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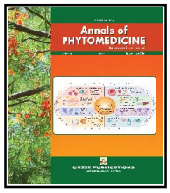

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Optimization of fermentation kinetics in native *Bacillus thuringiensis* isolates for enhanced crystal toxin production and sustainable plant protection

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Abstract

The cultivation of high quality medicinal plants is increasingly threatened by insect pests, necessitating the development of potent, residue-free biological control agents to preserve phytochemical integrity. This study focuses on the bioprospecting and bioprocess optimization of native *Bacillus thuringiensis* (Bt) isolates (VKK9 and VKK1) to maximize the production of entomopathogenic spores and crystal proteins. To enhance bioactive yields, peptone glucose salt medium (PGSM) was standardized by evaluating various carbon and nitrogen substrates alongside physical parameters. Starch and yeast extract were identified as the optimal substrates, achieving a maximum biomass of 6.345 g/l, viable spore counts of 3.56 × 107 spores/ml, and a total protein content of 138.5 mg/l at pH 7.0 and 30°C. Fermentation kinetics in an in-situ sterilizable bioreactor revealed a mass transfer coefficient (KLa) of 0.0367, an oxygen transfer rate (OTR) of 0.0204, and an oxygen utilization rate (OUR) of 0.10769 for the elite isolate VKK9, indicating superior metabolic efficiency and oxygen uptake during the transition from vegetative growth to sporulation. Growth curve analysis confirmed rapid multiplication with a generation time of 22 min, reaching peak protein yields (192.4 mg/l) at 48 h. Beyond their insecticidal potential, the isolates exhibited significant plant growth-promoting and antimicrobial activities, suggesting a multifunctional role in the rhizosphere of medicinal flora. These findings demonstrate that precise control of fermentation kinetics is essential for the scalable production of Bt based biotherapeutics, providing a sustainable alternative to chemical pesticides in the production of safe, high efficacy phytopharmaceuticals.

1. Introduction

The global shift toward "Green Medicine" has led to an exponential increase in the demand for high-quality medicinal plants and phytotherapeutics. However, the cultivation of these botanicals is often hampered by a wide array of insect pests, leading to significant yield losses and a reduction in the concentration of bioactive secondary metabolites (Amrutanand *et al.,* 2021; Shreedevasena *et al.,* 2024). Traditionally, chemical pesticides have been the primary tool for pest management; however, their use in phytomedicine is increasingly scrutinized. Chemical residues not only pose significant health risks to consumers but also interfere with the pharmacognostic profile of medicinal plants, often leading to the rejection of herbal raw materials in international markets (Devi and Kumar, 2024). Consequently, there is an urgent need for "bioprospecting" native microbial isolates that can offer ecofriendly, residue free protection for medicinal flora.

Bacillus thuringiensis (Bt) is a Gram-positive, soil-dwelling, spore-forming bacterium that has emerged as a cornerstone of biological pest management and a promising candidate for integrated phytomedicine strategies (Amrutanand *et al.,* 2021, Salam *et al.,* 2025; Medison *et al.,* 2025). Its uniqueness lies in the production of parasporal crystalline inclusions during the sporulation phase, known as delta-endotoxins (Cry and Vip proteins) (Jain *et al.,* 2016). These proteins exhibit highly specific entomopathogenic activity against lepidopteran, coleopteran, and dipteran pests while remaining entirely benign to non-target organisms, including humans and beneficial pollinators (Sieiro *et al.,* 2021). From a phytomedicinal perspective, the application of Bt based biopesticides ensures that medicinal plants remain free from synthetic toxicity, thereby preserving their therapeutic efficacy and safety profile (Avignone-Rossa *et al.,* 1992; Rossa and Mignone, 1993).

Despite the clear advantages of Bt, the industrial-scale transition to bio-based protection is often limited by the high cost of production and the variability in toxin yields among different bacterial strains (Abbate *et al.,* 2023). The physiological state of the bacterium and the surrounding nutritional environment are the primary determinants of the final concentration of spores and insecticidal toxins. Research indicates that aeration and oxygen transfer rates (OTR) are critical bioprocess parameters. A continuous and optimized oxygen supply has been shown to enhance endotoxin productivity, whereas limited aeration leads to truncated growth phases and poor biomass accumulation (Sarrafzadeh *et al.,* 2014). Furthermore, the mass transfer coefficient ($K_L a$) serves as a vital indicator of the efficiency of oxygen supply in stirred-tank bioreactors, directly influencing the metabolic vigor of the isolate.

