (2% methylamine tungstate), then after 1 min, the stain droplet was wicked to dryness using pieces of filter paper. The grid was then placed in a filter paper lined Petri dish and examined to observe the morphological features using TEM (Philips CM12 equipped with an analysis Docu Version 3.2 image analyse system).

RESULTS

Phenotypic characterization

The bacterial isolates were grown on a nutrient agar showing typical *Bacillus* morphology and were selected. The fully developed colonies are round, white, with regular

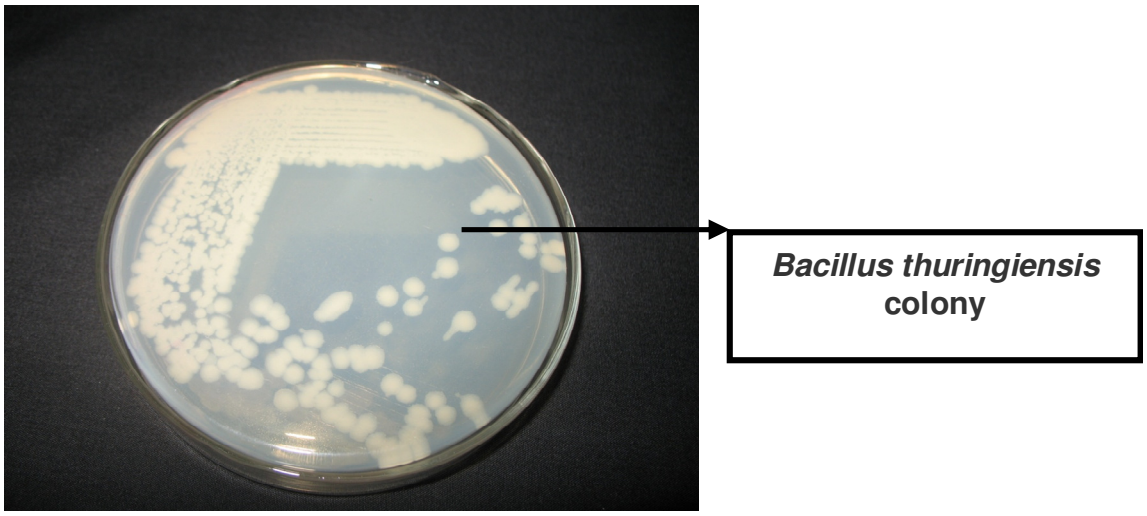

Figure 1. Colonies of *B. thuringiensis* on a nutrient agar plate.

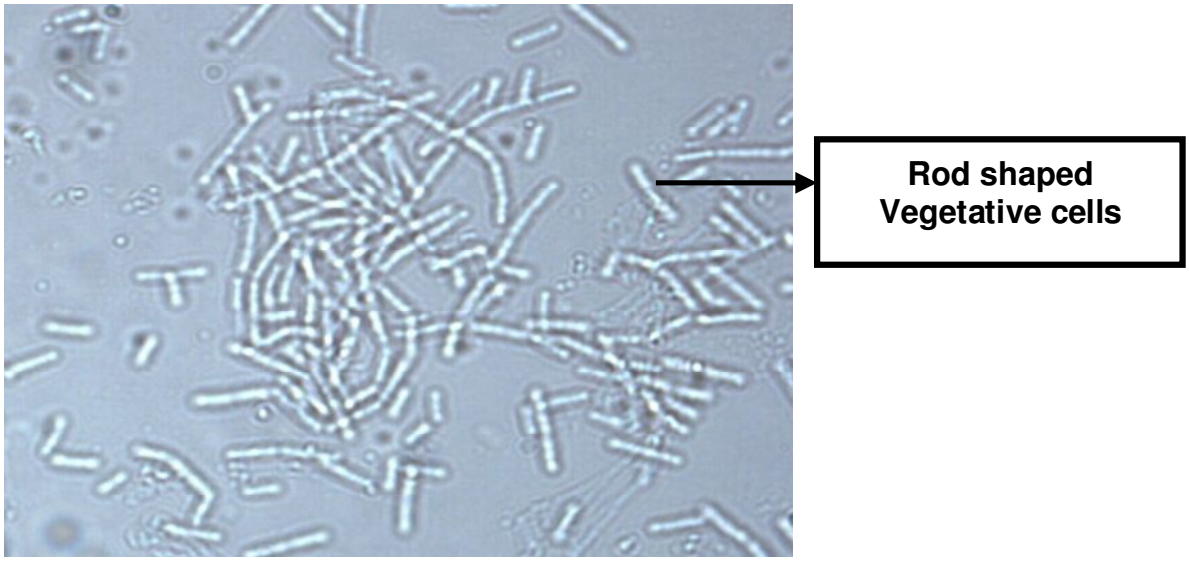

Figure 2. Vegetative cell morphology of *B. thuringiensis*.

margins (Figure 1). The vegetative cells are thin slender rods in short chains (Figure 2). Among the bacterial isolates, the isolate C4 was selected based on the SDS-PAGE protein profile to study the crystal morphology. The CBB stained Bt isolate C4 showed the presence of two types of crystal proteins, bipyramidal and spherical (Figure 4).

