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Biofilm formation in *Bacillus thuringiensis*: Current insights and ecological significance

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Abstract

Bacillus thuringiensis (Bt) is a widely used entomopathogenic bacterium known for its Cry toxin mediated insecticidal activity and its central role in biological pest management. In addition to toxin production, Bt has the capacity to form biofilms, structured microbial communities embedded within an extracellular polymeric matrix that enhance bacterial survival under diverse environmental conditions. Biofilm formation contributes to protection against abiotic stresses such as desiccation, temperature fluctuations, nutrient limitation and ultraviolet exposure, thereby supporting environmental persistence in soil and plant-associated habitats. Variability in biofilm-forming ability among Bt isolates suggests that this trait may influence ecological adaptability and persistence. Although biofilm formation does not directly alter toxin potency, it may indirectly affect field performance by promoting stability and prolonged survival of bacterial propagules. This review summarizes current knowledge on biofilm formation in Bt, outlines commonly used detection and visualization methods, and discusses its ecological significance. Key research gaps are highlighted to provide direction for future studies aimed at improving the effectiveness and sustainability of Bt-based pest management strategies.

Keywords: *Bacillus thuringiensis*, biofilm, entomopathogenic bacterium, extracellular polymeric matrix, biocontrol agents, pest management

Introduction

Biofilms represent structured communities of microorganisms attached to biotic or abiotic surfaces and embedded within a self-produced Extracellular Polymeric Substance (EPS). This matrix, mainly composed of polysaccharides, proteins, lipids and extracellular DNA, provides structural stability and protection to bacterial cells under adverse environmental conditions (Flemming *et al.*, 2016; Flemming and Wuertz, 2019; Alotaibi *et al.*, 2021) [16, 15, 2]. Biofilm formation is considered a survival strategy that enhances resistance to environmental stresses such as desiccation, nutrient limitation, temperature fluctuations and antimicrobial agents (Upadhyay *et al.* 2025; Gupta *et al.*, 2026; Rajagopalan *et al.*, 2026) [19, 39, 51]. In natural ecosystems, most bacteria exist predominantly in biofilm form rather than as free-living planktonic cells (Li *et al.*, 2021) [27].

Among entomopathogenic bacteria, *Bacillus thuringiensis* is widely recognized for its insecticidal properties and extensive use in biological pest management (Manjunatha *et al.* 2023) [32]. This Gram-positive, spore-forming bacterium produces parasporal crystalline proteins (Cry and Cyt toxins) during sporulation, which are toxic to various insect orders, particularly Lepidoptera (Venu *et al.* 2024; Manjunatha *et al.* 2026) [47, 52]. Due to its specificity, environmental safety and compatibility with integrated pest management programs, *B. thuringiensis* has been extensively utilized as a microbial biopesticide worldwide.

Beyond toxin production, the ecological fitness and persistence of *B. thuringiensis* in soil and plant environments are crucial for its effectiveness. In this context, biofilm formation has emerged as an important trait influencing bacterial survival, colonization ability and long-term stability. Biofilm-producing strains may exhibit enhanced resistance to environmental stresses (Zhao *et al.* 2025; Jalali *et al.* 2020; Jalali *et al.* 2024; An *et al.* 2024; Azizoglu *et al.* 2023) [3, 4, 23, 24, 49] and improved adherence to plant surfaces or insect gut tissues, potentially affecting their insecticidal performance.

Recent studies indicate that variability exists among indigenous isolates in their capacity to produce biofilm (Deeparani *et al.* 2025) [8], suggesting strain-dependent differences that may influence ecological adaptability and virulence.