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The nutrient composition of the fermentation media also plays a decisive role in the kinetics of growth and sporulation (Boniolo *et al.,* 2012). Carbon and nitrogen sources, such as glucose and yeast extract, act as the primary metabolic fuels. While adequate concentrations promote high yields, excess nutrients can lead to "catabolite repression," delaying sporulation and resulting in a heterogeneous population of low-potency spores (Anderson and Jayaraman, 2003). Recent trends in phytomedicine research emphasize the use of native, locally adapted Bt isolates, as these "indigenous bio-factories" often possess novel *cry* genes and secondary metabolites with broader antimicrobial and plant growth-promoting (PGP) activities.

Standardization of the fermentation process specifically the optimization of pH, temperature, and media substrates is essential to maximize the yield of these bioactive proteins. By utilizing precise fermentation kinetics, it is possible to synchronize the growth phases of native isolates to ensure a high-density harvest of terminal spores and crystal toxins. This not only improves the economic viability of biopesticides but also ensures a consistent dose-response relationship when applied to medicinal crops in the field (Masri *et al.,* 2020).

In view of the above, the present study was undertaken to bioprospect and optimize the fermentation conditions for native *B. thuringiensis* isolates (VKK9 and VKK1). The primary objective was to evaluate the impact of modified PGSM and standardized physical parameters (pH, temperature, and aeration) on the production of biomass and crystal proteins. By characterizing the fermentation kinetics, including OTR, OUR, and $K_L a$, this research aims to provide a robust framework for the mass production of Bt based biotherapeutics. Such advancements are crucial for the sustainable cultivation of medicinal plants, ensuring that the final phytopharmaceutical products are both potent and free from chemical contamination.

2. Materials and Methods

2.1 Microbial strains and maintenance

Two native isolates of *B. thuringiensis*, designated as VKK9 (NCBI accession no: ON974874) and VKK1 (NCBI accession no: ON974875), were obtained from the national facility for insect rearing and xenobiotic cum transgenic bioassay laboratory, ICAR-Indian Agricultural Research Institute (IARI), New Delhi. The isolates were maintained on nutrient agar (NA) slants at 4°C and were periodically subcultured to ensure genetic stability and viability for bioprocess optimization.

2.2 Inoculum development and seed culture

Primary cultures were initiated by inoculating a loopful of pure colony into 50 ml of luria bertani (LB) broth, followed by incubation in an orbital shaker at 30°C and 180 rpm for 24 h. To prepare the standardized seed culture for bioreactor studies, 10 ml of the primary culture was transferred into 1000 ml of tryptone soya broth (TSB) and incubated under identical conditions until the midlogarithmic phase was achieved.

2.3 Optimization of nutritional and physical parameters

A systematic one factor at a time (OFAT) approach was employed to standardize the modified PGSM media to maximize bioactive yield (Ozcengiz and Alaeddinoglu, 1991). For carbon and nitrogen sources, four carbon sources (starch, corn starch, glucose, and wheat flour) and four nitrogen sources (peptone, yeast extract, soybean flour, and a $(NH_4)_2SO_4$ + yeast mixture) were evaluated at a 2% concentration and for physical parameters, the impact of initial pH (6.5, 7.0, and 7.5) and incubation temperature (25°C, 30°C, and 35°C) was assessed based on total biomass and protein yield (Bradford, 1976).

2.4 Bioreactor configuration and fermentation conditions

Batch fermentation was conducted in a fermex biofmx 15 l *in-situ* sterilizable bioreactor (FermEx solutions LLP, Punjab, India). The vessel was filled with 10 ml of the optimized PGSM and sterilized at 121°C for 15 min. For process control, the temperature was maintained at 30°C, and the initial pH was adjusted to 7.0 using automated peristaltic pumps supplying 1 N HCl and 1 N NaOH and for aeration and agitation, dissolved oxygen (DO) was monitored *via* a DO_2 transmitter and maintained using a cascade control of agitation (300-1200 rpm) and an aeration rate of 1 VVM (volume of air per volume of liquid per minute).

