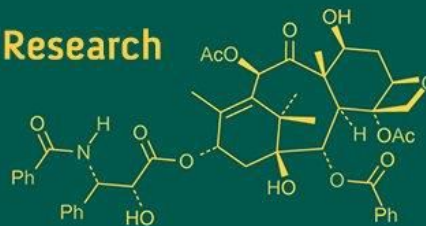
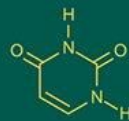
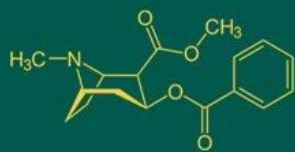


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 NAAS Rating (2025): 5.29
 IJABR 2025; 9(7): 1584-1590
www.biochemjournal.com
 Received: 20-04-2025
 Accepted: 24-05-2025

Deepa Rani N
 Department of Entomology,
 Dr. PDKV, Akola,
 Maharashtra, India

Dr. SK Bhalkare
 Department of Entomology,
 Dr. PDKV, Akola,
 Maharashtra, India

Dr. DB Undirwade
 Department of Entomology,
 Dr. PDKV, Akola,
 Maharashtra, India

Dr. US Kulkarni
 Department of Entomology,
 Dr. PDKV, Akola,
 Maharashtra, India

Dr. MP Moharil
 Department of Biotechnology,
 Dr. PDKV, Akola,
 Maharashtra, India

Corresponding Author:
Deepa Rani N
 Department of Entomology,
 Dr. PDKV, Akola,
 Maharashtra, India

Qualitative and quantitative evaluation of biofilm formation in indigenous *Bacillus thuringiensis* isolates

Deepa Rani N, Bhalkare SK, Undirwade DB, Kulkarni US and Moharil MP

DOI: <https://www.doi.org/10.33545/26174693.2025.v9.i7t.4996>

Abstract

Bacillus thuringiensis is a widely used entomopathogenic bacterium known for its biopesticidal potential. Beyond insecticidal activity, its ability to form biofilms enhances persistence, stress tolerance, and sporulation. In present study, twenty indigenous Bt isolates along with standard Btk HD-1 strain were evaluated for biofilm formation under static (non-agitated), farmer-friendly conditions across different time intervals (24 to 96 h). The study involved qualitative and quantitative assessments of biofilm formation, in different biofilm components (ring and pellicle). At 24 h, only SA-18 formed a ring. At 48 h, SY-4, SJa-2, SA-22, SDa-1, and HD-1 developed both ring and pellicle; SGd-1, SA-18, and SUD-2 formed ring only; and 12 other isolates formed pellicle only, while SBn-2 showed no visible biofilm. At 72 and 96 h, most isolates (including HD-1, SA-6, SA-20, SGd-1, SY-4, and others) produced both ring and pellicle, whereas SBn-2, SGn-4, and SAK-12 showed only pellicle formation. Based on intensity and coverage, 8 isolates were classified as strong ring biofilm producers and 10 isolates were classified as weak ring biofilm producers whereas 15 isolates were classified as strong pellicle biofilm producers and 6 isolates were classified as weak pellicle biofilm producers. The Isolate SA-18 showed the highest OD and biomass (60.5 mg) in ring component, while SAK-12 recorded the highest OD and biomass (113.5 mg) in pellicle component. These results reveal high variability in biofilm formation among Bt isolates, supporting their potential use as persistent and efficient biocontrol agents under simple and low-cost cultivation methods.

Keywords: *Bacillus thuringiensis*, biofilm, indigenous isolates, static culture, biocontrol potential

1. Introduction

Bacillus thuringiensis (Bt) is a facultative anaerobic, gram-positive, motile, spore-forming, rod-shaped bacterium (Martin and Travers., 1989) [10] widely used in biological pest control due to its production of insecticidal crystal proteins (Cry and Cyt toxins) that target specific insect pests, particularly those in the orders Lepidoptera, Diptera, and Coleoptera (Vidarthi *et al.*, 2002) [19]. These proteins are known for their high specificity and environmental safety, making Bt one of the most successful microbial biopesticides in the world. Bt accounts for over 90% of the global biopesticide market and is considered the most successful microbial pesticide used in agriculture, forestry, and public health (Sanchis and Bourguet., 2008) [15]. Beyond toxin production, Bt has the ability to form biofilms (Nay *et al.*, 2016). Bacterial biofilms are aggregates of bacteria embedded in extracellular polymeric substances (EPSs) produced by the bacteria themselves. The EPS matrix typically includes extracellular polysaccharides, proteins, lipids, and DNA, enabling the biofilm to adhere to a range of interfaces such as solid-liquid, solid-gas, liquid-liquid, and liquid-gas surfaces (Flemming *et al.*, 2016 [6]; Flemming and Wuerzt., 2019) [5]. Bacteria are capable of colonizing nearly all natural and synthetic surfaces by forming biofilms (Hall-Stoodley *et al.*, 2004 [7]; Sweet *et al.*, 2011 [18]; Flemming and Wuerzt., 2019) [5]. Compared with planktonic cells, the most important feature of bacterial biofilm is that it can help bacteria resist different environmental stresses such as ultraviolet (UV) radiation, antibiotic treatment, drying, temperature variation, oxidative stress, nutrient depletion, and pH change (Rinaudi and Giordano, 2010 [13]; Bernborn *et al.*, 2011 [2]; Koerdt *et al.*, 2011 [9]; Dang and Lovell, 2016) [3]. Many *B. thuringiensis* strains can form biofilms that float on the surface of liquid culture media (Wijman *et al.*, 2007 [20]; Auger *et al.*, 2009) [1, 16]. These surface biofilms have a distinct structure, typically featuring a dense ring that adheres to the vessel wall and encircles the