Understanding the production patterns, regulatory factors and detection methods of biofilm formation in *B. thuringiensis* is therefore essential for optimizing its application as a biocontrol agent. Although substantial research has been conducted on toxin production and insecticidal activity, comparatively less attention has been given to the biofilm-forming ability of indigenous isolates and its possible implications for insecticidal potential. This review aims to summarize current knowledge on biofilm formation in *B. thuringiensis*, the factors influencing its development, commonly employed detection techniques, and its ecological and applied significance in pest management

Biofilm formation in *Bacillus thuringiensis*

Biofilm formation represents a structured survival strategy that enables bacteria to persist under fluctuating environmental conditions. In *Bacillus thuringiensis*, biofilm development is a coordinated and regulated process involving cellular differentiation, extracellular matrix production, and surface colonization. *Bacillus thuringiensis* forms a floating biofilm at the air-liquid interface, consisting of two distinct structural components: a peripheral ring and a pellicle. The ring is a dense adherent structure attached to the walls of the culture vessel, whereas the pellicle is a surface-associated layer that spreads across the liquid

interface and integrates with the peripheral ring (El-Khoury *et al.* 2016) [13]. Although biofilm formation has been extensively characterized in other *Bacillus* species, increasing evidence suggests that Bt also possesses a comparable capacity for structured community formation.

Stages of biofilm development

Biofilm formation in *B. thuringiensis* follows a multistep progression (Sauer *et al.* 2022) [43]:

1. **Initial reversible attachment:** Reversible adhesion of planktonic cells to biotic or abiotic surfaces.
2. **Irreversible attachment:** Strengthened adhesion through production of extracellular polymeric substances (EPS).
3. **Microcolony formation:** Localized cell proliferation and aggregation.
4. **Maturation:** Development of a three-dimensional biofilm architecture embedded within an EPS matrix.
5. **Dispersion:** Release of cells to colonize new niches.

The extracellular matrix, primarily composed of exopolysaccharides, proteins, and extracellular DNA, provides structural integrity and acts as a protective barrier against environmental stress (Flemming *et al.*, 2016; Flemming and Wurtz, 2019) [16, 15]. Biofilm-associated cells often display altered physiological states compared to planktonic cells, including modified metabolic activity and enhanced stress tolerance (Li *et al.* 2021; Rinaudi and Giordano, 2010; Bernbom *et al.*, 2011; Koerdt *et al.*, 2011; Dang and Lovell, 2016) [7, 25, 27, 40, 53].

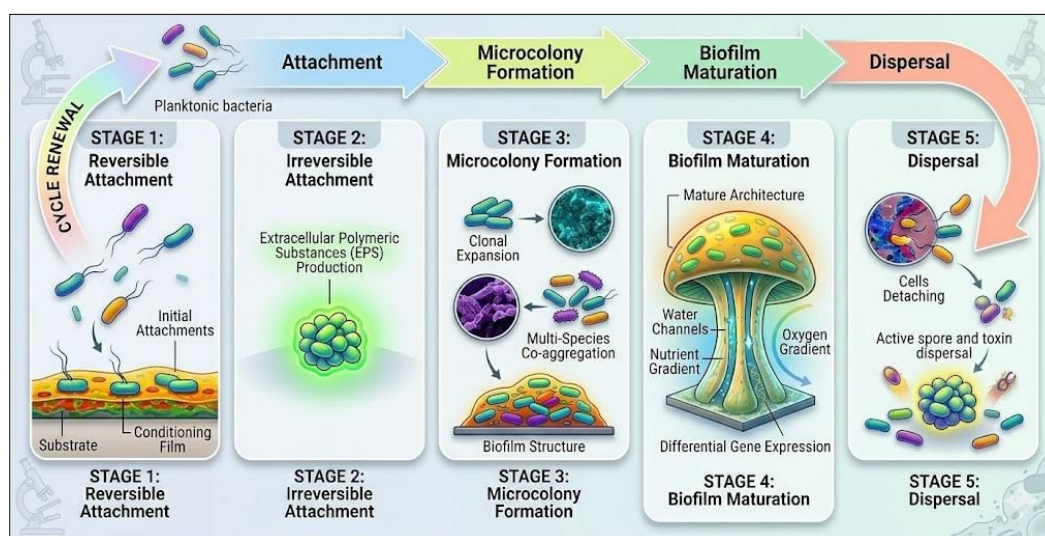


Fig 1. Stages of biofilm formation in *Bacillus thuringiensis* (Bt)