Motility test

In general, Bt strains are motile by peritrichous flagellum (Figure 3). However, non-motile Bt had also been reported (Damgaard et al., 1997). Recent studies showed that motility of Bt is an indirect indicator of virulence and biological activity of Bt strains (Bouillaut et al., 2005). The results of motility study indicated that all the Bt isolates are motile, indicating that these Bt isolates are virulent and may exhibit desired biological activity from the crop protection perspectives.

Haemolytic assay and nitrate reduction test

The study on the haemolytic activity of the Bt isolates revealed that all the isolates are haemolytic positive against human erythrocytes. All the isolates tested for nitrate reduction test exhibited nitrate reduction activity. Nitrogen is one of the macronutrient, the deficiency of which significantly affects crop production. Denitrification, the res-

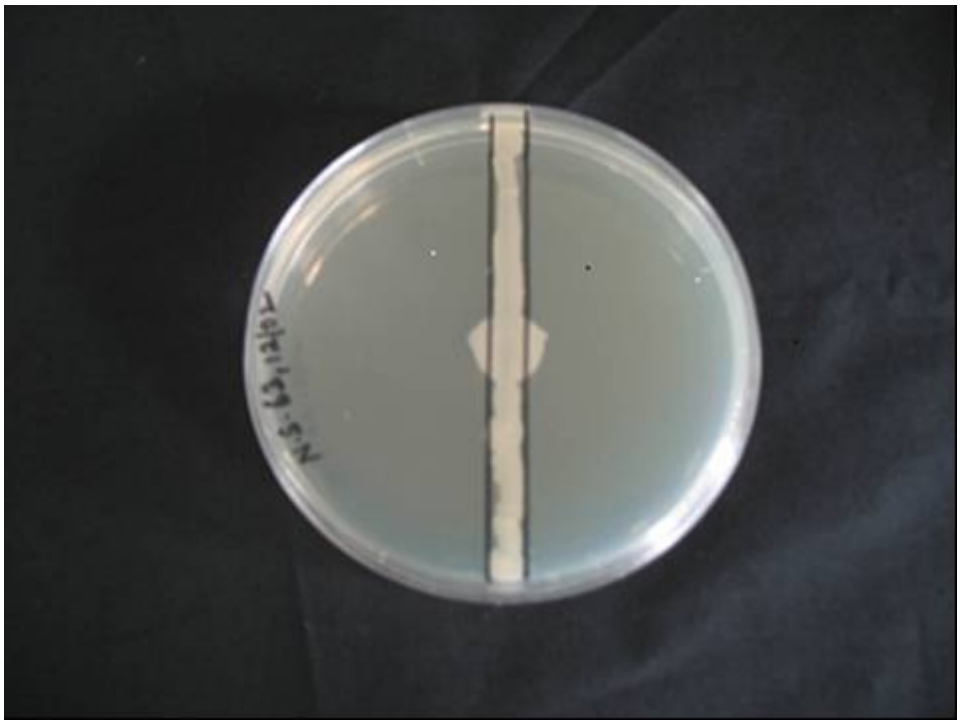

Figure 3. A motile strain of *B. thuringiensis* isolate.

piratory reduction of nitrate to gaseous products, is an important component of the nitrogen cycle, which influences soil fertility (Knowles, 1982). From the results it is assumed that these Bt isolates may play a role in biological nitrogen cycle and in improving soil fertility.

CBB staining and crystal protein morphology

Phase contrast microscopy is commonly used to detect the presence of parasporal inclusion bodies in the environmental isolates of Bt. CBB staining method offers two significant advantages over phase contrast microscopy; very small parasporal bodies are readily visible. Second, the presence of stained parasporal bodies are striking and instantaneously visible, much more so than with phase contrast microscopy (Rampersad and Ammons, 2002). It is evident from the result that the Bt isolate C4 harbours two types of crystal, the bipyramidal and spherical (Figure 4).