floating pellicle, which often exhibits visible protrusions (Fagerlund *et al.*, 2014) [4]. Conventional culturing of Bt typically involves shaking/incubation under aerated conditions. However, this requires costly infrastructure and high energy inputs (Rowe and Margaritis, 2004) [14]. Static culture, on the other hand, can simulate simple conditions suitable for farmer-level or resource-constrained applications. Such non-agitated systems are more accessible and can serve as farmer-friendly methods for microbial mass culturing.

The current study aims to screen indigenous Bt isolates for their biofilm production ability under static (non-agitated) conditions. Wet pellet weight was used as a direct indicator of biofilm formation, and OD₆₀₀ readings were recorded to observe growth dynamics under static incubation. This approach can help identify robust, locally adapted Bt strains for further development as biocontrol agents.

2. Materials and Methods

2.1 Bacterial Isolates

A total of twenty indigenous *Bacillus thuringiensis* (Bt) isolates collected from various agro-ecological zones of Maharashtra and maintained at the Department of Entomology and Biotechnology centre, Post Graduate Institute, Dr. PDKV, Akola, were used in this study. The standard strain *Bacillus thuringiensis* subsp. *kurstaki* HD-1 was included for comparison. The details of Bt isolates, used for evaluation of biofilm production are presented in (Table 1). The previously isolated Bt cultures were sub-cultured and maintained on nutrient agar plates at 4 °C for further use.

Table 1: List of Bt isolates used for evaluation of biofilm production pattern

S. No.	Bt isolate	NCBI Accession no.	Source
1	HD-1	5118	NCIM
2	PDKV-Bt-SA-6	ON331907.1	Department of Entomology (Dr. PDKV, AKOLA)
3	PDKV-Bt-SA-20	ON331908.1	
4	PDKV-Bt-SGd-1	ON331905.1	
5	PDKV-Bt-SY-4	ON331906.1	
6	PDKV-Bt-SA-18	OP209984	
7	PDKV-Bt-SAk-6	OP209985	
8	PDKV-Bt-SAk-9	OP209986	
9	PDKV-Bt-SGn-5	OP209988	
10	PDKV-Bt-SBn-2	OP209989	
11	PDKV-Bt-SGn-4	OP209987	
12	PDKV-Bt-SUd-2	PP265276.1	Biotechnology centre (Dr. PDKV, AKOLA)
13	PDKV-Bt-SJa-2	PP265269.1	
14	PDKV-Bt-SJa-1	PP265259.1	
15	PDKV-Bt-SHi-1	PP264549.1	
16	PDKV-Bt-SDa-2	PP264546.1	
17	PDKV-Bt-SDa-1	PP264537.1	
18	PDKV-Bt-SA-22	PP724411.1	
19	PDKV-Bt-SMa-1	PP724417.1	
20	PDKV-Bt-SAk-12	PP724433.1	
21	PDKV-Bt-SUd-1	PP724421.1	

2.2 Screening of *Bacillus thuringiensis* isolates for biofilm production under static culture condition

Biofilm development was evaluated under static (non-agitated) conditions using sterile glass test tubes (25 × 150 mm) containing 20 mL of sterile Luria-Bertani (LB) broth. Initially, precultures of each *Bacillus thuringiensis* (Bt) isolate were prepared by inoculating the isolates into LB broth and incubating overnight at 30±2 °C under shaking conditions. From these precultures, 5% (v/v) inoculum was

transferred into glass test tubes containing fresh sterile LB broth for biofilm production. The inoculated tubes were then incubated at 30±2 °C under static conditions (Fagerlund *et al.*, 2014) [4]. Observations on biofilm formation were recorded at 24, 48, 72, and 96 hours post-inoculation.

2.2.1 Qualitative Assessment of Biofilm Formation

At each time point (24 h, 48 h, 72 h, and 96 h), the test tubes were visually examined for the presence of ring (formed at the liquid-air interface) and pellicle (floating biofilm) structures. Based on the coverage, density, and strength of the biofilm, isolates were categorized as:

- Strong biofilm producers
- Weak biofilm producers
- No visible biofilm

2.2.2 Quantitative Estimation of Biofilm Formation

After 96 hours, both the ring and the pellicle part of biofilm were harvested separately into sterile microcentrifuge tubes (Mengmeng *et al.*, 2021). For ring recovery, the biofilm was manually scrapped from the sides of the tubes using a micropipette tip and transferred into microcentrifuge tube and the floating pellicle at the bottom was recovered using a micropipette and transferred to a separate microcentrifuge tube (Auger *et al.*, 2005). The samples were then centrifuged at 8000 rpm for 5 minutes, and the resulting pellet yield was recorded using a digital precision balance and their corresponding OD₆₀₀ values were determined using spectrophotometer.

