

RESEARCH ARTICLE

A Comparative study on sodium acetate and enrichment methods of *Bacillus thuringiensis* isolation

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Abstract

Bacillus thuringiensis is a very important soil bacterium that plays an immense role in managing insect pests in the agriculture field. These bacteria, as they reside in different habitats, have to be isolated in order to use them as a successful biological control agent. There are different methods of its isolation. Here, in our studies, we have compared two important methods of isolation of Bt from soil samples, viz., the sodium acetate method and the enrichment method. From the results, it's evident that the sodium acetate method has yielded the maximum number of Bt-like isolates as well as the maximum number of Bt colonies as compared to the enrichment method. The method has yielded 733 Bt-like isolates from which 26 Bt strains were confirmed. Whereas, the enrichment method contributed only 301 Bt-like colonies and of which there were only eight confirmed Bt strains. The statistical analysis using a paired t-test has also given evidence that the two methods are significantly different in isolating Bt strains. Hence, the sodium acetate methods can be confidently recommended for the isolation of Bt from soil samples.

Keywords: *Bacillus thuringiensis*, Sodium acetate, Enrichment method, Paired t-test.

Introduction

Bt is a naturally occurring gram-positive, rod-shaped, motile, facultative anaerobic and also spore-forming soil bacterium which, on a large scale, is an eco-friendly biopesticide against pests in agriculture and forestry (Gill *et al.*, 1992). The special characteristic of *Bt*, which differentiates it from the closely related *Bacillus* species (*B. cereus*, *B. anthracis*), is the presence of a parasporal crystal body which can be spotted near the spore and outside the exosporangium during the formation of the endospore (Andrews *et al.*, 1987). These strains show higher specificity, effectiveness and safety to vertebrates as well as the beneficial arthropods and even at very low doses can be very effective in controlling the insect pests (Roh *et al.*, 2007). The major constraint of the *Bt* formulations is the development of resistance to them by insect pests in course of time. The report says, about 21 species of insect pests have shown resistance to *Bt*, *Plodia interpunctella* being the first one to be recorded (McGaughey, 1985). The native strains may exhibit more effective and adaptable performance and in managing resistance development in local crop settings to control the insect pests than commercially available *Bt* formulations. So, it becomes important to search for novel strains of *Bt* that are native and can control insects that are relatively resistant to already known *Bt* formulations.

Different researchers have reported a variety of methods of Bt isolation. This includes the sodium acetate method, the modified sodium acetate method, the penicillin cycling method, the enrichment method, *etc.* The sodium acetate

method, which is a widely used method for the isolation of *Bt* was described by Travers *et al.* (1987). Similarly, a new enrichment method described by Patel *et al.* (2013) is also used for the isolation of *Bt*. In the present investigation, we have compared both methods and found that the sodium acetate method is the efficient method in isolating *Bt* as compared to the enrichment method.

Materials and Methods

Bt cultures

The reference strains, HD1 (*Btk*) and HD133 (*Bta*), were obtained from the *Bacillus* Genetic Stock Centre (BGSC), Ohio State University, USA.

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How to cite this article: Mathew, M., Ningthoujam, K., Daniel, G.R., Nirosha, V., Pathak, M., Suting, E.G. 2025. A Comparative study on sodium acetate and enrichment methods of *Bacillus thuringiensis* isolation. *Indian J. Hill Farm.*, **38**(1):45-48.

Source of support: Nil

Conflict of interest: None.

Received: 27/08/2024 **Revised:** 04/09/2025 **Accepted:** 04/09/2025

Sample collection

A total of 122 samples were collected from 10 districts, viz., Bishnupur, Churachandpur, Imphal East, Imphal West, Kakching, Kangpokpi, Noney, Senapati, Tamenglong and Thoubal of Manipur and four districts of Meghalaya, viz., Ri-Bhoi, West Khasi, East Garo, West Garo, South Garo, and North Garo. In which the majority were soil samples (96) and the remaining were water samples (26).

Bt isolation methods Sodium acetate method

One gram of soil is added to 10 mL Luria Bertani broth (LB), which is buffered with sodium acetate (0.25 M, pH 6.8) in 250 mL flasks. This was incubated for 4 hrs on a shaker at 30°C and 200 rpm. A 1-mL aliquot was centrifuged at 1000 rpm for 1 minute for the soil particles to settle. This was heated at 80°C for 10 minutes and then spread to Luria Bertani or Nutrient Agar plates and incubated for two days at 30°C. The plates were observed after two days for *Bt*-like colonies.

Enrichment method

In this method, 1 g of soil or 1-mL of the water sample was inoculated into 20 mL GYS sporulation media (GYS per litre: 1 g glucose, 2 g yeast extract powder, 2g $\text{NH}_4(\text{SO}_4)_2$, 0.06 g MnSO_4 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 g CaCl_2 , 5 g K_2HPO_4). This was incubated at 30°C for two days at 200 rpm on a rotary shaker. After two days, 1-mL aliquot was transferred into centrifuge tubes and centrifuged at 1000 rpm for one minute for the settlement of soil particles. The supernatant was then heated at 80°C for three minutes. This supernatant was then serially diluted to a dilution of 10^{-8} . The dilutions from 10^{-6} to 10^{-8} were spread to Luria Bertani media (LB) or Nutrient Agar media (NA) and incubated at 30°C for two days. After the incubation of two days, the plates were observed for the *Bt*-like colonies.

Statistical Analysis

The percentage of confirmed *Bt* colonies to other *Bt* like colonies on the isolation plate was compared. Statistical analysis was performed by paired *t*-test using SPSS to attain a significant difference between the percentage of *Bt* colonies isolated by the two methods.