Protein profile by SDS-PAGE analysis of the protein showed that there is a distinct variation in the protein profile of Bt isolates from cattle and goat faeces. In addition, each Bt isolates from cattle faeces exhibits a unique protein profile, with minium variation in the protein profile (Figure 5). In two of the isolates, C3 and C4, in addition to all other proteins, a common 135 kDa protein was observed. Generally a Bt strain exhibiting a protein profile of 130 to 140 kDa may have antilepidopteran activity. The presence of all other protein may be related to other biological activities, even as a probiotic in the cattle system. Similarly the protein profile of the isolates from the goat faeces showed similarity among their protein profile, yet with minor differences (Figure 6). One interesting observation is that all the isolates in the goat faeces sample showed the presence of a 135 kDa protein.

Electron microscope studies

The results of electron microscopic studies revealed the rod shaped structure of the Bt isolate C4 and the peritrichous flagellum (Figure 7). As the bacterium heading for sporulation, it concomitantly produces the parasporal crystal inclusion. As evident from the electron micrograph, the two types of crystals at the early stage of formation is apparent (Figure 8). Another interesting feature to be noted is that as the bacterium entered the sporulation phase, the flagella started to shed and progressed for cell lysis. This study confirms the findings of CBB staining results which indicates the presence of two types of crystals that is, bipyramidal and spherical. Earlier studies indicated that a Bt strain with bipyramidal crystals are toxic to lepidopteran larvae (Berhnard et al., 1997).

DISCUSSION

Since Bt has been isolated from grass foliage (Smith and Couche, 1991), it is assumed that the herbivores would

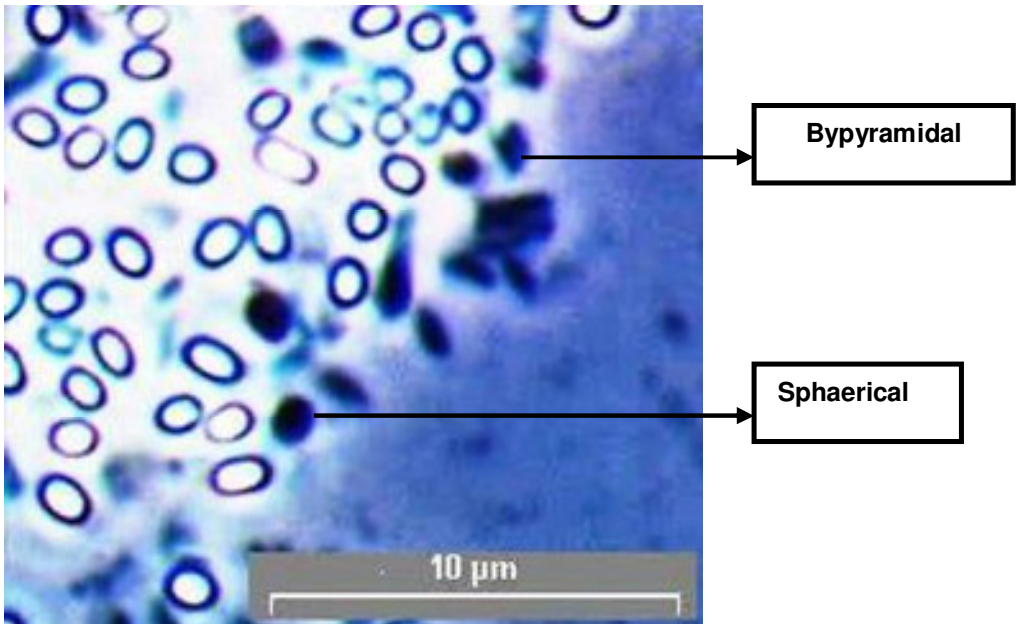

Figure 4. Phase contrast micrograph showing the *B.thuringiensis* crystal shapes.

