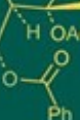
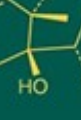
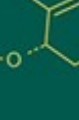
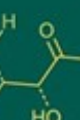
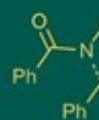


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Evaluation of different culture media for growth and sporulation of *Bacillus thuringiensis* PDKV-Bt-SY-4 under submerged and static fermentation conditions

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ABSTRACT

Bacillus thuringiensis (Bt) is an important microbial biopesticide used for the management of insect pests; however, high production costs and dependence on commercial formulations may limit its adoption. The present study evaluated different culture media and fermentation conditions for the growth and sporulation of the locally isolated strain *B. thuringiensis* PDKV-Bt-SY-4 as a preliminary step toward developing a practical and potentially low-cost Bt formulation. Six culture media, namely Nutrient Carbon Mineral (NCM), T3, NCM supplemented with L-tyrosine and CuSO₄ (NCM+), Nutrient Yeast Salt Medium (NYSM), glucose-based medium (GBM), and Tryptic Soy Broth (TSB), were evaluated under submerged and static fermentation conditions. Fifty millilitres of each medium were inoculated with 5% (v/v) seed culture and incubated at 28 °C for 5 days (120 h). Submerged fermentation was carried out at 180 rpm, whereas static fermentation was performed without agitation. Growth and sporulation were evaluated based on total viable cell count (TVCC) and viable spore count using the serial dilution and plate-count method. Under submerged fermentation, NYSM recorded the highest TVCC (3.20×10^8 CFU mL⁻¹) and viable spore count (1.50×10^8 CFU mL⁻¹). Under static fermentation, NCM recorded the highest TVCC (1.12×10^8 CFU mL⁻¹), while NCM+ recorded the highest viable spore count (1.32×10^8 CFU mL⁻¹). Although NYSM supported the highest growth and spore production under submerged conditions, NCM+ showed superior spore production under static conditions. Based on viable spore production and the practical advantage of eliminating continuous agitation, NCM+ under static fermentation was selected for further formulation studies. The findings provide a basis for further development of a potentially cost-effective *B. thuringiensis* formulation.

Keywords: *Bacillus thuringiensis*, PDKV-Bt-SY-4, Culture media, Submerged fermentation, Static fermentation, Biopesticide, Viable spore count

Introduction

Bacillus thuringiensis (Bt) is a Gram-positive, spore-forming entomopathogenic bacterium that is widely used as a microbial insecticide for the management of economically important insect pests (Bravo *et al.*, 2007; Palma *et al.*, 2014) [1, 2]. During sporulation, Bt produces parasporal crystalline proteins, mainly Cry and Cyt toxins, which show insecticidal activity against susceptible insects belonging to several orders, particularly Lepidoptera, Coleoptera and Diptera (Bravo *et al.*, 2007; Palma *et al.*, 2014) [1, 2]. Following ingestion by a susceptible insect, these toxins are activated under the alkaline conditions of the midgut and bind to specific receptors on the midgut epithelial cells. This interaction results in disruption of the cell membrane, cellular damage and, ultimately, death of the insect (Bravo *et al.*, 2007; Pardo-López *et al.*, 2013) [1, 3]. The specificity of Bt toxins, together with their comparatively favourable environmental profile, has contributed to the widespread use of Bt in biological and integrated pest management programmes (Palma *et al.*, 2014) [2].

The effectiveness of a Bt-based biopesticide is influenced by several factors, including the bacterial strain, composition of the culture medium, fermentation conditions, level of spore production and formulation characteristics (Duraismy *et al.*, 2023; Khedher *et al.*, 2011) [4, 5]. Optimizing these factors is therefore important for obtaining preparations with a high viable spore concentration, effective insecticidal activity and adequate storage stability (Duraismy *et al.*, 2023; Khedher *et al.*, 2011) [4, 5]. Among these factors, the selection of a suitable culture medium and fermentation condition is particularly important because they directly influence bacterial growth and sporulation (Duraismy *et al.*, 2023; Khedher *et al.*, 2011) [4, 5]. Therefore, the present study was undertaken to evaluate different culture media for growth and sporulation of *B.*

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thuringiensis PDKV-Bt-SY-4 under submerged and static fermentation conditions, with the aim of identifying a suitable production condition for subsequent development of a Bt-based biopesticide formulation.

