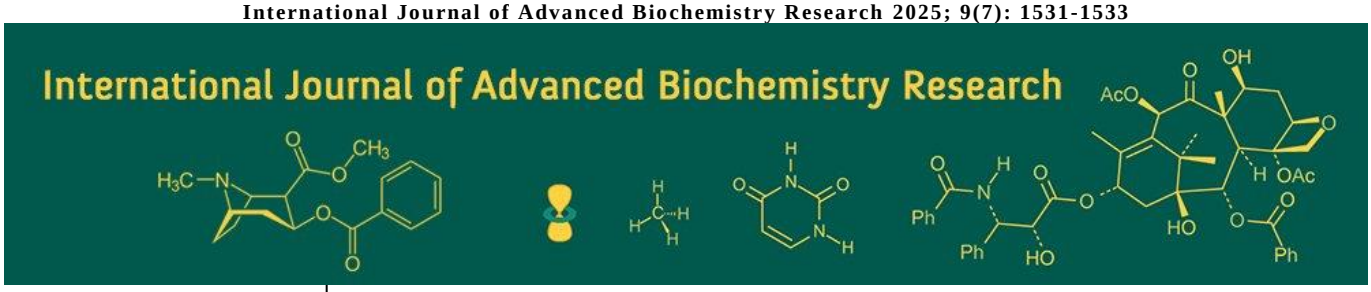

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Culturing and CFU-evaluation of melanin enriched PDKV *Bacillus thuringiensis* isolates in modified Luria Bertani Broth

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Abstract
Quantification of colonies for melanin-fortified PDKV *Bacillus thuringiensis* strains from aqueous suspension concentrate (AS) formulations was carried out on Luria Bertani agar using an electronic colony counter. Among the AS variants assessed, PDKV *Bt* SGN-4 containing melanin recorded the highest spore count, achieving 19.1 × 10^8 CFU/ml. This was followed by PDKV *Bt* SA-22 with melanin (11.6 × 10^8 CFU/ml), PDKV *Bt* SAK-12 (5.3 × 10^8 CFU/ml), and the standard strain *Btk* HD-1 (8.1 × 10^8 CFU/ml). Except for SAK-12, the remaining strains surpassed the spore density threshold of the standard *Bt kurstaki* 5% AS formulation, typically estimated at 1 × 10^8 spores/ml. The results underscore the suitability of LB agar as a reliable growth medium for supporting the proliferation of new PDKV *Bt* isolates.

Keywords: *Bacillus thuringiensis*, melanin enriched strains, PDKV *Bt* isolates, aqueous suspension formulation, colony forming unit (CFU)

1. Introduction
Bacillus thuringiensis (*Bt*) *a* biological control agent, is widely used in the pest management programmes in various modes. The organism was first discovered in silkworms by Japanese sericultural engineer Shigetane Ishiwata (1901) [2]. *Bt* is a gram-positive, spore forming bacteria known for its ability to produce crystal proteins (Cry). *Bt* is the most widely used as microbial insecticide, and many toxic proteins have been applied to agricultural pest control (Kumar *et al.*, 2008) [3]. The parasporal crystal proteins and spores, which constitute the key insecticidal agents in *Bacillus thuringiensis*, rapidly lose activity upon exposure to sunlight. This degradation is primarily caused by the generation of hydroxyl radicals and hydrogen peroxide under UV and ionizing radiation, which leads to protein cross-linking and loss of bioactivity. Several research groups have successfully developed melanin-producing mutants of *Bacillus thuringiensis*, particularly from subspecies *kurstaki*, through repeated exposure to ultraviolet (UV) irradiation. These melanin overproducing mutants exhibited significantly enhanced resistance to UV radiation compared to their parental wild-type strains (Saxena *et al.*, 2002) [5].

Numerous studies and commercial applications have shown *Bt*-based biopesticides are effective under field conditions when applied correctly. *Bt* does not persist long in the environment, reducing ecological risks compared to synthetic pesticides. When used with proper strategies (like rotation or combination with other control methods), *Bt* can be part of IPM programs the present study focused on developing different formulations of *Bacillus thuringiensis* (*Bt*) strains enriched with melanin, which were originally isolated from the Department of Entomology, PDKV. These formulations were assessed for their growth and viability using Luria Bertani agar medium. Since UV radiation is known to degrade Cry proteins and reduce,The effectiveness of *Bt*, enhancing UV resistance in these strains is of critical importance. To address this, the study also aimed to evaluate the colony forming ability (CFU/ml) of novel Dr. PDKV, Akola *Bt* strains formulated as aqueous suspension concentrates, thereby determining their viability and potential stability under environmental conditions.

2. Materials and Methods

This research aimed to assess the viability and growth characteristics of melanin enriched *Bacillus thuringiensis* (*Bt*) formulations developed from PDKV isolates using Luria Bertani medium. The bacterial strains utilized in this investigation were previously isolated and cultured by the Department of Entomology. Experimental work was carried out in the entomology laboratory at Dr. Panjabrao Deshmukh Krishi Vidyapeeth (M.S), during the academic year 2024-2025.

2.1 Culturing of *Bt* strains

3 indigenous *Bacillus thuringiensis* isolates, along with the standard reference strain *Btk*-HD-1, previously evaluated for insecticidal efficacy by the Department of Entomology, were re isolated and preserved on Luria Bertani broth for the present investigation. These innovative PDKV *Bt* strains were formulated into experimental microbial insecticide preparations within the Entomology Laboratory at Dr. PDKV, Akola. All bacterial isolates included in the study were cultured using LB medium following the methodology described by Abida Bibi *et al.*, (2013). Following the formulation process, the concentration of viable spores was determined and expressed as colony-forming units per milliliter (CFU/ml).