Regulatory and physiological aspects

Biofilm formation in members of the *Bacillus cereus* group is controlled by complex regulatory networks. Although the regulatory pathways in Bt are not as comprehensively studied as in *Bacillus subtilis*, homologous systems such as Spo0A-dependent signaling and quorum sensing mechanisms have been reported to influence surface attachment and community behaviour (Verplaetse *et al.* 2017) [54]. These regulatory systems coordinate the balance between vegetative growth, sporulation, and biofilm development. Interestingly, biofilm formation and sporulation may represent alternative survival strategies under nutrient limitation. In some strains, enhanced biofilm

production has been associated with improved persistence, whereas in others, sporulation predominates. This suggests that ecological adaptation may determine the preferred survival pathway in different Bt isolates.

Strain-Dependent variation in biofilm production

Considerable variation exists among various Bt strains in their biofilm-forming capacity (Hussain and Oh, 2017, Lim *et al.*, 2021; Zhao *et al.* 2024) [21, 30, 50]. Studies have categorized isolates as weak, moderate, or strong producers based on biomass accumulation in microtiter assays (Deeparani *et al.* 2025) [8]. Such variability may be

attributed to genetic diversity, environmental origin, and differential regulation of extracellular matrix components.

Factors influencing biofilm formation in *Bacillus thuringiensis*

Biofilm formation in *Bacillus thuringiensis* is not a static trait but a dynamic response influenced by environmental, nutritional, and strain-specific factors. Variations in growth conditions can significantly alter Extracellular Polymeric Substance (EPS) production, surface adhesion capacity, and overall biofilm architecture. Understanding these influencing factors is essential for interpreting differences among indigenous isolates and optimizing their field performance.

Temperature

Temperature is a key environmental determinant regulating biofilm formation in *Bacillus thuringiensis*. Most Bt isolates exhibit optimal biofilm development within the mesophilic range (approximately 28-37 °C), which coincides with their optimal growth temperature. At this range, enhanced enzymatic activity and nutrient metabolism promote active cell proliferation and increased production of Extracellular Polymeric Substances (EPS), facilitating rapid microcolony formation and matrix development.

Deviation from the optimal temperature can significantly influence biofilm architecture and stability. At lower temperatures, reduced metabolic rates and enzyme kinetics may limit EPS synthesis and delay biofilm maturation. In contrast, elevated temperatures beyond the optimal range may disrupt membrane integrity, impair protein stability, and affect adhesion mechanisms, thereby reducing biofilm biomass. Temperature also modulates bacterial surface properties, including hydrophobicity and the expression or functionality of surface appendages such as flagella and pili (Garrett *et al.* 2008) [17], which are critical for initial attachment.

Furthermore, temperature influences the physicochemical characteristics of the EPS matrix (Pagan *et al.* 2015) [37], including polysaccharide viscosity and gel-forming properties, which contribute to biofilm cohesion and mechanical stability. In Bt, temperature may additionally regulate the balance between vegetative growth, sporulation, and biofilm formation, suggesting an adaptive physiological response to environmental stress. Thus, temperature not only governs growth kinetics but also shapes the structural integrity and persistence of Bt biofilms.

pH

Environmental pH affects bacterial surface charge, membrane stability, and enzyme activity, thereby influencing attachment efficiency (Agarwal *et al.* 2011) [1]. Neutral to slightly alkaline conditions generally favour

biofilm development, as optimal enzymatic function supports exopolysaccharide (EPS) production and stable surface adhesion. Extreme acidic or alkaline conditions can reduce adhesion efficiency and impair matrix synthesis, leading to decreased biofilm biomass and structural instability. The physicochemical properties of EPS, including viscosity and gel strength, are also pH-dependent and contribute to biofilm cohesion (Nostro *et al.* 2012; Chaieb *et al.* 2007) [34, 25].

Since agricultural soils and phylloplane environments exhibit variable pH conditions, this factor is particularly important in determining the ecological adaptability and persistence of indigenous Bt isolates.