2.3 Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean±standard error (SE). Differences in biofilm formation among the *Bacillus thuringiensis* isolates were analysed using one-way Analysis of Variance (ANOVA). The statistical analysis was conducted using GraphPad Prism version 9.0. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results and Discussion

3.1 Qualitative Assessment of Biofilm Formation

Biofilm development by *Bacillus thuringiensis* (Bt) isolates was visually evaluated through the observation of pellicle and ring structures in static culture tubes at different incubation intervals (24, 48, 72, and 96 hours). At 24 hours, isolate SA-18 alone exhibited ring formation. At 48 hours, isolates SY-4, SJa-2, SA-22, SDa-1, and the standard strain HD-1 produced both ring and pellicle structures. Three isolates SGd-1, SA-18, and SUd-2 formed only ring, while 12 isolates viz., (SA-6, SA-20, SAK-6, SAK-9, SGn-5, SGn-4, SJa-1, SHi-1, SDa-2, SMA-1, SAK-12 and SUd-1) exhibited only pellicle formation. Whereas, SBn-2 showed no visible biofilm.

At 72 hours, 18 isolates viz., (SA-6, SA-20, SGd-1, SA-18, SAK-6, SAK-9, SGn-5, SY-4, SUd-2, SJa-2, SJa-1, SHi-1, SDa-2, SDa-1, SA-22, SMA-1, SUd-1) including HD-1, produced both ring and pellicle structures, while three isolates (SBn-2, SGn-4, and SAK-12) produced only pellicle. At 96 hours, the same three isolates (SBn-2, SGn-4, and SAK-12) continued to produce only the pellicle part, while the remaining eighteen isolates consistently developed both ring and pellicle biofilms.

Based on the intensity and coverage of biofilm formation, eight isolates (HD-1, SGd-1, SA-18, SAK-6, SAK-9, SY-4,

SJa-2, and SA-22) were categorized as strong ring biofilm producers, while ten isolates (SA-6, SA-20, SGn-5, SUD-2, SJa-1, SHi-1, SDa-2, SDa-1, SMA-1, and SUD-1) exhibited weak ring formation. With respect to pellicle development, fifteen isolates (HD-1, SA-6, SA-20, SGd-1, SAK-6, SAK-9, SY-4, SUD-2, SHi-1, SDa-2, SDa-1, SA-22, SAK-12, and SUD-1) demonstrated strong pellicle formation, while six isolates (SA-18, SGn-5, SBn-2, SGn-4, SJa-1, SJa-2, and SMA-1) were identified as weak pellicle producers (Table 2; Figure 1).

The results obtained in the present study regarding the variability in biofilm formation among different *Bacillus thuringiensis* isolates over time are in agreement with earlier findings. Karina *et al.* (2014)^[8] and Mengmeng *et al.* (2021) also reported considerable heterogeneity in the biofilm-forming ability among various Bt isolates. Similarly, Xingchen Zhao *et al.* (2024)^[21] observed that the Bt strain

SA-11 exhibited strong biofilm formation, whereas ABTS-351 showed weak to moderate formation, and ABTS-1857 failed to form biofilm altogether. Nay El-Khoury *et al.* (2016)^[12] studied biofilm development in Bt strains over time intervals from 16 to 96 hours and observed distinct structures ring and pellicle during the progression. According to their findings, no visible ring or pellicle was observed at 16 hours. By 20 hours, a thin pellicle surrounded by a ring began to appear, and at 24 hours, the biofilm developed a well-defined architecture characterized by a thick ring and dense pellicle layer. With prolonged incubation (36, 48, and 72 hours), both ring and pellicle structures became more prominent and well-developed, indicating dynamic changes in biofilm architecture over time. These observations align with our findings, where ring and pellicle formation varied significantly among isolates and intensified with longer incubation durations.