2.5 Analytical procedures for kinetic parameters

Samples (100 ml) were withdrawn at 6, 12, 24, 48, and 72 h intervals for kinetic analysis. For biomass and spore enumeration, dry cell weight (DCW) was determined by centrifuging 50 ml of broth at 10,000 rpm for 5 min, followed by drying the pellet at 50°C. Spore counts were performed using the heat shock method (80°C for 10 min), followed by serial dilution and plating on NA. For total bioactive protein estimation, the pellet was rinsed with 0.1 N NaCl to remove debris, and the crystal protein concentration was quantified using the Bradford Method (1976), with bovine serum albumin (BSA) as the standard. For oxygen transfer kinetics, the mass transfer coefficient ($K_L a$) was determined using the dynamic method. The oxygen transfer rate (OTR) and oxygen utilization rate (OUR) were calculated using the following equations:

$$OTR = K_L a\ (C^*L - C)$$

$$OUR = OTR - dC/dt$$

where C*L is the saturated dissolved oxygen concentration and C is the actual DO content.

2.6 Growth curve and generation time analysis

Growth kinetics were monitored using the bioscreen C automated microbiology growth curve analysis system. Replicates were inoculated with 5% v/v inoculum and monitored hourly at 600 nm. The growth rate constant (k) and generation time (g) were calculated as:

$$k = \frac{\log(Xt) - \log(Xo)}{0.301 \times t}$$

$$g = \frac{1}{k}$$

2.7 Secondary metabolite and microscopic characterization

The presence of bipyramidal crystal proteins and terminal spores was confirmed using phase contrast microscopy (100x) and coomassie brilliant blue staining. Additionally, preliminary screening for antimicrobial activity against phytopathogens and plant growth-promoting traits (phosphate solubilization and IAA production)

was performed to assess the isolates' multifunctional potential in phytomedicine.

2.8 Statistical analysis

All experiments were performed in triplicate. Data were subjected to analysis of variance (ANOVA), and mean values were compared using student's t-test at a 5% (p<0.05) level of significance using SPSS software.

3. Results

3.1 Bioprospecting and media optimization for bioactive yields

The optimization of nutritional parameters revealed that the yield of spores and crystal proteins is highly dependent on the complexity of the carbon and nitrogen sources. Among the carbon substrates evaluated, starch yielded the highest biomass (6.306 g/l) and a superior viable spore count of 4.5×10^7 spores/ml. In contrast, while glucose supported rapid vegetative growth, it resulted in a lower total protein content (123.73 mg/l) compared to starch (140.10 mg/l).

Nitrogen source evaluation identified yeast extract as the most efficient substrate for secondary metabolism. It produced a maximum cell yield of 7.23 g/l and a significant viable spore count of 2.5×10^9 spores/ml. The combination of $(NH_4)_2SO_4$ and yeast also showed high sporulation frequency (5.61×10^{-1}), confirming that organic nitrogen is essential for the synthesis of entomopathogenic proteins (Table 1).

Table 1: Optimization of nutritional sources for maximum bioactive yield

Source	Total cell count	Viable spore count (spore/ml)	Sporulation frequency	Total protein estimation (mg/l)	Cell yield (g/l)
Carbon source					
Starch	6.7×10^8	4.5×10^7	3.48×10^{-2}	140.108	6.306
Corn starch	1.59×10^7	2.24×10^6	1.40×10^{-1}	112.652	5.403
Glucose	5.39×10^7	6.82×10^6	1.26×10^{-1}	123.734	5.987
Wheat bran flour	2.73×10^6	1.98×10^5	7.25×10^{-2}	105.543	4.567
Nitrogen source					
Peptone	2.1×10^9	3.66×10^8	1.74×10^{-1}	134.728	6.568
Yeast	5.2×10^9	2.5×10^9	4.80×10^{-1}	155.986	7.236
Soya bean flour	7.5×10^8	3.2×10^7	4.26×10^{-2}	96.439	5.567
$(NH_4)_2SO_4$ + yeast	8.4×10^9	4.72×10^9	5.61×10^{-1}	137.328	6.875

3.2 Impact of physical parameters on sporulation

The physiological response of the native isolates to pH and temperature was highly specific. A neutral pH of 7.0 was found to be optimal, yielding 6.21 g/l of biomass and 132.30 mg/l of protein. Deviations to pH 6.5 or 7.5 resulted in a marginal decline in protein synthesis. Regarding temperature, 30°C was identified as the critical threshold for maximum productivity; increasing the temperature to 35°C led to a drastic reduction in biomass (4.01 g/l) and viable spores (2.63×10^5 spores/ml), indicating thermal sensitivity in the sporulation signaling pathway of these native isolates (Table 2 and Figure 1).