Nutrient availability

Nutrient composition is one of the most significant determinants of biofilm production (Kumar *et al.* 1998; Prakash *et al.* 2003; Tilahun *et al.* 2016) [26, 38, 45]. Carbon sources such as glucose have been reported to stimulate EPS synthesis, thereby enhancing biofilm biomass (Bühler *et al.* 1998; Rochex *et al.* 2007) [47, 55]. Conversely, nutrient limitation may trigger stress-induced survival responses that promote community formation. Nitrogen limitation, in particular, can shift bacterial physiology toward protective strategies such as biofilm formation or sporulation (Delaquis *et al.* 1989; Rochex and Lebeault, 2007) [9, 47]. The interaction between nutrient stress and regulatory signaling pathways may therefore determine the extent of biofilm development in different isolates.

Surface characteristics

Surface hydrophobicity and texture significantly influence initial bacterial attachment. Hydrophobic surfaces generally promote stronger adhesion due to enhanced physicochemical interactions (Pagan *et al.* 2015; Donlan 2002) [37, 11]. In laboratory assays, polystyrene microtiter plates are commonly used; however, in natural environments, Bt may encounter plant cuticles, soil particles, or insect gut surfaces, each presenting distinct attachment challenges. Thus, biofilm formation observed under laboratory conditions may not always directly reflect field performance, highlighting the need for cautious interpretation.

Strain-Dependent variation

Substantial heterogeneity exists among indigenous Bt isolates with respect to biofilm-forming capacity (Deeparani *et al.* 2025) [8]. Such variation may arise from: Genetic diversity, differences in regulatory pathways, ecological adaptation, environmental origin. Isolates recovered from soil, rhizosphere, or insect cadavers may exhibit differential production patterns, potentially reflecting adaptation to specific ecological niches.

Table 1: Factors Influencing Biofilm Formation in Bt Isolates

Factor	Favorable Condition	Possible Mechanism	Observed Outcome
Temperature	28-37 °C	Optimal metabolic and enzymatic activity	Increased EPS production
pH	Neutral-slightly alkaline	Improved cell surface interaction	Enhanced attachment
Carbon source	Moderate glucose availability	Stimulation of EPS synthesis	Increased biomass
Nitrogen limitation	Reduced nitrogen	Stress-induced survival response	Biofilm enhancement
Surface type	Hydrophobic surfaces	Stronger physicochemical adhesion	Higher colonization
Strain variability	Indigenous diversity	Genetic and ecological differences	Weak to strong producers

(Deeparani *et al.* 2025) [8].

Although several environmental factors influencing biofilm formation have been identified, their relative contribution varies among isolates. Furthermore, most studies have been conducted under controlled laboratory conditions, and field-level validation remains limited. Therefore, future investigations should aim to integrate environmental simulation models with standardized quantification techniques to better predict performance in agricultural ecosystems.

Detection and quantification of biofilm in *Bacillus thuringiensis*

Accurate detection and quantification of biofilm formation are essential for comparing production patterns among *Bacillus thuringiensis* isolates. A variety of qualitative and quantitative techniques have been employed, each with specific advantages and limitations. Selection of an

appropriate method depends on the objective of the study, required sensitivity, and available laboratory facilities.

Microtiter plate (Crystal Violet) assay

The microtiter plate assay remains the most widely used method for screening biofilm formation in Bt isolates. In this assay, bacterial cultures are incubated in polystyrene wells, allowing surface-attached biofilm to develop. Following incubation, non-adherent cells are removed, and attached biomass is stained with crystal violet. The retained dye is solubilized and quantified spectrophotometrically (Basak 2013; O'Toole 2011; Djordjevic *et al.* 2002) [10, 36, 56]. This method provides: Rapid screening of multiple isolates, quantitative comparison using optical density values and classification into weak, moderate, and strong biofilm producers. However, it primarily measures total biomass (cells + matrix) and does not distinguish between viable and non-viable cells.