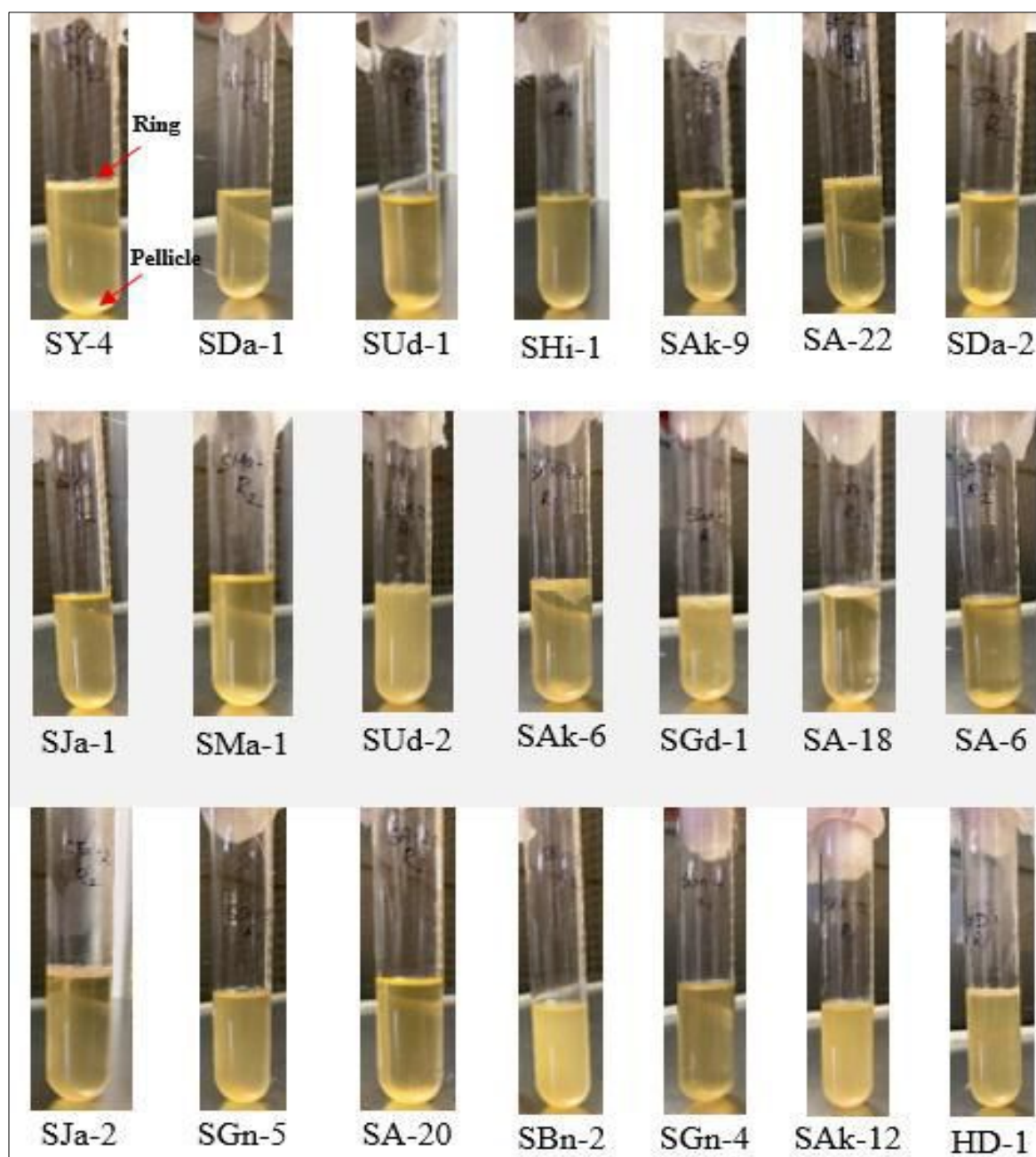


Fig 1: Biofilm formation by different *Bacillus thuringiensis* isolates after 96 hours of static incubation in LB broth. Pellicle formation is observed at the bottom, and ring biofilm at the air-liquid interface along the test tube wall. The image highlights variation in biofilm development among isolates, including the standard strain Bt-HD-1.

Table 2: Screening of *Bacillus thuringiensis* isolates for biofilm production

S. no	Bt isolates	24 h		48 h		72 h		96 h	
		Ring	Pellicle	Ring	Pellicle	Ring	Pellicle	Ring	Pellicle
1	HD-1	-	-	+	+	++	++	++	++
2	PDKV-Bt-SA-6	-	-	-	+	+	++	+	++
3	PDKV-Bt-SA-20	-	-	-	+	+	++	+	++
4	PDKV-Bt-SGd-1	-	-	+	-	++	++	++	++
5	PDKV-Bt-SA-18	+	-	+	-	++	+	++	+
6	PDKV-Bt-SAK-6	-	-	-	+	++	++	++	++
7	PDKV-Bt-SAK-9	-	-	-	+	++	++	++	++
8	PDKV-Bt-SGn-5	-	-	-	+	+	+	+	+
9	PDKV-Bt-SBn-2	-	-	-	-	-	+	-	+
10	PDKV-Bt-SY-4	-	-	+	+	++	++	++	++
11	PDKV-Bt-SGn-4	-	-	-	+	-	+	-	+
12	PDKV-Bt-Sud-2	-	-	+	-	+	++	+	++
13	PDKV-Bt-SJa-2	-	-	+	+	++	+	++	+
14	PDKV-Bt-SJa-1	-	-	-	+	+	+	+	+
15	PDKV-Bt-Shi-1	-	-	-	+	+	++	+	++
16	PDKV-Bt-SDa-2	-	-	-	+	+	++	+	++
17	PDKV-Bt-SDa-1	-	-	+	+	+	++	+	++
18	PDKV-Bt-SA-22	-	-	+	+	++	++	++	++
19	PDKV-Bt-SMa-1	-	-	-	+	+	+	+	++
20	PDKV-Bt-SAK-12	-	-	-	+	-	++	-	++
21	PDKV-Bt-SUD-1	-	-	-	+	+	++	+	++

++ Strong biofilm + Weak biofilm-No biofilm

3.2 Quantitative Estimation of Biofilm Formation

To evaluate the biofilm yield potential of *Bacillus thuringiensis* (Bt) isolates, the ring and pellicle components were harvested separately following 96 hours of static incubation. The biofilm materials were gently collected using a sterile pipette and centrifuged at 8000 rpm for 5 minutes to obtain biomass pellets. Quantitative assessment included measuring the wet weight of the biomass (pellet weight) and the optical density at 600 nm (OD_{600}) to estimate cell density. For each isolate, two sets of parameters were recorded: (i) Ring portion-pellet weight and OD_{600} value, and (ii) Pellicle portion-pellet weight and OD_{600} value.