Table 2: Optimization of pH and temperature for maximum spore and protein content

Parameters	Total cell count	Viable spore count (spore/ml^{-1})	Sporulation frequency	Protein estimation (mg/l)	Cell yield (g/l)
pH					
6.5	3.8×10^8	4.7×10^7	1.23×10^{-1}	129.764	5.921
7.0	4.69×10^8	2.83×10^7	6.03×10^{-2}	132.301	6.215
7.5	4.17×10^8	3.21×10^7	7.69×10^{-2}	130.783	6.027
temperature					
25°C	5.91×10^7	6.17×10^6	1.04×10^{-1}	119.632	5.315
30°C	6.72×10^8	3.56×10^7	5.29×10^{-2}	138.517	6.345
35°C	5.21×10^6	2.63×10^5	5.04×10^{-2}	114.972	4.012

A significant correlation was observed between pH fluctuations and DO levels. During the first 12 h, the pH rose to 8.5 due to initial protein deamination, followed by a sharp decline to 5.0 between 24 to 48 h, coinciding with organic acid production during rapid vegetative multiplication. The stabilization of pH back to 7.0 at 72 h coincided with the completion of sporulation and the release of crystal proteins.

3.3 Bioreactor fermentation and oxygen transfer kinetics

Fermentation in the 15 L bioreactor using isolate VKK9 provided deep insights into the relationship between oxygen mass transfer and protein synthesis. The mass transfer coefficient (KLa) was calculated at 0.0367, ensuring an OTR of 0.0204. The OUR reached

0.10769, reflecting high metabolic activity during the midlogarithmic phase (Table 3 and Figure 2).

3.4 Growth dynamics and generation time

Automated growth curve analysis revealed distinct kinetic profiles for the two isolates. Isolate VKK9 demonstrated superior growth vigor with a generation time (1/k) of 22 min. Its lag phase was notably short (3 h), entering the logarithmic phase rapidly. Isolate VKK1, while also productive, exhibited a longer doubling time of 360 min. and a more extended lag phase of 5 h (Table 4 and Figure 3).