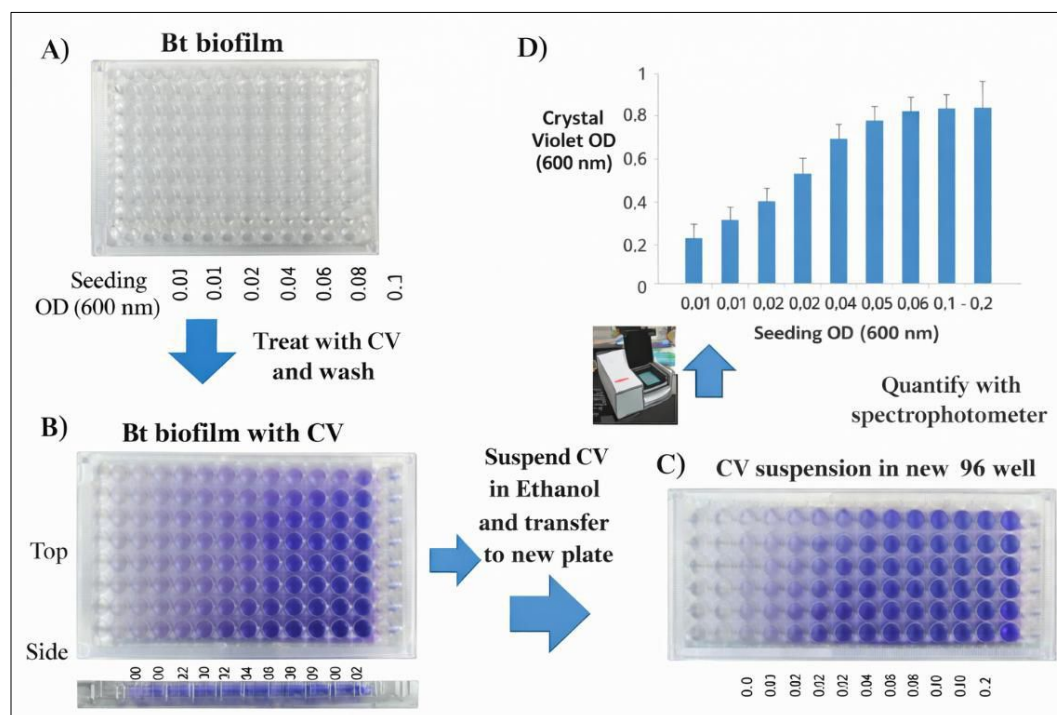


Fig 2: Schematic representation of the crystal violet microtiter plate assay for biofilm quantification

Biofilm formation in 96-well plates followed by washing, crystal violet staining, solubilization of bound dye, and spectrophotometric measurement of Optical Density (OD) for quantitative assessment of biofilm biomass. Adapted from Wilson *et al.*, 2017 [48].

Congo red agar (CRA) method

Congo red agar is a qualitative method used to detect exopolysaccharide production, a key component of the

biofilm matrix. Bt isolates producing significant extracellular polymeric substances often form black, dry, crystalline colonies on CRA plates. Although simple and cost-effective, this method: Provides only qualitative information, is influenced by medium composition and incubation conditions and may not correlate perfectly with quantitative biofilm biomass. Still, it is useful for preliminary screening of indigenous isolates.