Materials and Methods

The study was conducted to evaluate different media for the growth and sporulation of *Bacillus thuringiensis* PDKV-Bt-SY-4 under submerged and static fermentation conditions. The strain isolated and cultured from Department of Entomology and maintained at Laboratory of Molecular Biology and Nanotechnology, were used in the study. The study was conducted in Laboratory of Molecular Biology and Nanotechnology, Biotechnology Centre, Department of Agril. Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola (M.S.) during the year 2025-2026.

Culturing of Bt strains

The local *Bacillus thuringiensis* isolate PDKV-Bt-SY-4, previously isolated and screened for insecticidal activity in the Biotechnology Centre, was used in the present study. The isolate was subculture and maintained on *Luria* broth medium. *Luria* broth medium, as described by Travers *et al.* (1987) [6], was used for the routine cultivation and maintenance of the *B. thuringiensis* isolate throughout the study.

Culture media used for fermentation

Different culture media were evaluated to determine their suitability for the growth and sporulation of *Bacillus thuringiensis* PDKV-Bt-SY-4 under static and submerged fermentation conditions. The media evaluated included Nutrient Carbon Mineral (NCM) medium, T3 medium, NCM supplemented with tyrosine and CuSO₄, Nutrient Yeast Salt Medium (NYSM), Glucose-based medium (GBM), and Tryptic Soy Broth (TSB). The respective media were prepared according to the formulations reported by Talkhan *et al.* (2013) [7], Travers *et al.* (1987) [6], Bora *et al.* (2024) [8], Khedher *et al.* (2011) [5], and Duraisamy *et al.* (2023) [4] as shown in Table 1.

Culture maintenance

The local isolate PDKV-Bt-SY-4, previously isolated and screened for insecticidal activity in the Biotechnology Centre, Department of Agril. Botany and Department of Entomology, was used for the present study. The culture was subculture and maintained on *Luria* Broth (LB) medium at 4 °C under aseptic conditions. The maintained culture was periodically subculture at every 15 days to ensure viability and purity before use in subsequent experiments. Freshly grown

cultures were used for seed culture preparation and fermentation experiments.

Seed culture preparation of *Bacillus thuringiensis* PDKV-Bt-SY-4

The maintained culture of PDKV-Bt-SY-4 was used for the preparation of seed culture. A loopful of the actively growing culture was aseptically transferred into 50 mL of sterile *Luria* Broth (LB) medium in a 250 mL Erlenmeyer flask. The inoculated medium was incubated at 28 °C for 12 h or overnight under shaking conditions at 180 RPM to obtain an actively growing culture with OD value more than 0.6. The resulting seed culture was used as the inoculum for subsequent fermentation experiments at a 5% (v/v) inoculum level.

Inoculation of seed culture into different fermentation media

The prepared seed culture of PDKV-Bt-SY-4 was used to inoculate the selected fermentation media. 50 mL of each medium were dispensed into sterile 250 mL Erlenmeyer flasks and inoculated aseptically with 5% (v/v) seed culture. An uninoculated flask containing the respective medium was maintained as a control. Each treatment was maintained in replication to ensure the reliability and reproducibility of the observations.

The inoculated flasks were incubated at 28 °C for 5 days under two fermentation conditions. For submerged fermentation, the cultures were incubated on a rotary shaker at 180 rpm, whereas for static fermentation, the cultures were incubated without agitation. The cultures were subsequently evaluated for total viable cell count (TVCC) and viable spore count to compare the growth and sporulation of PDKV-Bt-SY-4 among the different media and fermentation conditions.