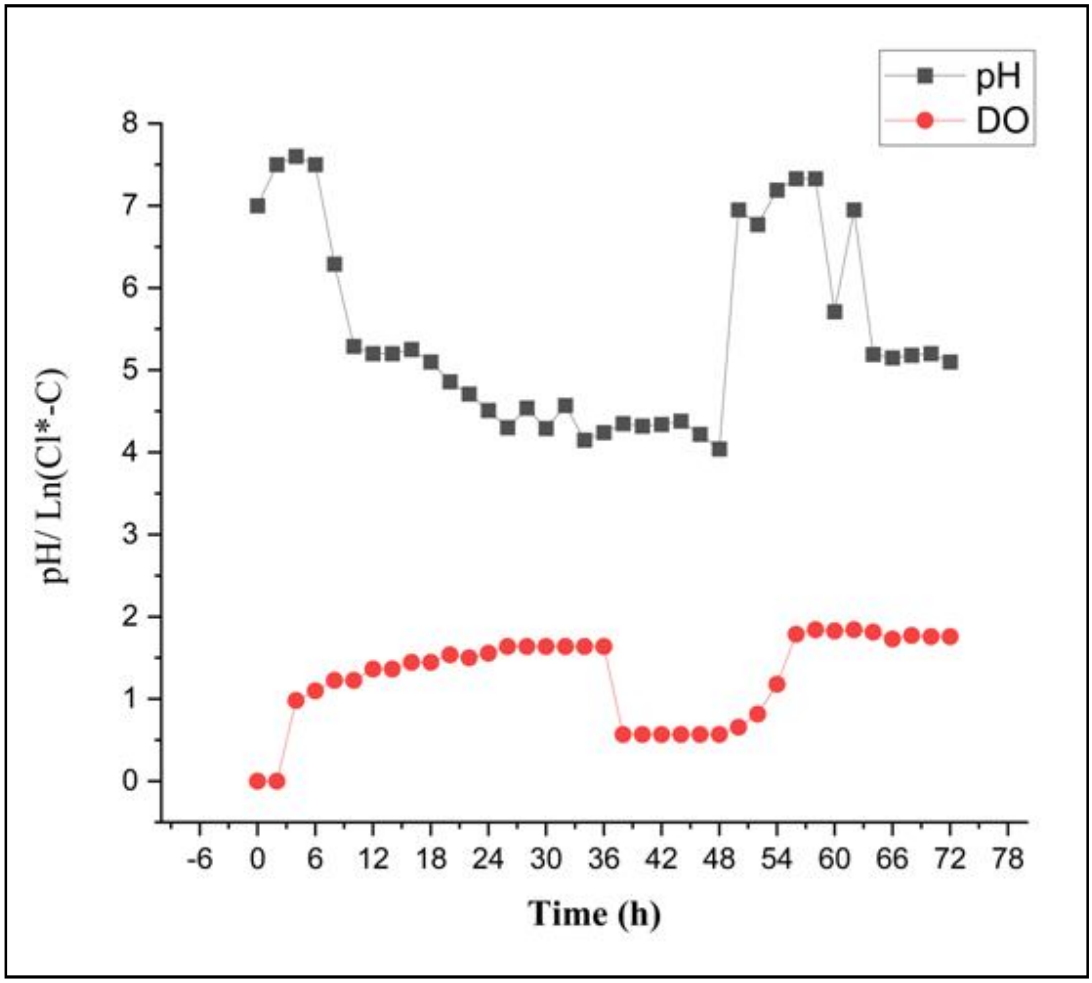

Figure 1: Relationship between pH/Ln (CL*-C) and time during fermentation.

Table 3: Fermentation kinetics parameters

Fermentation kinetics parameters	Values
Biomass concentration (g/l)	7.824 g/l
K_La, mass transfer coefficient	0.0367
CL*	7.71 mg/l (Min. DO of water @30°C and atm)
OTR	0.0204
dC/dt	- 0.08729
OUR	0.10769
specific oxygen uptake rate, QO_2	0.013764
Productivity	2.67 mg/l of total protein
	108.66 mg/l of biomass yield
Specific growth rate	0.044222