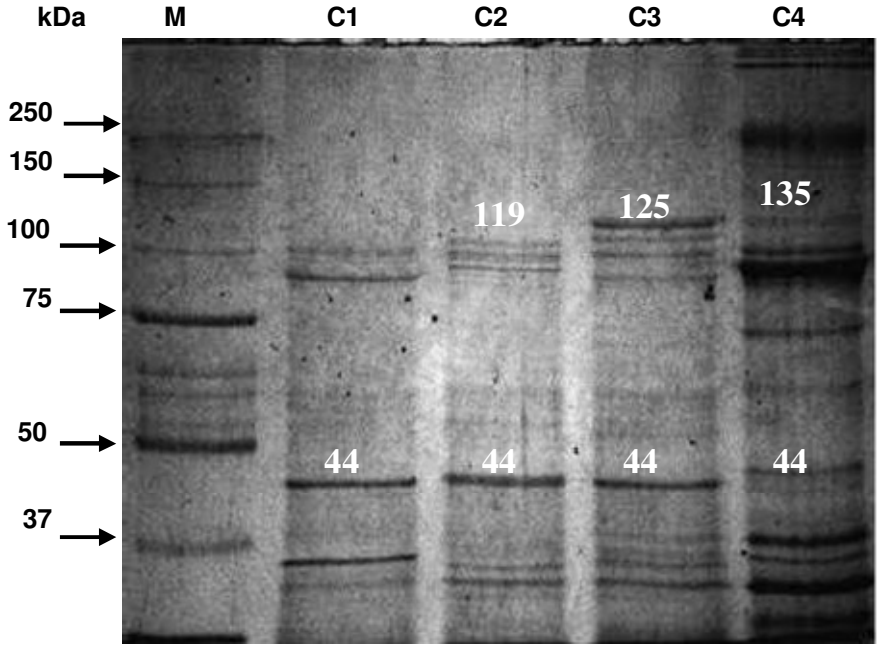

Figure 5. SDS PAGE analysis of the *B.thuringiensis* isolates (cow).

have acquired Bt as a natural event during grazing. Another possible mechanism of entry of Bt into the herbivores is through the animal feeds, as these animal feeds are also sources of Bt (Meadows et al., 1992). The third mode of Bt introduction into the animals may be through the drinking water from natural ponds and rivers. The differences in the protein profile in SDS-PAGE may be attributed to the presence of more than one Bt strain, as the herbivores acquire Bt from different sources. Thus, there is likely to be a regular introduction of new strains of *B. thuringiensis* into the animal system. In addition there is a potential for emergence of diverse population of Bt brought about by natural process of plasmid transfer (Meadows et al., 1992) in the stomach microenvironment of herbivorous animals.

From this study, we hypothesis that the Bt spores may

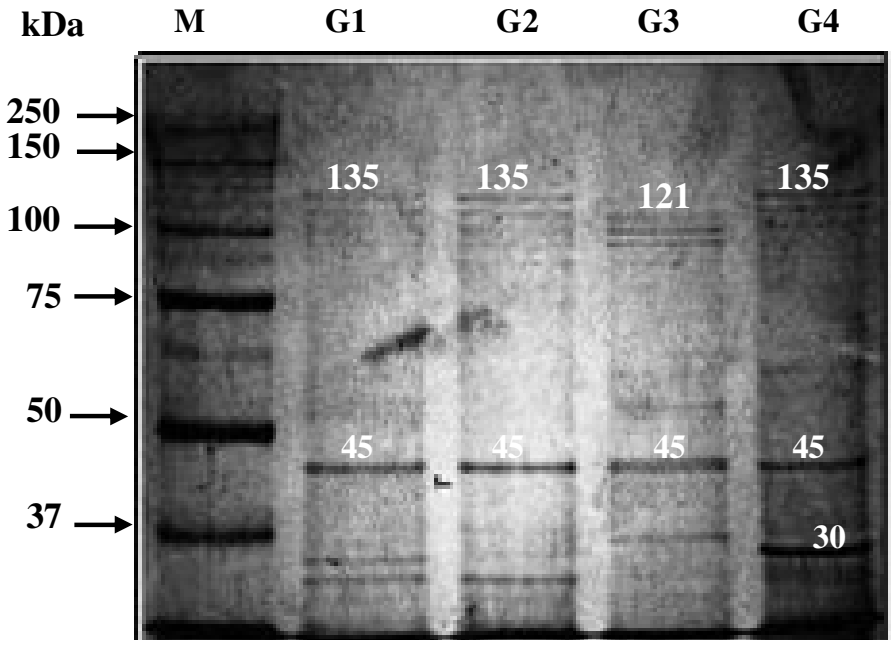

Figure 6. SDS PAGE analysis of *B.thuringiensis* isolates (goat).