2.1.1 Preparation of *Bacillus thuringiensis* formulations

A sterile inoculating loop was used to aseptically transfer pure colonies of *Bacillus thuringiensis* strains namely SA-22, SAK-12, SGN-4, along with the standard reference strain HD-1 into 200 ml of sterilized Luria Bertani (LB) broth contained in Erlenmeyer flasks. These inoculated flasks were incubated on a rotary shaker set at 280 rpm and maintained at a temperature of 32 °C for 24 hours to allow optimal bacterial growth and initial sporulation. After incubation, the broth was allowed to cool to approximately 60°C, at which point 100 µL of an actively sporulating inoculum was introduced into each culture flask. This inoculum was prepared by taking a loopful of 72-hour-old, highly sporulated *Bt* culture and suspending it in 5.0 ml of sterile saline solution. The suspension was thoroughly vortexed to ensure even distribution of spores and vegetative cells. A smear from the suspension was further examined under a microscope to verify the purity and quality of the culture. In order to stimulate melanin biosynthesis in the *Bt* strains, L-tyrosine at a concentration of 3 g/L and copper sulphate ($CuSO_4$) at 50 mg/L were added to the LB medium. The flasks were then subjected to a secondary incubation at 42 °C on a rotary shaker running at 160 rpm for a prolonged duration of 120 hours to support extended fermentation and pigment production. Following the fermentation process, biopesticide formulations were prepared using one Liter of freshly cultured *Bt* broth.

To enhance shelf life and maintain the biological efficacy of the product during storage, formulation stabilizers and preservatives were added comprising glycerol (10 ml), boric acid (10 g), corn starch (35 g), and methyl-para-hydroxybenzoate (10 g). The entire formulation was mixed thoroughly by shaking, its pH was measured and recorded, and the final product was stored in a dark room at ambient temperature to prevent photodegradation and maintain microbial viability over time.

2.2 Estimation of CFU counts

To evaluate the viable spore concentration in the prepared *Bacillus thuringiensis* formulation, a measured quantity of the formulation was first diluted to the required application strength using 100 ml of sterile distilled water. Serial dilutions were then performed under aseptic conditions. From these, a suitable dilution was chosen and uniformly spread on Luria Bertani agar plates. The plates were incubated at a controlled temperature of approximately 30 ± 2 °C for a period of 24 hours. After incubation, the number of visible colonies was counted using a calibrated colony counter. The number of viable spores was expressed as colony-forming units per ml (CFU/ml). This value was calculated by multiplying the total number of colonies observed by the corresponding dilution factor and then dividing the result by the volume of the sample plated, expressed in ml. The procedure followed standard microbiological techniques, ensuring accurate quantification of viable *Bt* spores present in the formulated biopesticide (Saberi *et al.*, 2020) [4].

3. Results and Discussion

The data in Table 1 presents the colony-forming unit (CFU/ml) counts for various melanin enriched PDKV *Bacillus thuringiensis* (*Bt*) formulations. Among the aqueous suspension concentrates evaluated, the SGN-4 melanin enriched AS formulation strain exhibited the highest spore concentration, reaching 19.1×10^8 CFU/ml. This was followed by the SA-22 melanin enriched AS formulation with a count of 11.6×10^8 CFU/ml. The SAK-12 melanin enriched AS formulation recorded a CFU of 5.3×10^8/ml, while the standard reference strain, *Btk* HD-1, showed a spore count of 8.1×10^8 CFU/ml.

Table 1: Spore count of melanin enriched PDKV *Bt* aqueous suspension concentrate (AS) formulations

S. No	PDKV *Bt* AS formulations	Spore count (CFU/ml)
1	PDKV *Bt* SA-22 with melanin enriched AS formulation	11.6×10^8
2	PDKV *Bt* SAK-12 with melanin enriched AS formulation	5.3×10^8
3	PDKV *Bt* SGN-4 with melanin enriched AS formulation	19.1×10^8
4	*Btk* HD-1 standard AS formulation	8.1×10^8

The insecticidal potential of *Bacillus thuringiensis* is largely attributed to the production of crystal (Cry) proteins, rather than the number of viable spores (CFUs). While spore counts indicate the viability of the bacterial culture, they do not necessarily reflect its bioefficacy. The effectiveness of *Bt* formulations depends more on the type, amount, and structural stability of the Cry proteins synthesized during sporulation. Hence, evaluating Bt efficiency should focus primarily on the yield and biological activity of these toxins, rather than relying solely on spore concentration as an indicator (Bravo *et al.*, 2011) [1].

4. Conclusion

Among the four evaluated strains, the SGN-4 melanin-enriched aqueous suspension (AS) formulation exhibited the highest colony forming unit count, which may be attributed

to its elevated concentration of biologically active components. Overall, the PDKV *Bt* formulations demonstrated superior CFU counts (CFU/ml) in comparison to the commercial standard *Bt kurstaki* 5% AS formulation, available in the market, which typically contains 1×10^8 spores/ml. These findings highlight the Melanin enrichment in *Bacillus thuringiensis* formulations enhances strain stability by protecting Cry proteins from UV-induced degradation. This improves the persistence and field efficacy of *Bt*-based biopesticides under sunlight exposure.

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