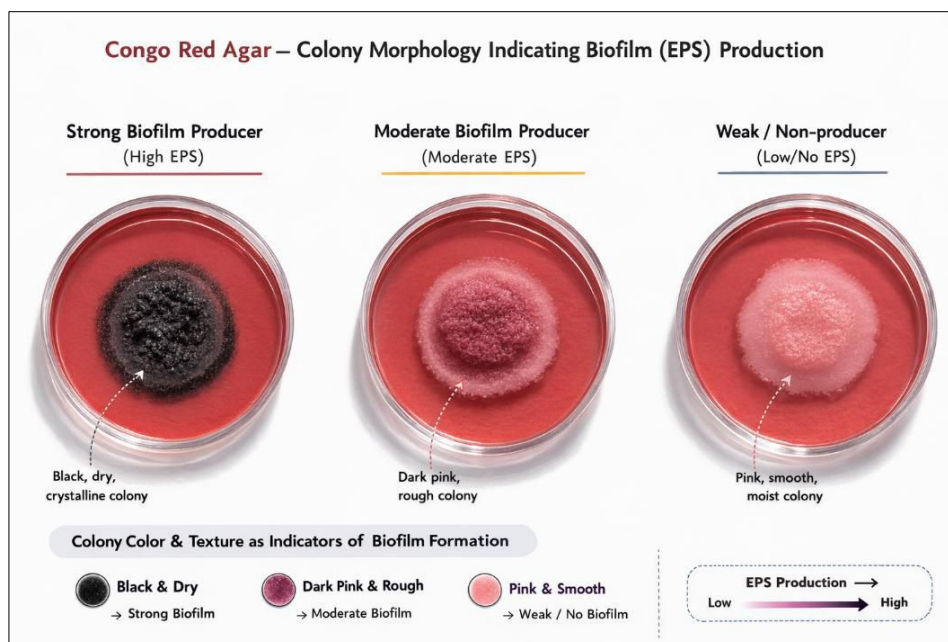


Fig 3: Morphological characteristics of biofilm-forming isolates on Congo Red Agar medium.

Black, dry crystalline colonies indicate strong biofilm producers; dark pink rough colonies indicate moderate producers; and pink smooth colonies indicate weak or non-biofilm producers.

Microscopic techniques

Microscopic approaches allow visualization of biofilm architecture and structural organization.

- a) **Light Microscopy:** Used for preliminary visualization of surface attachment.
- b) **Scanning Electron Microscopy (SEM):** Provides detailed imaging of biofilm morphology, matrix

distribution, and cell aggregation. SEM studies in Bt have demonstrated dense multilayered structures in strong biofilm-producing strains (Baum *et al.* 2009; Holling *et al.* 2014) [20].

- c) **Confocal Laser Scanning Microscopy (CLSM):** CLSM enables three-dimensional visualization of biofilms, often combined with fluorescent staining to differentiate live and dead cells (Jakobs *et al.* 2000; Nwaneshiudu 2012) [22, 35]. These methods provide structural insights but require specialized equipment and technical expertise.