Estimation of colony-forming units (CFU/mL)

The viable bacterial population of *Bacillus thuringiensis* PDKV-Bt-SY-4 was estimated by the serial dilution and plate count method. A 0.9% sterile saline solution was used as the diluent for preparing serial ten-fold dilutions of the fermentation broth. An appropriate dilution was selected, and a measured aliquot was aseptically spread onto suitable agar medium. The inoculated plates were incubated under suitable conditions, and colonies developed on plates containing a countable number of colonies were enumerated. The viable bacterial population was expressed as colony-forming units per millilitre (CFU/mL) and calculated using the dilution factor and volume of sample plated (Cappuccino and Welsh, 2017).

Calculation

$$\text{CFU/ml} = \frac{(\text{Number of colonies} \times \text{Dilution factor})}{\text{Volume of culture plate (in ml)}}$$

Estimation of total viable cell count (TVCC) and viable spore count

The total viable cell count (TVCC) and viable spore count of PDKV-Bt-SY-4 were determined by the serial dilution and plate count method. Sterile 0.9% saline solution was used as the diluent for preparing serial ten-fold dilutions of the fermentation broth.

For TVCC estimation, no heat treatment was applied. Appropriate serial dilutions of the culture were directly plated onto suitable agar medium. Following incubation, the colonies developed on countable plates were enumerated and expressed as CFU/mL, representing the

total viable population, including vegetative cells and heat-resistant spores.

For viable spore enumeration, a separate aliquot of the fermentation broth was subjected to heat treatment at 80 °C for 10 min to eliminate the vegetative cells while retaining viable heat-resistant spores. The heat-treated sample was subsequently serially diluted using sterile 0.9% saline solution and plated onto suitable agar medium. After incubation, the colonies that developed were counted and expressed as CFU/mL (viable spores). The use of an 80 °C heat treatment for 15 min before serial dilution and plating has been reported for the selective enumeration of viable *Bt* spores.

Table 1. Evaluation of six different media for better Total viable cell growth and spore

SN	Medium	Reference
1.	NCM- Nutrient Carbon Mineral	Talkhan <i>et al.</i> 2013
2.	T3 medium	Travers <i>et al.</i> 1987.
3.	NCM+Inducer(CuSO ₄ + L-Tyrosine)	Modified from Talkhan <i>et al.</i> (2013)
4.	NYSM- Nutrient Yeast Salt Medium	Bora <i>et al.</i> 2024
5.	GBM- Glucose Based Medium	Khedher <i>et al.</i> 2011
6.	TSB- Tryptic Soy Broth	Duraisamy <i>et al.</i> 2023

Results and Discussion

Total viable cell count under submerged and static fermentation

The total viable cell count (TVCC) of *Bacillus thuringiensis* PDKV-Bt-SY-4 varied considerably among the different culture media and fermentation conditions. The results are presented in Table 2.

Under submerged fermentation (180 rpm), the highest TVCC was recorded in NYSM medium (3.2×10^8 CFU/mL), followed by T3 medium (9.5×10^7 CFU/mL), GBM (7.7×10^7 CFU/mL), NCM medium (7.3×10^7 CFU/mL), NCM+ medium (7.2×10^7 CFU/mL), and TSB (1.9×10^7 CFU/mL). Thus, NYSM supported the highest bacterial growth under submerged fermentation.

Under static fermentation, the highest TVCC was observed in NCM medium (1.12×10^8 CFU/mL), followed by NCM+ (7.6×10^7 CFU/mL), NYSM (6.6×10^7 CFU/mL), T3 (4.9×10^7 CFU/mL), TSB (4.1×10^7 CFU/mL), and GBM (3.6×10^7 CFU/mL).

Overall, submerged fermentation resulted in higher TVCC than static fermentation in four of the six media, namely T3, NYSM, GBM, and TSB. The most pronounced difference was observed in NYSM, where the TVCC under submerged fermentation (3.2×10^8 CFU/mL) was substantially higher than that under static fermentation (6.6×10^7

CFU/mL). In contrast, NCM showed better bacterial growth under static conditions (1.12×10^8 CFU/mL) than under submerged conditions (7.3×10^7 CFU/mL). These results indicate that both culture medium and fermentation condition influenced the growth of *B. thuringiensis* PDKV-Bt-SY-4, with NYSM under submerged fermentation showing the highest TVCC among the treatments evaluated. (Table 2 & Graph 1)

Viable spore count under submerged and static fermentation

The viable spore count of *Bacillus thuringiensis* PDKV-Bt-SY-4 varied among the different culture media and fermentation conditions. The results are presented in Table 3.