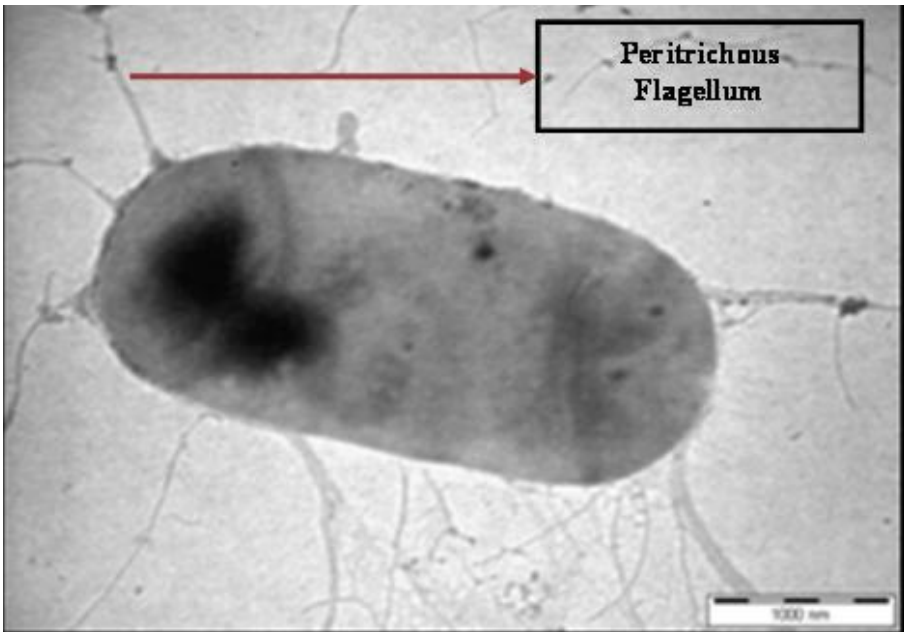

Figure 7. Transmission electron microgram of *B. thuringiensis* isolate C4.

be protected from inactivation by ultraviolet radiation, as the organism is protected under the cover of the faecal matter. As these faeces are left in the open atmosphere, they are subjected to decomposition and drying which does not affect the viability of the Bt spores. This hypothesis is substantiated by the protein profiling studies, which showed diverse proteins in different isolates. Further, the presence of two different types of crystal proteins such as the bipyramidal and sperical crystals in the Bt isolate C4 indicates that the spores in the animal faeces are protected from the harsh environment. The traditional farming practices include the application of dried faecal matter, called farmyard manure (FM) as a source of crop nutrients indirectly enriches the soil with beneficial microbes including Bt. As evident from the results of nitrate reduction activity, these Bt isolates may

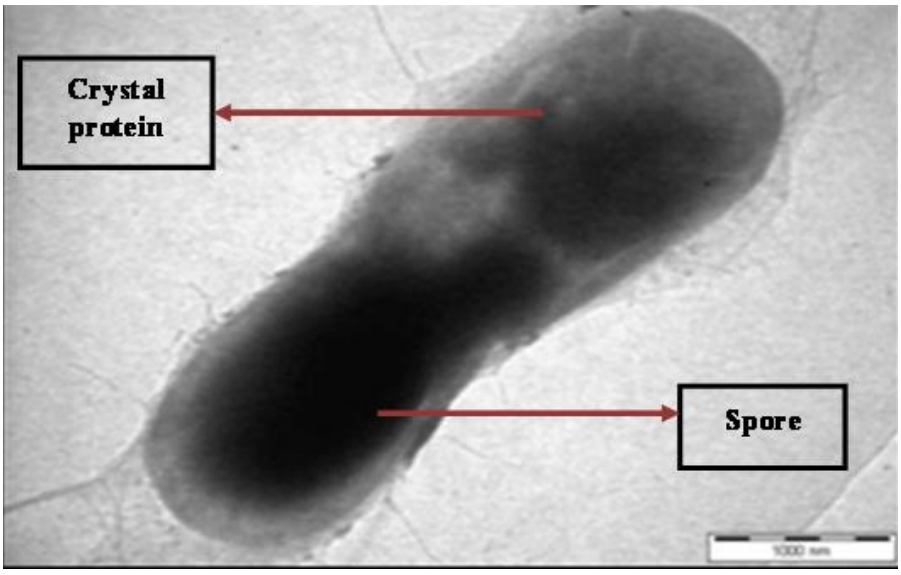

Figure 8. Transmission electron micrograph of *B. thuringiensis* isolate C4 with spore and crystal protein.

play a key role in improving soil health and nutrient status. This study assumes importance in terms of providing insights on the biological role of Bt in the herbivorous animals and the biosafety of feeding the animals with fodder from transgenic plants especially the maize, and rice straw. On safety considerations, only *B. anthracis*, a closely related organism has been pathogenic to cattle and so far Bt has not been implicated as an animal pathogen. Hence it is assumed that the presence of Bt as a gut microflora may play the role of a probiotic in the animal system. In addition, studies with greenhouse workers involved in mixing and spraying of Bt based bio-pesticides indicated that, despite the occurrence of Bt spores in their faeces, these did not produce gastro-intestinal symptoms (Jensen et al., 2002). Thus Bt is considered to be safe to non-target organisms and plays a critical role in sustainable crop production.

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