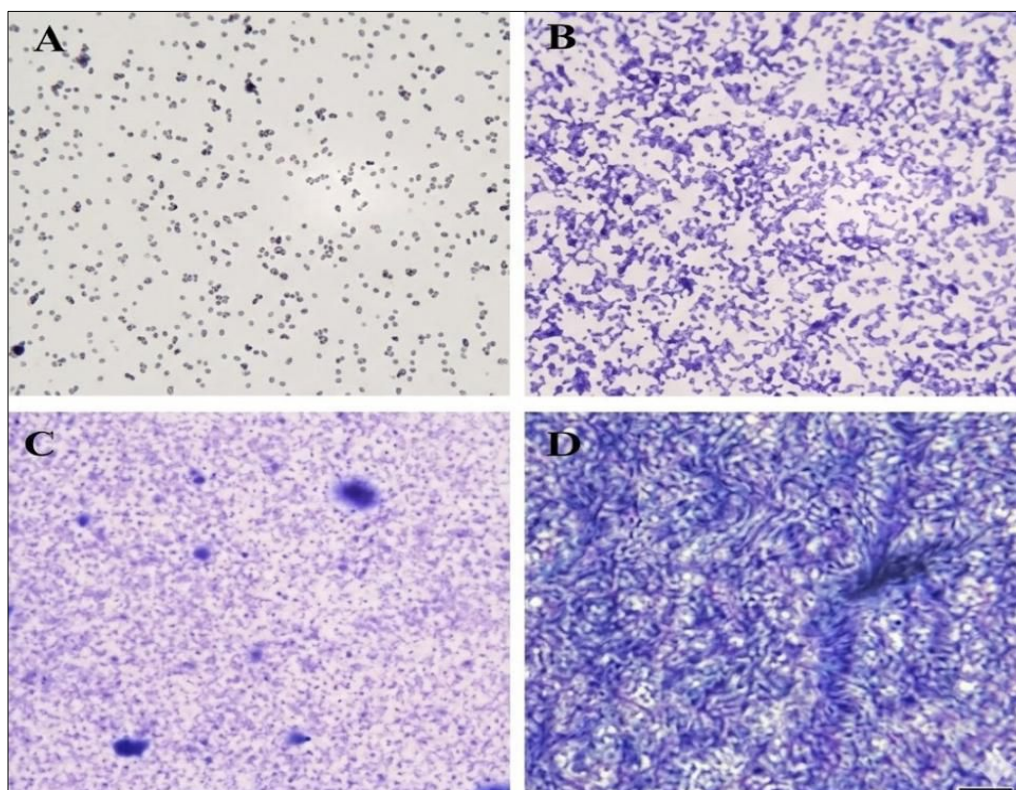


Fig 4: Light microscopic visualization of biofilm development over time

Light microscopy images showing progressive stages of biofilm formation in a static culture system following crystal violet staining. Biofilm development is illustrated at early (A), initial attachment (B), intermediate (C), and mature (D) stages after inoculation. Adapted from Wilson *et al.* 2017^[48].

Table 2: Comparative overview of methods used for detection and quantification of biofilm formation.

Method	Type	Quantitative/Qualitative	Advantages	Limitations
Microtiter plate assay	Biomass staining	Quantitative	Simple, reproducible, high throughput	Measures total biomass only
Congo Red Agar	EPS detection	Qualitative	Cost-effective	Medium-dependent variability
SEM	Structural analysis	Qualitative	High-resolution imaging	Expensive, destructive
CLSM	3D biofilm imaging	Semi-quantitative	Live/dead differentiation	Requires fluorescent staining
Dry weight method	Biomass estimation	Quantitative	Simple approach	Low precision

(Wilson *et al.* 2017)^[48].

Ecological and applied significance of biofilm formation in *Bacillus thuringiensis*

Biofilm formation in *Bacillus thuringiensis* (Bt) is not merely a laboratory phenotype but represents an ecologically relevant survival strategy that enhances environmental persistence, stress tolerance, and potentially insecticidal performance. Understanding the ecological and applied implications of biofilm production is essential for improving the field efficacy of Bt-based biopesticides.

Environmental persistence and stress tolerance

In natural agroecosystems, Bt is exposed to multiple abiotic stresses, including: UV radiation, Desiccation, Temperature fluctuations, Nutrient limitation. Biofilm-associated Bt cells are embedded within an extracellular polymeric matrix composed of polysaccharides, proteins, and extracellular DNA. This matrix acts as a protective barrier that: reduces UV penetration, minimizes water loss, buffers sudden environmental changes (Semenyuk *et al.* 2014)^[44]. Consequently, biofilm-forming isolates may survive longer on plant surfaces compared to planktonic cells (Li *et al.* 2024; Zhao *et al.* 2025)^[28, 49]. Enhanced persistence directly influences the duration of insecticidal activity under field conditions.

Survival on Phylloplane and rhizosphere

Bt naturally inhabits soil and plant-associated environments. Biofilm formation facilitates: stable attachment to leaf surfaces (phylloplane), colonization of root zones (rhizosphere), aggregation in soil microhabitats. On the phylloplane, biofilm structures protect cells from UV exposure and washing by rain (Elhariry 2011; Zhao *et al.* 2024)^[12, 50]. In the rhizosphere, biofilms enhance nutrient acquisition and interaction with plant exudates. This ecological adaptability may explain why certain indigenous isolates exhibit better field persistence than commercial strains.

