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Towards sustainable vector control: Innovative substrate identification for developing eco-friendly, cost-effective, and highly potent biopesticides

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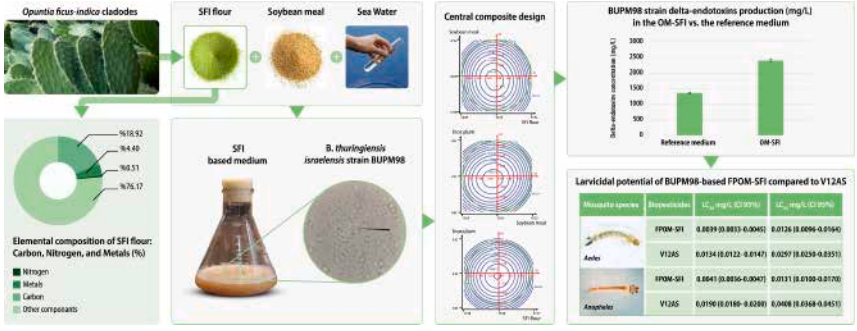
GRAPHICAL ABSTRACT



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ABSTRACT

The unavailability of biological insecticides for mosquitoes' control in Tunisia and their high cost make chemical pesticides the most used solution. In the present study, the development of a bio-sourced media based on agriculture by-product and free biological material was conducted for the generation of commercially valuable biopesticide from BUPM98 *Bacillus thuringiensis israelensis* strain. Through the physico-chemical characterization of the spineless *Opuntia ficus-indica* cladodes flour (SFI flour), an important total organic carbon rate (18.92 %) was detected. Thus, SFI flour was used as a potential carbon source for *B. thuringiensis* cells growth and delta-endotoxin production. A bio-sourced media based on diluted sea water, SFI flour and soybean meal, was optimized for BUPM98 delta-endotoxin production through the response surface methodology. The adjusted medium improved the production by 58 % compared to the reference medium. Moreover, an additional improvement of 22.85 % in delta-endotoxin synthesis was achieved through cultivating BUPM98 in 1 L shake flasks with baffles under optimal conditions. This enabled the biopesticides production in the novel medium (FPOM-SFI) to reach

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2405.5 ± 45 mg/L. Interestingly, the FPOM-SFI(BUPM98) achieved a more than threefold activity than VectoBac®12AS. In fact, it revealed LC_{50} values of 0.0039 mg/L and 0.0041 mg/L, against *Aedes aegypti* and *Anophele gambiae* larvae, respectively. These findings underscore the substantial potential of the BUPM98 based biopesticide produced in an almost bio-sourced medium for mosquito management in Tunisia as well as in North African countries, as an economical and eco-friendly alternative of chemical insecticides. FPOM-SFI(BUPM98) could be a sustainable and affordable pest control solution.

1. Introduction

Mosquitoes are considered as one of the main groups of arthropods that cause nuisance and public health problems (Tabbabi et al., 2019). Generally, three mosquito genera form the main disease vectors: *Anopheles*, *Aedes* and *Culex* (Tabbabi and Daaboub, 2017a). In recent decades, climate change and globalization have promoted mosquito-borne diseases (MBDs) geographic expansion to new areas, such as North African countries, where some of these MBDs were unusual or even unknown (Nebbak et al., 2022). *Bacillus thuringiensis* subspecies *israelensis* is one of the most effective biolarvicide against mosquitoes (Pitton, 2024). It is an aerobic gram-positive spore-forming bacterium capable of producing insecticidal crystal proteins, also known as delta-endotoxins. Its production occurs during sporulation and has specific toxicity when ingested by insect larvae (Duarte Neto et al., 2020). This bacterium is recommended by the World Health Organization (WHO) owing to the very specific activity of its delta-endotoxins on Dipteran larvae (Wu et al., 2021). It is hyperactive against 72 species of mosquitoes including 21 *Anopheles* spp., 21 *Aedes* spp., and 17 *Culex* spp (Lu et al., 2023). However, in many countries, the mosquito borne diseases are still a serious problem. In fact, the production of *B. thuringiensis*-based biopesticide, particularly in terms of the spore-crystal complex, is an expensive bioprocess and the progress in this field is limited, failing to meet market demands for cost-effective commercial bioinsecticides (Devidas et al., 2014; Zghal et al., 2018; Yapo et al., 2020). This process mainly depends on the composition of the culture medium and fermentation conditions. The growth, spore production, and toxicity of this bacterium are significantly influenced by the availability and the composition of the nutrient sources, including carbon, nitrogen, and macronutrients (Fayad et al., 2022). One of the main challenges of large-scale production is that raw materials represent 35–59 % of the total production cost, which limits the widespread use of this biopesticide (dos Santos et al., 2024). Commonly used nutrient sources in these media include various peptones, extracts, and hydrolysates, are expencive for industrial-scale production and face negative market acceptance due to their origin as animal byproducts (Nohata and Kurane, 1997; Devidas et al., 2014). Therefore, the media optimization is crucial for the bioprocess development to produce affordable biological agents. These optimization efforts are very important to meet the needs microbial insecticides' quantities.