3.2.1 Yield Assessment of Bt Isolates in Ring Biofilm

3.2.1.1 Ring Biofilm Pellet Biomass

The ring-associated biomass (Table 3; Figure 2) exhibited considerable variation among the tested *Bacillus thuringiensis* (Bt) isolates. Among the 18 isolates that produced visible ring structures, the pellet biomass ranged from 11.50 ± 1.50 mg to 60.50 ± 27.50 mg. The standard strain, HD-1, recorded a ring biomass of 26.00 ± 12.00 mg. Notably, the indigenous isolate SA-18 yielded the highest biomass (60.50 ± 27.50 mg), which was approximately 2.33 times greater than that of HD-1. This was followed by SJa-2 (51.50 ± 23.50 mg, 1.98 times) and SY-4 (35.50 ± 10.50 mg, 1.36 times). Isolates such as SGd-1 (25.00 ± 8.00 mg), SGn-5 (24.50 ± 0.50 mg), and SAK-6 (24.00 ± 1.00 mg) produced ring biomass comparable to that of the standard strain. Conversely, isolates including SGn-4, SBn-2, and SAK-12 exhibited no measurable ring biomass (0.00 ± 0.00 mg), suggesting poor or absent growth in ring form. Statistical analysis revealed a significant difference among the treatments ($p < 0.05$).

3.2.1.2 Ring Biofilm OD_{600} Analysis

The optical density (OD_{600}) values for the ring biomass of Bt isolates, as presented in Table 3 and Figure 3, ranged from 0.00 to 2.28. The standard strain HD-1 recorded an OD_{600} of 1.97. Among the indigenous isolates, SA-18 (2.28), SJa-2

(2.02), and SY-4 (1.98) recorded higher OD_{600} values than HD-1, indicating a greater cell density, which corresponded well with their respective ring pellet biomasses. Isolates such as SGd-1 (1.93) and SGn-5 (1.92) showed OD_{600} values comparable to the standard strain, suggesting similar biofilm-forming ability. In contrast, isolates like SA-22 (0.54), SMa-1 (0.62), SGn-4 (0.00), SBn-2 (0.00), and SAK-12 (0.00) exhibited markedly lower or negligible OD_{600} values, indicative of weak or absent ring biofilm development under static conditions.

Table 3: Yield Assessment of Bt Isolates in Ring Biofilm

Bt isolates	Pellet yield (mg/ring) Mean $\pm$ SE**	OD_{600} value (Cell density per ring)
Bt-HD-1	26.00 ± 12.00	1.97
PDKV-Bt-SA-6	17.50 ± 6.50	1.42
PDKV-Bt-SA-20	13.50 ± 7.50	0.75
PDKV-Bt-SGd-1	25.00 ± 8.00	1.93
PDKV-Bt-SA-18	60.50 ± 27.50	2.28
PDKV-Bt-SAK-6	24.00 ± 1.00	1.87
PDKV-Bt-SAK-9	22.50 ± 4.50	1.65
PDKV_Bt_SGn-5	24.50 ± 4.00	1.92
PDKV_Bt_SBn-2	0.00 ± 0.00	0
PDKV-Bt-SY-4	35.50 ± 10.50	1.98
PDKV-Bt-SGn-4	0.00 ± 0.00	0
PDKV_Bt_SUD_2	17.50 ± 5.50	1.41
PDKV_Bt_SJa_2	51.50 ± 23.50	2.02
PDKV_Bt_SJa_1	17.00 ± 3.00	1.38
PDKV_Bt_SHi_1	19.50 ± 7.50	1.63
PDKV_Bt_SDa_2	16.00 ± 6.00	1.12
PDKV_Bt_SDa_1	14.00 ± 7.00	0.84
PDKV_Bt_SA-22	11.50 ± 1.50	0.54
PDKV_Bt_SMa-1	12.50 ± 3.50	0.62
PDKV-Bt-SAK-12	0.00 ± 0.00	0
PDKV-Bt-SUD-1	23.50 ± 8.50	1.82
F value ($df=5,12$)	2.29	
P value (<0.05)	0.03	

*Mean = Mean of three replications **S.E. = Standard Error

3.2.2 Yield Assessment of Bt Isolates in Pellicle Biofilm

3.2.2.1 Pellicle Biofilm Pellet Biomass

The quantitative assessment of pellicle biomass among the *Bacillus thuringiensis* isolates exhibited marked variability, as presented in Table 4 and Figure 2. The pellicle pellet weight ranged from 25.50±21.00 mg to 113.50±1.00 mg across the tested isolates. The reference strain, HD-1, yielded a pellicle biomass of 105.00±3.00 mg. Among the indigenous isolates, SAK-12 recorded the highest pellicle biomass (113.50±1.00 mg), which was 1.08 times greater than the standard strain. This was followed by SAK-6 (106.50±2.00 mg, 1.01 times). Other isolates such as SAK-9 (91.00±4.00 mg), SHi-1 (76.00±5.00 mg), and SGd-1 (73.50±6.00 mg) also demonstrated substantial biomass production. In contrast, isolates including SA-18 (25.50±21.00 mg) and SJa-2 (40.50±2.00 mg) produced significantly lower pellicle biomass, suggesting a weaker capacity for pellicle development. Statistical analysis confirmed that the differences observed among the treatments

were statistically significant ($p < 0.05$), indicating true variability in pellicle-forming ability across the Bt isolates.