Results

When a total of 122 samples were considered for the isolation of *Bt*, the sodium acetate method yielded 733 *Bt*-like colonies. Among these, 26 isolates were confirmed as *Bt*. Whereas the enrichment method yielded only 301 *Bt*-like colonies and out of these, only eight were confirmed to be *Bt* (Table 1). Screening of *Bt* isolates obtained was done using morphology, phase contrast microscopy and PCR detection of *cry* genes. These were also confirmed as *Bt* after partial sequencing of 16s rRNA and NCBI BLAST. Hence, a greater number of *Bt* strains were isolated using the sodium acetate method as compared to the enrichment method.

Table 1 : Sampling locations of different districts of Manipur and Meghalaya

Sl. No.	District	Manipur	
		No. of Block/village	Sample type-Soil (S), Water (W)
1	Bishnupur	16	S/W
2	Churachandpur	2	S
3	ImphalEast	2	S
4	ImphalWest	6	S/W
5	Kakching	3	S
6	Kangpokpi	3	S
7	Noney	6	S/W
8	Tamenglong	3	S
9	Thoubal	2	S
10	Senapati	2	S
Meghalaya			
1	West Khasi hills	11	S/W
2	East Garo hills	14	S/W
3	North Garo hills	4	S/W
4	South Garo hills	2	S/W
5	West Garo hills	6	S
6	Ri-Bhoi	21	S/W

A significant difference was observed in the number of *Bt* colonies obtained by the sodium acetate method (Table 2). In some of the soil samples, the *Bt* colonies were only obtained using the sodium acetate method, whereas the enrichment method failed to yield *Bt* (Fig. 1).

Discussions

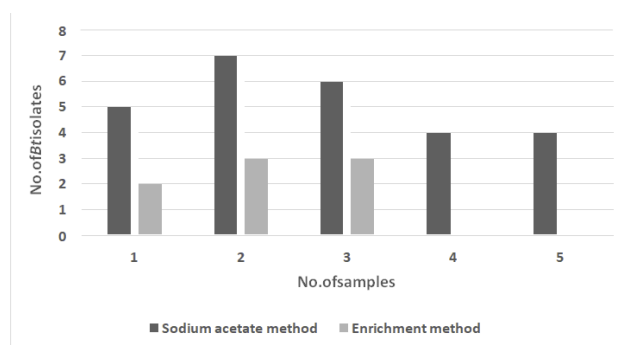
The present investigation has confirmed that the sodium acetate method is efficient in isolating *Bacillus thuringiensis* as compared to the enrichment method. This was found contradictory to the studies of Patel *et al.* (2013), who confirmed that the enrichment method was better in isolating more *Bt* strains as compared to the sodium acetate method. Patel *et al.* decided to prepare a new medium for *Bt* isolation, as a single method is not suitable to isolate microorganisms from different ecological niches. In their study, they have shown that the enrichment method yielded higher percentages of *Bt* colonies (55–75%) in many soil samples and a higher frequency of crystal inclusions, Cry protein bands, and *cry/cyt* genes. Also, they were able to isolate *Bt* from 44 out of 58 samples. They also observed a larger size of the *Bt* colony compared to other *Bacillus* species. The growth kinetics studies of different *Bacillus*

Table 2 : Comparison of different methods of Bt isolation

solation methods	Total samples examined	Bt like isolates	Bt-positive samples	Bt index
Sodium acetate method	122	733	26	0.03
Enrichment method	122	301	8	0.02

Table 3 : Comparison of the two methods of isolation using the paired t-test

Method of isolation	Sodium acetate method	Enrichment method	t value	p-value
	Mean	Mean		
Number of Bt isolates	5.20	1.60	14.697	0.0001

**Fig 1 :** Statistical analyses of Bt isolation by the sodium acetate method and the enrichment method from soil samples (p-value <0.05)

species revealed a higher rate of germination of *Bt* and was higher than compared of other *Bacillus* species.

In our study, the enrichment method was not successful in isolating *Bt* from some of the soil samples. In contrast, the sodium acetate method yielded *Bt*-like colonies from all samples, with the maximum number of *Bt* strains. This is the most widely used method of isolation; the majority of the investigators have used the sodium acetate method to isolate *Bt*, which includes Travers *et al.* (1987), who demonstrated that this method allowed the selective germination of spores. The method was also used by Aramideh *et al.* (2010) and showed that most of the isolates were obtained in 0.25M concentration (56.25%). Anitha *et al.* (2011) used this method to isolate 63 local isolates of *Bt* from the cotton rhizosphere soils. Hence, it is seen that the method is the most preferred method of *Bt* isolation (Table 3).

In the sodium acetate method, LB medium is used and is buffered with sodium acetate (pH 6.8). Shnayderman *et al.* (2005) have found that *Bt* growth rate is higher than *B. subtilis* and *E. coli* in LB medium at 30°C. Moreover, many scientists have reported that the sodium acetate method is the best method for isolation. *Bt*, like Ejiofor and Johnson (2002), in their studies have found that the sodium acetate method is a selective method that is highly sensitive as compared to other techniques, like enrichment techniques, because enrichment techniques have a lower detection limit of 10³ bacteria per gram of soil.

Conclusion

The present investigation has shown that the sodium acetate method is the most suitable method for isolating *Bt* strains as compared to the enrichment method. Since, sodium acetate method has yielded the maximum number of *Bt* colonies (26) from the soil samples used. Whereas the enrichment method failed to yield *Bt* from many of the soil samples and only a few *Bt* strains (eight) were found. The statistical analysis also proved that there is a significant difference in the number of *Bt* colonies exhibited by both methods. However, a single method is not enough to isolate a bacterium like *Bt*, which is omnipresent. Hence, there should be continued studies in isolating this bacterium from different ecological niches.

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