Recently, the low-cost production of *B. thuringiensis,* achieved through the optimization of culture conditions using cheaper raw material has drawn a great deal of attention (Duarte Neto et al., 2020). The use of nonconventional sources has yielded a new knowledge in this field. The cost of raw materials for *B. thuringiensis* production is significantly affected by their regional availability. Using locally abundant, low-cost substrates, such as agricultural byproducts or industrial waste, can reduce production costs. For example, dos Santos et al. (2024) showed that pulp wash from the orange juice industry in Brazil is an affordable substrate for biopesticide production. In Indonesia, Suryadi et al. (2019) demonstrated that coconut and sugar cane enhance *B. thuringiensis* toxicity against *Ae. aegypti* larvae. Similarly, Sujani et al. (2018) used local substrates like coconut water and rice bran in Sri Lanka to develop a medium that reduced production costs by up to 293 times. In Côte d'Ivoire, Yapo et al. (2020) utilized sugar cane molasses to create an affordable medium enriched with yeast extract. These examples emphasize the importance of regional sourcing, though their efficiency may be reduced in areas with limited supply or high transportation costs.

Nowadays, Tunisia is considered to be a favourable location for mosquito emergence, particularly, in wadis, lakes and sebkhas (Sijoumi's sebkha), which are good places for mosquito breeding and spread. The high cost and unavailability of biological insecticides in Tunisia have led to the widespread use of chemical pesticides (Daaboub et al., 2008). Over more than forty years of intensive use, this has caused environmental pollution and the development of resistance in some mosquito species (Daaboub et al., 2008; Tabbabi and Daaboub, 2017b; Ben Cheikh et al., 1998; Lamontagne, 2004). In this context, *Opuntia ficus-indica*, or prickly pear, is a widespread succulent plant found in arid regions, including Mediterranean countries and North Africa (Arba, 2022). It covers about 25,000 ha in Tunisia (Arba, 2022) and is rich in carbohydrates, fiber, vitamins (C and B), and minerals like calcium and potassium (Boutakiout, 2015). Due to its abundance and composition, it holds potential as a nutrient source for *B. thuringiensis*. Using it as a low-cost raw material for *B. thuringiensis*-based biopesticides could offer significant scientific, economic, and technological benefits, which this study aims to investigate.

This work addresses the critical need for an affordable bio-larvicide for mosquito control in Tunisia by investigating the optimization of a cost-effective culture medium using locally available resources. Specifically, the study evaluates the impact of prickly pear cladodes flour on the production of toxins, spore formation, and larvicidal toxicity, comparing the efficacy of the locally produced bio-larvicide with a commercially available formulation, VectoBac® 12AS, in controlling *Ae. aegypti* and *An. gambiae* larvae. This work aims to contribute to the development of sustainable and economically viable biological pest control solutions.

2. Materials and methods

2.1. Culture medium

The LB (Luria Bertani Broth) medium was used for the preparation of the pre-inoculum (Sambrook et al., 1989). A previously-optimized complex medium (Ghribi et al., 2007) was taken as a reference production medium. Cladodes of *Opuntia ficus-indica* species were collected from Sidi Bouzid region, Tunisia, to be used as an alternative source of carbon for *B. thuringiensis israelensis* production. Commercial soybean meal, containing 46 % proteins, was kindly provided by a local mill of animal foods (ALCO Affes Group, Sfax, Tunisia). Sea water was sampled from the Mediterranean-sea coast of Sfax, Tunisia,(Ghribi et al., 2007).

