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Influence of pH Variations on Biofilm Formation of *Bacillus thuringiensiskurstaki* HD1 on Polystyrene Surfaces

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ABSTRACT: Biofilm formation enhances microbial survival, growth, nutrient access, biocide resistance, productivity, interactions, and stability. *Bacillus thuringiensis kurstaki* (Btk) HD1 is used as a biopesticide against lepidopterans (moths and butterflies). Thus, the objective of this paper is to evaluate the influence of pH variations on biofilm formation of *Bacillus thuringiensis kurstaki* HD1 on polystyrene surfaces across a range of pH levels from 1 to 14. The results demonstrated a clear pH-dependent trend in biofilm production. Biofilm formation was optimal between pH 4 and pH 9, with the highest production at pH 4. In contrast, extreme pH values (1, 10, 11, 12, 13, and 14) resulted in reduced biofilm formation. The study highlights the significant influence of pH on biofilm formation by Btk HD1, with optimal biofilm production occurring within a pH range of 4 to 9. These findings suggest that maintaining environmental pH within this range could enhance the efficacy and stability of Btk HD1 as a biopesticide.

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Bacillus thuringiensis (Bt) was initially discovered in 1901 by Ishiwata Shigetane, a Japanese biologist, who found it in diseased silkworms (Ibrahim *et al*., 2010). Berliner noted the presence of a crystal in Bt in 1915, but its function remained unknown until a later time(Sansinenea, 2012). The first commercial product of the bacterium, known as 'Sporeine', was available in France in 1938(Sansinenea, 2012). However, it wasn't until the 1950s that Bt was recognized for its potential as a biopesticide. The discovery of a more effective strain of *B. thuringiensis kurstaki* (HD1) by Dulmage in 1970 led to its widespread use as a biological pest control agent(Sansinenea, 2012). Since then, Bt has become a widely used biopesticide.

Biofilms were initially observed by Antonie van Leeuwenhoek, but the theoretical framework for understanding their formation did not emerge until 1978. It is now recognized that biofilms are ubiquitous, found in aquatic environments, industrial water systems, various natural habitats, and medical devices crucial to public health (Donlan and

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Costerton, 2002). Biofilms consist of organized communities of microorganisms arranged in a complex structure that adheres to either inert or living surfaces (Silva *et al*., 2014). Almost any material exposed to bacteria-containing fluids can serve as a substrate for biofilm formation. Factors such as surface roughness, chemistry, and the presence of conditioning films influence how bacterial cells attach to a surface (Goller and Romeo, 2008). Bacteria forms biofilms as a response to environmental challenges like UV radiation, desiccation, nutrient scarcity, extreme pH, temperature extremes, high salt levels, elevated pressure, and antimicrobial substances(Muhammad *et al*., 2020). The formation of biofilms by bacteria such as *Bacillus thuringiensis kurstaki* (Btk) HD1 is of particular interest due to the organism's widespread application as a biopesticide. Thus, biofilm formation can influence its stability, effectiveness, and longevity as a biological control agent.

Various factors influence bacterial biofilm development, including temperature, pH, oxygen levels, hydrodynamics, osmolarity, specific ions, nutrients, and biotic environmental factors. Exploring these factors is essential for optimizing the application and performance of bacteria(Goller and Romeo, 2008). pH is a critical environmental parameter that can significantly alter bacterial physiology and biofilm development(Hostacká *et al.*, 2010). Despite the importance of pH in microbial ecology, its specific impact on the biofilm formation of Btk HD1 remains underexplored. Thus, the objective of this paper is to evaluate the influence of pH variations on biofilm formation of *Bacillus thuringiensis kurstaki* HD1 on polystyrene surfaces.

MATERIALS AND METHODS

*Spore lysis Stage:*1 mL of overnight cultures of Btk HD1 strains was inoculated into LB medium and incubated at 30°C with shaking at 150 rpm for 36 hours until the OD600 values were approximately 0.9. The culture medium was then examined microscopically to observe the production of spores and crystals (spore-lysis stage). Subsequently, the culture was centrifuged at 5000 rpm for 5 minutes at 4°C, and the pellets were washed three times with autoclaved distilled water.

*pH adjustment***:** Before autoclaving, the pH of the LB medium was set to values between 1 and 14 using NaOH or HCl. The medium was then autoclaved to ensure sterility. The Btk HD1 bacterial pellet was subsequently mixed with the autoclaved, pH-adjusted LB medium for further experimentation.

Biofilm Assay: Biofilm formation was assessed using crystal violet staining. Specifically, 100 µL of the Btk HD1 sample was inoculated into 96-well plates and incubated for 36 hours at 30°C without shaking. Following incubation, the plate was inverted to discard planktonic cells and gently washed with sterile distilled water three times before being allowed to air dry. Then, 150 µL of a 0.1% crystal violet solution was added to each well and allowed to stand at room temperature for 10-15 minutes. Excess dye was rinsed off by washing the plates with distilled water three times and allowing them to air dry overnight. To solubilize the crystal violet, 200 µL of 95% ethanol was added to each well of the microtiter plate and incubated for 10-15 minutes at room temperature. The optical density (OD) of each well was measured at 595 nm using a spectrophotometer.