Under submerged fermentation (180 rpm), the highest viable spore count was recorded in NYSM (1.5×10^8 CFU/mL), followed by NCM (1.14×10^8 CFU/mL), NCM+ (5.4×10^7 CFU/mL), GBM (3.7×10^7 CFU/mL), T3 (3.6×10^7 CFU/mL) and TSB (3.5×10^7 CFU/mL). Thus, NYSM supported the highest spore production under submerged fermentation.

Under static fermentation, the highest viable spore count was observed in NCM+ (1.32×10^8 CFU/mL), followed by NCM (9.6×10^7 CFU/mL), T3 (3.3×10^7 CFU/mL), NYSM (3.1×10^7 CFU/mL), GBM (2.4×10^7 CFU/mL) and TSB (2.2×10^7 CFU/mL). Therefore, NCM+ exhibited the highest spore production under static conditions. A comparison of the two fermentation conditions showed that NYSM and NCM supported higher viable spore production under submerged fermentation, whereas NCM+ showed considerably higher spore production under static fermentation. The difference was particularly evident in NYSM, where the viable spore count under submerged fermentation (1.5×10^8 CFU/mL) was much higher than that observed under static fermentation (3.1×10^7 CFU/mL). In contrast, NCM+ recorded a higher viable spore count under static fermentation (1.32×10^8 CFU/mL) than under submerged fermentation (5.4×10^7 CFU/mL).

Among all the treatments evaluated, the highest overall viable spore count was recorded in NYSM under submerged fermentation (1.5×10^8 CFU/mL), while the lowest count was observed in TSB under static fermentation (2.2×10^7 CFU/mL). The results indicate that spore production was influenced by both the culture medium and the fermentation condition. NYSM was more favourable for spore production under submerged conditions, whereas NCM+ performed better under static conditions. (Table 3 & Graph 2)

Table 2. Total viable cell counts of *Bacillus thuringiensis* PDKV-Bt-SY-4 in different culture media under submerged and static fermentation

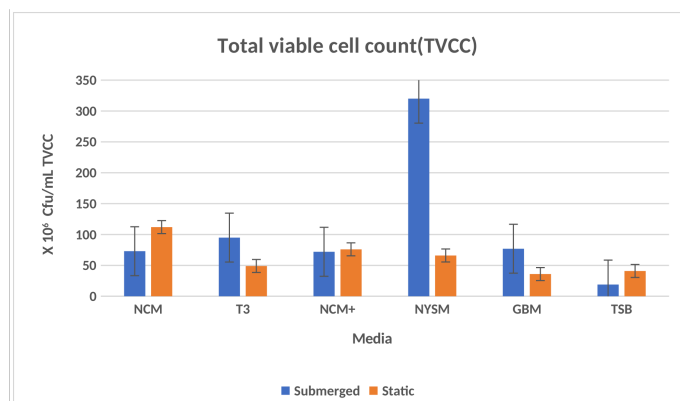
SN	Medium	Submerged (Cfu/mL)	Static (Cfu/mL)
1.	NCM	7.3 X 10 ⁷	1.12 X 10 ⁸
2.	T3	9.5 X 10 ⁷	4.9 X 10 ⁷
3.	NCM+	7.2 X 10 ⁷	7.6 X 10 ⁷
4.	NYSM	3.2 X 10 ⁸	6.6 X 10 ⁷
5.	GBM	7.7 X 10 ⁷	3.6 X 10 ⁷
6.	TSB	1.9 X 10 ⁷	4.1 X 10 ⁷

Table 3. Viable spore count of *Bacillus thuringiensis* PDKV-Bt-SY -4 in different culture media under submerged and static fermentation