Impact on insecticidal potential

Although the insecticidal activity of Bt primarily depends on Cry toxins and spore ingestion by larvae, biofilm formation may indirectly influence toxicity through: improved survival until ingestion, enhanced sporulation stability (Semenyuk *et al.* 2014)^[44], increased retention on plant surfaces (Zhao *et al.* 2024)^[50], protection of toxin crystals from environmental degradation (Ajijah *et al.* 2023)^[57]. Some studies suggest that strong biofilm-forming strains show improved persistence, which may translate into prolonged

Dry weight and biomass estimation

In some studies, biofilm biomass is scraped from surfaces, dried, and weighed to determine total mass (Trulear *et al.* 1982; Characklis 1990)^[46, 6]. While simple, this approach s

insect mortality (Li *et al.* 2021)^[27] under semi-field conditions. However, the relationship between biofilm intensity and direct toxicity remains complex and requires further investigation. While biofilm formation enhances environmental stability, it does not necessarily increase intrinsic toxin potency; rather, it contributes to sustained exposure of larvae to viable spores and toxin crystals.

Implications for biopesticide formulation

Biofilm-forming ability may be exploited in developing improved Bt formulations. Potential applications include: Selection of strong biofilm-producing indigenous isolates for formulation, Incorporation of biofilm-promoting additives, Development of slow-release or surface-retentive formulations. Biofilm-associated cells may exhibit improved shelf stability and field persistence, thereby reducing the frequency of application. However, excessive biofilm formation could also pose challenges in large-scale fermentation and downstream processing (Flach *et al.*, 2014)^[14], indicating the need for balanced selection criteria.

Role in resistance management

Sustained persistence of Bt in the environment due to biofilm formation may influence resistance dynamics in target pests. Prolonged exposure at sublethal concentrations could theoretically accelerate resistance development (Liang *et al.*, 2012; Gong *et al.*, 2022; Margus *et al.* 2024)^[29, 18, 33] if not managed properly. Therefore, integrating biofilm-forming Bt strains into integrated pest management (IPM) programs requires careful monitoring to ensure long-term efficacy.

Research gaps and future perspectives

Although biofilm formation in *Bacillus thuringiensis* has gained increasing attention, several important gaps remain in understanding its ecological and applied relevance. Most available studies have focused on laboratory-based characterization of biofilm production using standard *in vitro* assays, whereas systematic evaluation of its relationship with insecticidal efficacy against target pests such as *Plutella xylostella* under semi-field and field conditions remains limited. The extent to which biofilm-forming ability contributes to prolonged spore persistence, Cry toxin stability, and sustained larval mortality requires further investigation.

Additionally, considerable variability exists in methodological approaches used for biofilm quantification, making cross-study comparisons difficult. Standardization

of assay conditions and classification criteria would improve reproducibility and reliability of data. While regulatory pathways governing biofilm formation are well characterized in model bacteria, the molecular mechanisms controlling adhesion, extracellular polymeric substance production, and their interaction with sporulation processes in Bt remain insufficiently explored.

From an applied perspective, the potential exploitation of strong biofilm-forming indigenous isolates for improved formulation stability and surface retention has not been systematically assessed. Furthermore, the ecological consequences of enhanced environmental persistence, including its possible implications for resistance development in target pests, warrant careful evaluation. Future research integrating physiological, molecular, ecological, and formulation-based approaches will be essential to fully elucidate the role of biofilm formation in enhancing the field performance and sustainability of Bt-based biopesticides.

Conclusion

Biofilm formation is an important adaptive trait in *Bacillus thuringiensis* that enhances environmental persistence and survival under adverse conditions. While Bt is primarily recognized for its Cry toxin-mediated insecticidal activity, its ability to form structured biofilms may indirectly contribute to improved spore stability, surface adherence, and prolonged exposure to target pests. Variability in biofilm-forming capacity among isolates highlights its potential relevance in strain selection for biocontrol applications. However, a clear mechanistic link between biofilm formation and enhanced field efficacy remains to be fully established. Future studies integrating biofilm characterization with toxicity assays and molecular analyses will be essential to optimize Bt-based pest management strategies.

Disclaimer (Artificial intelligence)

Author(s) hereby declares that NO generative AI technologies such as Large Language Mode (Chat GPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

Competing interests

Authors have declared that no competing interests exist.

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