3.2.2.2 Pellicle Biofilm OD₆₀₀ Analysis

The OD₆₀₀ values measured for the pellicle component of *Bacillus thuringiensis* isolates demonstrated considerable variation, ranging from 0.34 to 3.95 (Table 4; Figure 3). The standard strain HD-1 recorded an OD₆₀₀ of 3.02, serving as a benchmark for comparison. Notably, SAK-12 (3.95) and SAK-6 (3.22) surpassed the standard strain, indicating higher cell density and more robust pellicle formation. These were followed by isolates SAK-9 (2.85) and SHi-1 (2.62), which also recorded strong growth profiles. In contrast, isolates SA-18 (0.34) and SJa-2 (0.43) exhibited markedly lower OD₆₀₀ values in the pellicle phase. Interestingly, despite their reduced pellicle density, these isolates had earlier demonstrated significant biomass accumulation in the ring component, suggesting a preferential biofilm formation near the liquid-air interface rather than in the submerged pellicle layer.

Table 4: Yield Assessment of Bt Isolates in Pellicle Biofilm

Bt isolates	Pellet yield (mg/pellicle) Mean*±SE**	OD ₆₀₀ value (Cell density per pellicle)
Bt-HD-1	105.00±3.00	3.02
PDKV-Bt-SA-6	66.00±10.00	1.65
PDKV-Bt-SA-20	62.50±16.00	0.97
PDKV-Bt-SGd-1	73.50±6.00	2.32
PDKV-Bt-SA-18	25.50±21.00	0.34
PDKV-Bt-SAK-6	106.50±2.00	3.22
PDKV-Bt-SAK-9	91.00±4.00	2.85
PDKV_Bt_SGn-5	57.00±18.00	0.77
PDKV_Bt_SBn-2	69.00±8.00	1.92
PDKV-Bt-SY-4	64.50±12.00	1.36
PDKV-Bt-SGn-4	63.00±14.00	1.12
PDKV_Bt_SUd_2	72.50±7.00	2.05
PDKV_Bt_SJa_2	40.50±20.00	0.43
PDKV_Bt_SJa_1	56.00±19.00	0.63
PDKV_Bt_SHi_1	76.00±5.00	2.62
PDKV_Bt_SDa_2	58.00±17.00	0.86
PDKV_Bt_SDa_1	67.00±9.00	1.74
PDKV_Bt_SA-22	65.50±11.00	1.42
PDKV_Bt_SMa-1	63.50±13.00	1.12
PDKV-Bt-SAK-12	113.50±1.00	3.95
PDKV-Bt-SUd-1	63.00±14.00	1.14
F value (df=5,12)	2.08	
P value (<0.05)	0.042	

*Mean = Mean of three replications **S.E. = Standard Error

The present study revealed significant variability in biomass production among *Bacillus thuringiensis* (Bt) isolates, both in the ring and pellicle phases. Certain isolates showed high biomass accumulation in one form but not the other, underscoring strain-specific growth patterns likely influenced by physiological and environmental factors Sonia Paytubi *et al.*, 2017^[17]. A strong positive correlation was observed between OD₆₀₀ values and wet pellet weights across both biomass phases, validating OD₆₀₀ as a reliable indicator of biomass yield. High-performing isolates such as PDKV-Bt-SAK-6, PDKV-Bt-SAK-9, and PDKV-Bt-SGd-1 consistently recorded elevated OD₆₀₀ values along with substantial biomass in both the ring and pellicle components. Conversely, PDKV-Bt-SAK-12 exhibited negligible ring formation yet demonstrated the highest pellicle OD₆₀₀ and biomass, suggesting a preference for bottom-associated growth, likely governed by isolate-specific traits. The standard strain HD-1 maintained balanced growth across both components, supporting its continued use as a reference

standard. The observed differences in biomass distribution patterns between ring and pellicle phases highlight the diverse biofilm-forming abilities of Bt isolates.

These findings are consistent with earlier studies. Nay El-Khoury *et al.* (2016)^[12] reported a proportional relationship between OD₆₀₀ and biomass (spores and vegetative cells), with pellicle OD₆₀₀ values approximately 1.7 times higher than ring OD₆₀₀ after 72-96 hours of incubation. Similarly, Mengmeng *et al.* (2021) observed increasing OD₆₀₀ values correlating with biomass yield during the exponential growth phase in Bt strains HD1 and HD73. Karina *et al.* (2014)^[8] further demonstrated OD₆₂₀ as a reliable metric for biofilm quantification in 40 environmental Bt strains, categorizing several strains as strong biofilm producers based on their OD values. Overall, integrating OD₆₀₀ analysis with biomass measurements offers a comprehensive understanding of isolate growth behaviour and provides a practical approach for selecting high-yielding Bt strains for biocontrol and industrial applications.