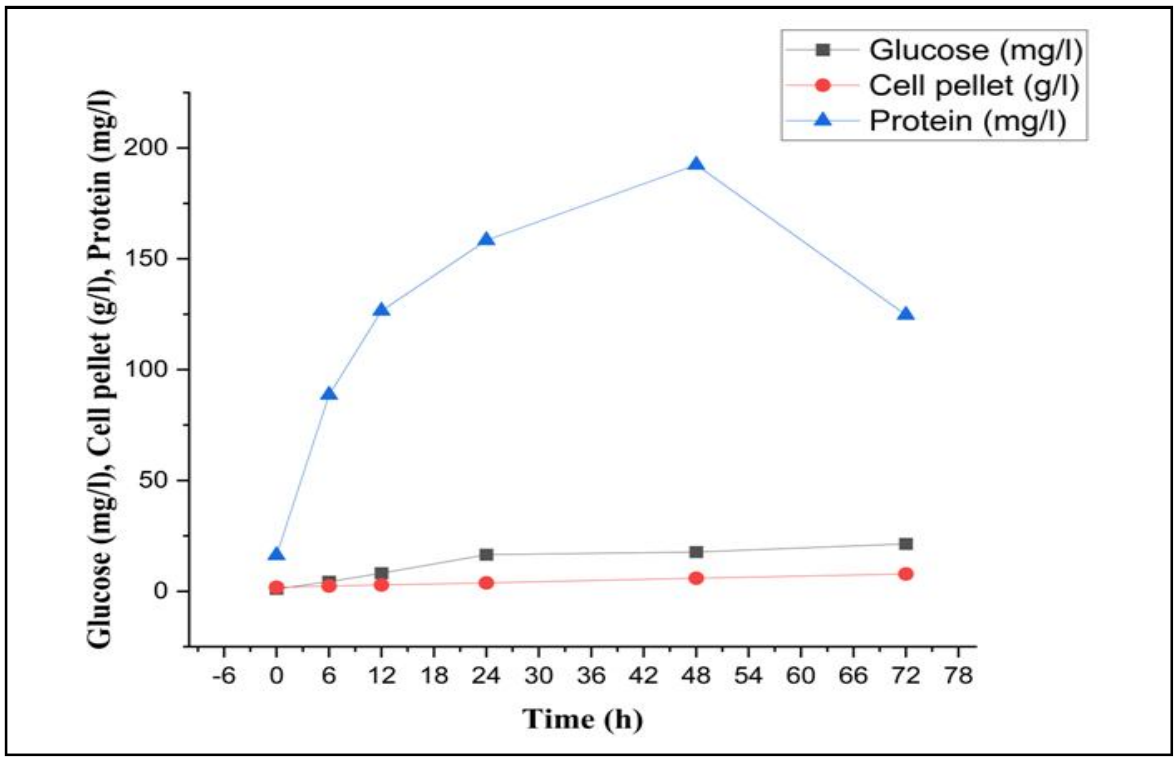

Figure 2: Cell yield, total protein content and glucose content of fermentation product during 72 h of fermentation.

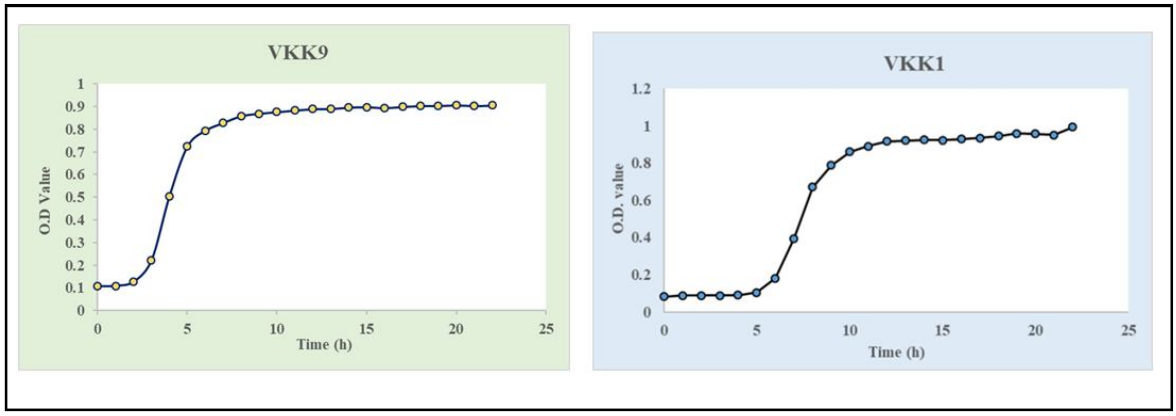

Figure 3: Growth curve of Bacillus strains at different time interval (a) VKK9 strain (b) VKK1 strain.

Table 4: Comparative growth parameters of native Bt isolates

Parameter	Isolate VKK9	Isolate VKK1
Specific growth rate (μ)	0.118	0.136
Growth rate constant (K)	2.644	2.870
Generation time (1/k)	22 min	20 min

3.5 Characterization of bioactive metabolites

Phase-contrast microscopy confirmed the presence of distinct bipyramidal crystal proteins and ellipsoidal terminal spores at the end of 72 h. Total protein estimation via the Bradford method showed that while biomass peaked at 72 h (7.824 g/l), the concentration of bioactive crystal proteins reached its maximum at 48 h (192.39 mg/l). This indicates that the harvesting window for maximum entomopathogenic potency is during the late stationary phase, prior to extensive proteolysis in the culture broth.

4. Discussion

4.1 Media optimization and metabolic synchronization

The bioprospecting of native *B. thuringiensis* isolates offers a transformative approach to the sustainable cultivation of medicinal

plants. As the global phytopharmaceutical industry moves toward zero residue standards, the role of indigenous microbial bio-factories like VKK9 and VKK1 becomes paramount. Our findings underscore that bioefficacy is a direct consequence of the metabolic synchronization achieved through optimized fermentation (Sikdar *et al.,* 1991; Osman *et al.,* 2015).