SN	Medium	Submerged (Cfu/mL)	Static (Cfu/mL)
1.	NCM	1.14 X 10 ⁸	9.6 X 10 ⁷
2.	T3	3.6 X 10 ⁷	3.3 X 10 ⁷
3.	NCM+	5.4 X 10 ⁷	1.32 X 10 ⁸
4.	NYSM	1.5 X 10 ⁸	3.1 X 10 ⁷
5.	GBM	3.7 X 10 ⁷	2.4 X 10 ⁷
6.	TSB	3.5 X 10 ⁷	2.2 X 10 ⁷

Conclusion

The comparative evaluation of different culture media under submerged and static fermentation conditions was carried out to identify a suitable medium and fermentation condition for the efficient production of *Bacillus thuringiensis* PDKV-Bt-SY-4. The cultures were incubated for 5 days (120 h) at 28 °C, with submerged fermentation carried out at 180 rpm, while static fermentation was performed without agitation (0 rpm). The performance of the different media was evaluated based on total viable cell count (TVCC) and viable spore count.



Graph 1. Evaluation of Total viable cell counts of *Bacillus thuringiensis* PDKV-Bt-SY-4 in different culture media under submerged and static fermentation.

Under submerged fermentation, NYSM recorded the highest TVCC (3.2×10^8 CFU/mL) and viable spore count (1.5×10^8 CFU/mL). In comparison, under static fermentation, NCM recorded the highest TVCC (1.12×10^8 CFU/mL), while NCM supplemented with L-tyrosine and CuSO₄ (NCM+) recorded the highest viable spore count (1.32×10^8 CFU/mL). Although NYSM showed the highest overall growth and spore production under submerged conditions, the difference between submerged and static fermentation was not consistently large across all media. In particular, NCM and NCM+ showed relatively comparable performance between the two fermentation conditions.

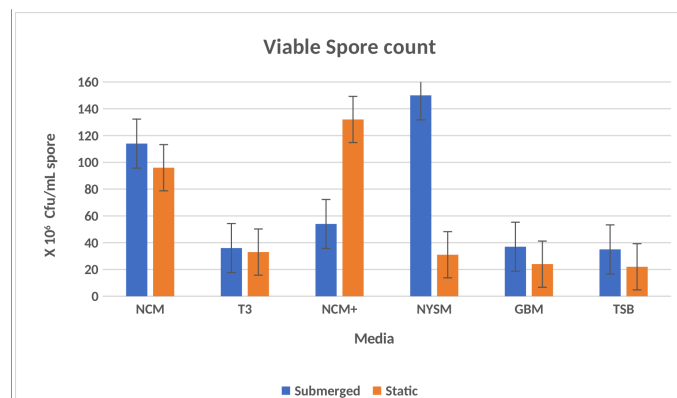
From a practical and economic perspective, static fermentation has an important advantage because it does not require continuous agitation, thereby reducing energy consumption, equipment requirements, and overall production costs. Since the objective of the present study was to develop a cost-effective and farmer-friendly *B. thuringiensis* production system, avoiding mechanical agitation wherever possible would be beneficial. Therefore, when the difference in growth and sporulation between submerged and static fermentation is relatively small, static fermentation can be preferred over submerged fermentation for further development. Based on the results obtained under static fermentation, NCM+ showed the highest viable spore count (1.32×10^8 CFU/mL), while NCM showed the highest TVCC (1.12×10^8 CFU/mL). Since spore production is particularly important for the development of a stable *B. thuringiensis* biopesticide, NCM+ under static fermentation was considered the most promising condition for further studies, subject to confirmation through statistical analysis and subsequent experiments.

Thus, considering biological performance together with the economic advantage of eliminating agitation, static fermentation was considered

a suitable approach for the subsequent development of the *B. thuringiensis* PDKV-Bt-SY-4 formulation. The selected static fermentation condition can potentially simplify the production process and reduce production costs while maintaining satisfactory bacterial growth and spore production.

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Graph 2. Evaluation of viable spore counts of *Bacillus thuringiensis* PDKV-Bt-SY-4 in different culture media under submerged and static fermentation

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