Table 1: Assessment of bacterial adherence through the microtiter plate method

Biofilm formation	Mean optical density (OD) values
Non/weak	< 0.120
Moderate	0.120–0.240
High	> 0.240 (Oufrid *et al*., 2015)

RESULTS AND DISCUSSION

Our study investigated the impact of pH variation on biofilm formation by Btk HD1 on polystyrene surfaces. Biofilm development was quantitatively assessed by measuring the optical density (OD) at 595 nm after 36 hours of incubation. The results revealed pH-dependent trends in biofilm production, as indicated by the mean OD values and their standard deviations (SD): pH1 = 0.087 (SD 0.004), pH2 = 0.123 (SD 0.042), pH3 = 0.143 (SD 0.040), pH4 = 0.247 (SD 0.055), pH5 = 0.140 (SD 0.053), pH6 = 0.140 (SD 0.053), pH7 = 0.163 (SD 0.035), pH8 = 0.157 (SD 0.0306), pH9 = 0.183 (SD 0.021), pH10 = 0.100 (SD 0.040), pH11 = 0.107 (SD 0.0306), pH12 = 0.110 (SD 0.046), pH13 = 0.110 (SD 0.026), and pH14 = 0.083 (SD 0.0306). Notably, biofilm formation exhibited an optimal pH range between pH 4 and pH 9, with pH 4 showing the highest biofilm production. Extreme pH values ((pH values of 1, 10, 11, 12, 13, and 14) generally resulted in reduced biofilm formation (Fig. 1 and Fig. 2).

Optimal biofilm formation was observed within the pH range of 4 to 9, with pH 4 exhibiting the highest biofilm production. This suggests that moderately acidic to neutral conditions favor biofilm development by Btk HD1. Conversely, extreme pH values, such as those at pH 1, pH 10, pH 11, pH 12, pH 13, and pH 14, generally resulted in reduced biofilm formation. This trend highlights the sensitivity of biofilm production

to highly acidic or highly alkaline environments, which likely disrupt cellular processes critical for biofilm establishment and maintenance.

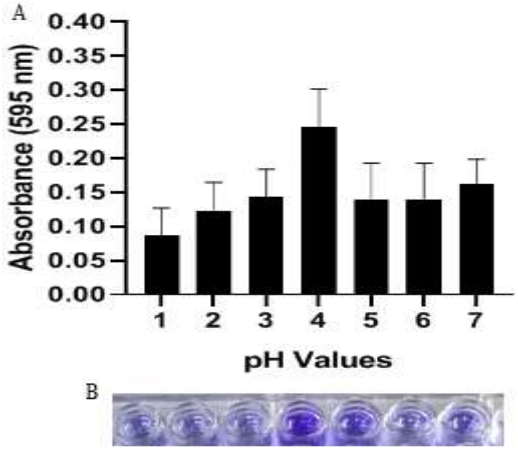

Fig. 1: The development of biofilm by Btk HD1 on polystyrene 96-well plates at acidic pH (A) was quantified by measuring the optical density of stained biofilms at 595 nm. (B) Biofilm formation on the surface of the polystyrene microtiter plate was visualized with 0.1% crystal violet staining after 36 hours of incubation.

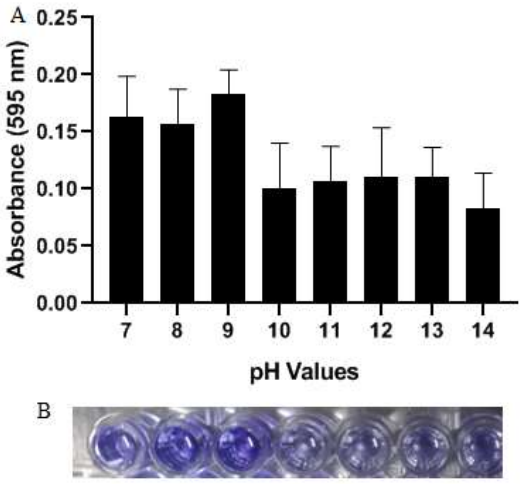

Fig. 2: The development of biofilm by Btk HD1 on polystyrene 96-well plates at alkaline pH (A) was quantified by measuring the optical density of stained biofilms at 595 nm. (B) Biofilm formation on the surface of the polystyrene microtiter plate was visualized with 0.1% crystal violet staining after 36 hours of incubation.

These findings align with previous research indicating that environmental pH can significantly impact bacterial growth. For instance, studies on other *Bacillus* species have demonstrated similar pH-dependent bacterial growth patterns, where neutral to slightly acidic conditions promote bacterial growth, while extreme pH levels inhibit it (Nur *et al.,* 2017; Sanjaya *et al*, 2023). Moreover, our findings are consistent with previous studies demonstrating the critical role of pH in biofilm formation by various bacterial species. For example, Tango et al. (2018) reported that biofilm formation by *Staphylococcus aureus* is significantly influenced by pH, with reduced biofilm formation observed at highly acidic and highly alkaline conditions. Similarly, D'Urzo *et al*. (2014) found that biofilm formation by *Streptococcus agalactiae* was maximal at acidic pH of 5 and declined sharply at neutral pH.

The optimal pH range for biofilm formation observed in our study (pH 4 to 9) aligns with the physiological pH range of many natural environments where Btk HD1 is likely to be deployed as a biopesticide (Mahapatra *et al.,* 2022; Satapute *et al*., 2012). This suggests that maintaining the environmental pH within this range could enhance the stability, effectiveness, and longevity of Btk HD1 biofilms, thereby improving its performance as a biological control agent. Moreover, the highest biofilm formation at pH 4 suggests that slightly acidic conditions may favor the adhesion and colonization of Btk HD1 on surfaces, which could be advantageous in agricultural settings where soil pH can vary.

Conclusion: In conclusion, the pH of the environment is a crucial factor influencing biofilm formation by Btk HD1. Our study highlights the importance of considering pH when deploying Btk HD1 as a biopesticide, to maximize its efficacy and sustainability. Future research should focus on exploring the molecular mechanisms underlying pH-dependent biofilm formation in Btk HD1 and investigating the influence of other environmental factors on its biofilm-forming capacity.

Declaration of Conflict of Interest: The authors declare no conflict of interest.

Data Availability Statement: Data are available upon request from the corresponding author

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