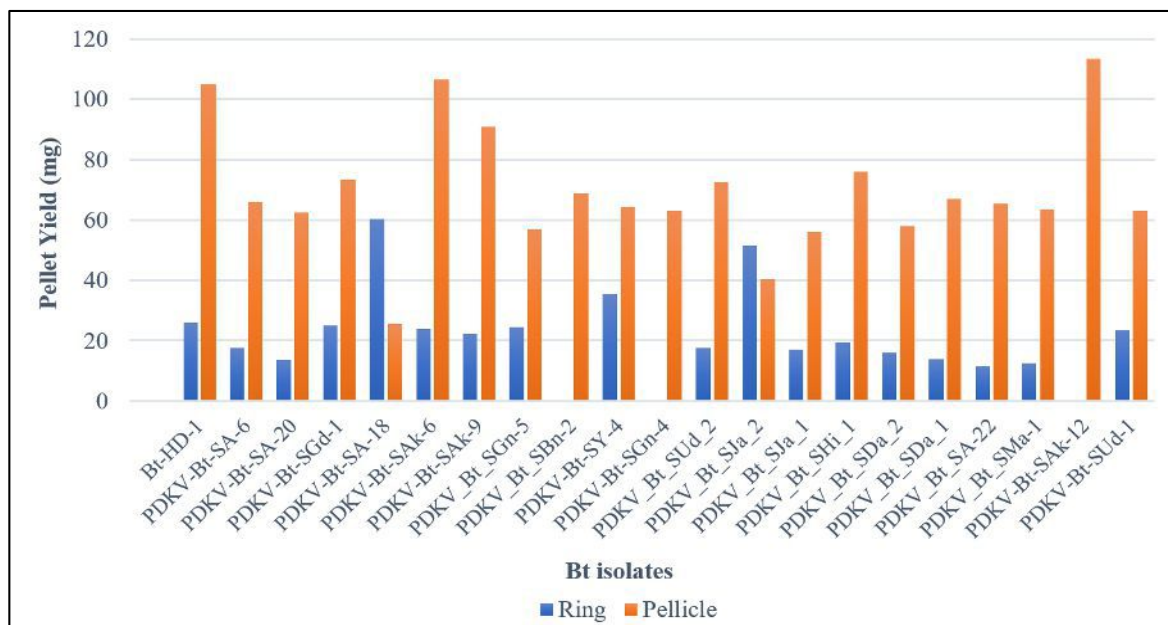


Fig 2: Comparison of Pellet Yield of Bt Isolates in Ring and Pellicle components of biofilm

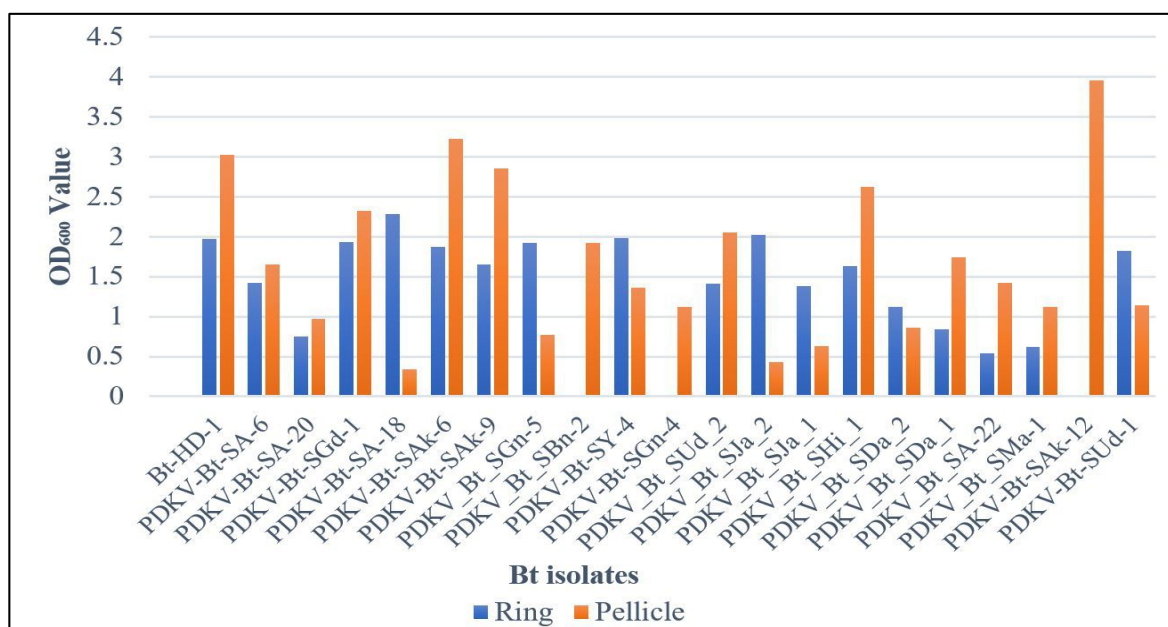


Fig 3: Comparison of Cell Density (OD₆₀₀) of Bt Isolates in Ring and Pellicle components of biofilm

4. Conclusion

The present study revealed considerable variability in the biofilm growth behaviour of indigenous *Bacillus thuringiensis* isolates under static conditions, with distinct preferences for either ring or pellicle formation. This dual-component analysis provided a comprehensive understanding of isolate-specific biomass production strategies. Quantitative parameters such as wet pellet biomass and OD₆₀₀ values served as reliable indicators of cell density and growth potential. A strong positive correlation between OD₆₀₀ and biomass weight across both biofilm forms validated the use of optical density as a rapid screening tool for high-performing isolates. Among the tested indigenous isolates, PDKV-Bt-SAK-6, PDKV-Bt-SAK-9, and PDKV-Bt-SGd-1 consistently demonstrated superior or comparable biomass accumulation and OD₆₀₀ values in both ring and pellicle phases compared to the standard strain HD-1, indicating their robust adaptability and potential for enhanced bioinsecticide production. In contrast, isolate like PDKV-Bt-SAK-12

exhibited exclusive pellicle-dominant growth, highlighting the strain-specific nature of biofilm development. These findings underscore the importance of integrated qualitative and quantitative screening for identifying efficient Bt isolates. Such strains hold promise for cost-effective, static culture-based production strategies, offering a farmer-friendly alternative to conventional agitated fermentation systems.