The significant increase in spore and protein yields when using starch and yeast extract highlights the importance of balanced C:N ratios. Unlike synthetic media, organic nitrogen sources like yeast extract provide essential vitamins and growth factors that act as precursors for delta-endotoxin synthesis (Avignone-Rossa *et al.,* 1992). In this study, the maximum protein yield of 192.40 mg/l achieved with optimized substrates suggests a robust transition from the vegetative phase to the stationary phase, where crystal protein synthesis is most intensive (Mohd-Salleh *et al.,* 1980). The high yield of delta-endotoxins is not merely a marker of fermentation efficiency but a prerequisite for maintaining the phytochemical profile of the target medicinal crops. By effectively managing pest pressure without the use of synthetic pesticides, these optimized Bt formulations prevent 'chemical stress-induced' alterations in the plant's secondary metabolism. This ensures that the levels of key bioactive constituents, such as alkaloids, flavonoids, and terpenoids, remain consistent with the plant's natural genetic expression, a cornerstone of standardized phytotherapy. This is critical for phytomedicine, as high-potency formulations allow for lower application rates on medicinal crops, preserving the integrity of the plant's natural rhizosphere (Vu *et al.,* 2009).

4.2 Fermentation kinetics and oxygen transfer dynamics

The mass transfer coefficient (K_La) of 0.0367 and the OUR of 0.10769 recorded for isolate VKK9 indicate highly efficient aerobic metabolism. In bioreactors, oxygen often becomes a limiting factor as biomass increases; maintaining a high OTR of 0.0204 is essential to prevent the accumulation of inhibitory metabolic by-products like acetic acid (Duarte Neto *et al.,* 2020).

The sharp decline in DO observed between 36 to 48 hours correlates with the peak of crystal protein production (192.39 mg/l), suggesting that oxygen demand is intrinsically linked to the synthesis of bioactive Cry proteins (Gong *et al.,* 2012). For a phytomedicine focused production line, these kinetic parameters provide a blueprint for achieving the batch to batch consistency required for standardized herbal drug protection.

4.3 Biosynergy and growth kinetics

A standout observation was the isolates' multifunctional potential. While the Cry proteins manage lepidopteran pests, the isolates' antimicrobial metabolites concurrently suppress soil-borne pathogens, while their plant growth promoting traits may enhance the biosynthesis of host-plant secondary metabolites (Eski *et al.,* 2017). This "biosynergy" ensures the medicinal plant is protected from biotic stress while its pharmacological potency is potentially enhanced. Furthermore, the pharmacological significance of utilizing native isolates like VKK9 extends beyond simple crop protection. The potential enhancement of host-plant secondary metabolites through PGP traits, often mediated by the secretion of microbial auxins or systemic resistance induction, can lead to a higher concentration of therapeutic compounds. For instance, increased biomass and physiological health in the medicinal host often correlate with a more robust synthesis of compounds responsible for antioxidant, anti-inflammatory, or antimicrobial activities. This biosynergy transforms the Bt isolate from a mere biopesticide into a biostimulant that fortifies the therapeutic grade of the raw herbal material.

Growth curve analysis revealed that VKK9 possesses a shorter lag phase and faster generation time (22 min.) compared to VKK1, making it an ideal candidate for industrial-scale "rapid fermentation" cycles. The ability to reach peak biomass in 72 h with a yield of 7.824 g/l demonstrates that native isolates, under optimized conditions (pH 7.0; 30°C), can outperform standard commercial strains (Saberi *et al.,* 2023).

5. Conclusion

The precise control of fermentation parameters, specifically oxygen transfer and pH stabilization, is the key to unlocking the full potential of native Bt isolates. By integrating these optimized bio-processes into the cultivation of medicinal plants, we can ensure the production of safer, chemical-free raw materials that meet the rigorous quality standards of the international phytopharmaceutical market.

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Authors' contribution statement

Raman Gopalakrishnan: carried out the experiments, data analysis and draft preparation, wrote the original draft**; Vinay Kumari Kalia**: conceptualized and designed the methodology; **Minakshi Grover and Karuppiah Manikandan**: reviewed and edited the manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, we used Grammarly to improve the language. After using this tool/service, we reviewed and edited the content as needed and take(s) full responsibility for the content of the publication

Conflict of interest

The authors declare that there are no conflicts of interest.

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