5. Acknowledgements

This study is a part of thesis research of first author submitted to Department of Entomology, Post Graduate Institute, Dr. PDKV Akola. The authors are grateful to Department of Entomology, Post Graduate Institute, Dr. PDKV, Akola, MS, India for providing all the necessary facilities for conducting the present research work. Authors are also grateful to the student's advisory committee members for their critical comments and valuable suggestions during the study.

6. References

- Auger S, Ramarao N, Faille C, Fouet A, Aymerich S, Gohar M. Biofilm formation and cell surface properties among pathogenic and nonpathogenic strains of the *Bacillus cereus* group. *Appl Environ Microbiol*. 2009;75:6616-6618.
- Bernbom N, Vogel BF, Gram L. *Listeria monocytogenes* survival of UV-C radiation is enhanced by presence of sodium chloride, organic food material and by bacterial biofilm formation. *Int J Food Microbiol*. 2011;147:69-73.
- Dang H, Lovell C. Microbial surface colonization and biofilm development in marine environments. *Microb Mol Biol Rev*. 2016;80:91-138.
- Fagerlund A, Dubois T, Okstad OA, Verplaetse E, Gilois N, Bennaceur I, *et al*. SinR controls enterotoxin expression in *Bacillus thuringiensis* biofilms. *PLoS ONE*. 2014;9:e87532.
- Flemming HC, Wuertz S. Bacteria and archaea on Earth and their abundance in biofilms. *Nat Rev Microbiol*. 2019;17:247-260.
- Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: An emergent form of bacterial life. *Nat Rev Microbiol*. 2016;14:563-575.
- Hall-Stoodley, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol*. 2004;2:95-108.
- Karina Garcia J, Bravo A, Diaz J, Gutierrez D, Torres PV, Gomez de Leon P. Variability of *Bacillus thuringiensis* strains by ERIC-PCR and biofilm formation. 2014;70:10-18.
- Koerdt A, Orell A, Trong Khoa P, Mukherjee J, Wlodkowski A, Karunakaran E. Macromolecular fingerprinting of *Sulfolobus* species in biofilm: A transcriptomic and proteomic approach combined with spectroscopic analysis. *J Proteome Res*. 2011;10:4105-4119.
- Martin PAW, Travers RS. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl Environ Microbiol*. 1989;55:2437-2442.
- Li M, Shu C, Ke W, Li X, Yu Y, Guan X, *et al*. Plant Polysaccharides Modulate Biofilm Formation and Insecticidal Activities of *Bacillus thuringiensis* Strains. *Front Microbiol*. 2021;12.
- El-Khoury N, Majed R, Perchat S, Kallassy M, Lereclus D, Gohar M. Spatio-Temporal Evolution of Sporulation in *Bacillus thuringiensis* Biofilm. *Front Microbiol*. 2016;7.
- Rinaudi LV, Giordano W. An integrated view of biofilm formation in rhizobia. *FEMS Microbiol Lett*. 2010;304:1-11.
- Rowe G, Margaritis A. Bioprocess design and economic analysis for the commercial production of environmentally friendly bioinsecticides from *Bacillus thuringiensis* HD-1 kurstaki. *Biotechnol Bioeng*. 2004;86(4):377-384.
- Sanchis V, Bourget D. *Bacillus thuringiensis*: applications in agriculture and insect resistance management. *Agron Sustain Dev*. 2008;28:11-20.
- Auger S, Ramarao N, Faille C, Fouet A, Aymerich S, Gohar M. Biofilm Formation and Cell Surface Properties among Pathogenic and Nonpathogenic Strains of the *Bacillus cereus* Group. *Appl Environ Microbiol*. 2009;75(20):6616-6618.
- Paytubi S, Cansado C, Madrid C, Balsalobre C. Nutrient Composition Promotes Switching between Pellicle and Bottom Biofilm in *Salmonella*. *Front Microbiol*. 2017;8.
- Sweet MJ, Croquer A, Bythell JC. Development of bacterial biofilms on artificial corals in comparison to surface-associated microbes of hard corals. *PLoS One*. 2011;6:e21195.
- Vidyarthi AS, Tyagi RD, Valero JR, Surampalli RY. Studies on the production of *B. thuringiensis* based biopesticides using wastewater sludge as a raw material. *Water Res*. 2002;36:4850-4860.
- Wijman JG, De Leeuw PP, Moezelaar R, Zwietering MH, Abee T. Air-liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. *Appl Environ Microbiol*. 2007;73:1481-1488.
- Zhao X, Hofte M, Spanoghe P, Rajkovic A, Uyttendaele M. Biofilm-forming Ability of *Bacillus thuringiensis* Strains from Biopesticides on Polystyrene and their Attachment on Spinach. *J Food Protect*. 2024;87(8):100321.