The 500 mL and 1000 mL flasks with four baffles, containing 50 mL and 100 mL of culture medium, respectively, were used in this study. The preparation and incubation of the shake flasks were achieved as previously described by Ghribi et al. (2007). The pH was adjusted to 7.0. Before sterilization at 121 °C for 20 min, 1 g/L of $CaCO_3$ was added in each shake flask to maintain the pH stability.

2.2. Carbon source preparation and physico-chemical analysis

In order to facilitate its manual manipulation, thorns of *Opuntia ficus-indica* cladodes were eliminated by means of a brief heating using the flame of the Bunsen burner. Then, plant materials were dried in atmospheric conditions. The dried products were ground in a spice grinder,

sieved through a 250 μm sieve to obtain the spineless *Opuntia ficus-indica* cladodes flour (SFI flour). The SFI flour was kept in bottles until analysis.

After digestion of the SFI flour sample with 1.25 % Sulfuric acid (H2SO4) and 1.25 % sodium hydroxide (NaOH) solutions, the crude fiber of SFI flour was determined through AOAC Official Method 978.10. Moisture content was assessed using AOAC method 925.09 by oven drying the sample at 110 ∘C for 4 h. The fat content was determined by AOAC method 920.39. SFI flour sample was treated with n-Hexane for 6 h in a Soxhlet (Abutaha and AL-Mekhlafi, 2024). The resulting extract was then dried using BUCHI rotating evaporator R-100 and the extraction yield was determined gravimetrically. All AOAC methods referenced in this study follow the guidelines outlined by Latimer (2012). The nitrogen content was estimated by the Kjeldahl method (Kjeldahl, 1883) and converted to protein content using the conversion factor 6.25 (Jiang et al., 2014). The determination of total organic carbon was carried out with Shimadzu TOC analyzer TOC-VCPH according to standard methods as mentioned by Keskes et al. (2020). Atomic absorption spectroscopy was used to analyze the metal composition (Pb^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Cr^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}): firstly, the samples were attacked by hot acid solutions (HCl, HNO_3), then filtered and finally identified by atomic absorption spectroscopy technique via a "Perkin Elmer AAnalyst 200 atomic absorption spectrometer" device. The total sugars were determined by the Dubois method as described by Gao et al. (2022). Thus the reducing sugar concentrations were estimated by the dinitrosalicylic acid (DNS) method (Lam et al., 2021). The glucose content was estimated using the glucose (GOPOD Format) − kit enzymatic assay.

2.3. Delta-endotoxin production optimization

2.3.1. Bacterial strains and inoculum preparation

B. thuringiensis israelensis strain BUPM98 belongs to the Biopesticides laboratory collection and was characterized by Zghal et al. (2018). The acrystalliferous strain 4Q7 was originally obtained from *Bacillus* Genetic Stock Centre, Ohio State University, Columbus, Ohio, USA and used as negative control.

The inoculum was prepared by transferring cells from nutrient agar plates into 3 mL of LB medium and incubated overnight at 30 °C. Aliquots (0.5 mL) were used to inoculate 50 mL of the LB medium. The optical density (OD) at 600 nm was determined after being shake at 200 rpm in a shaker set over a 6-hours incubation at 30 °C. The culture broth was used to inoculate the studied media to start with an initial OD_{600} of 0.15. Then, the flasks were incubated the flasks for 72 h at 30 °C in the shaker set at 200 rpm.

2.3.2. Design of the experiments

The experimental design methodology was applied to optimize *B. thuringiensis israelensis* delta-endotoxin production by means of the response surface methodology. The present study adopted the central composite design using the following selected variables: X1: SFI flour (g/L), X2: soybean meal (g/L), and X3: initial inoculum OD_{600}. Each variable was assessed at five coded levels (−1.68, −1, 0, +1 and +1.68) (Table 1).

An hybrid design of 18 experiments was generated by Nemrod-W (Version 2007 software) (Mathieu et al., 2000). A total of 18 experiments were conducted including 2x2x2 full factorial design experiments (runs N°1 to 8), 6 axial points (runs N°9 to 14) and 4 replicates in the domain center (runs N°15 to 18) to estimate the variability of the experimental results (Table 2). As shown in Table 2, the response values (Ŷ) used in each trial were the average of three separate experimental results.

Table 1
Experimental range of the three variables studied using the central composite design in terms of actual and coded factors.

Variables	−1.68	−1	0	+1	+1.68
X1: SFI flour (g/L)	22.68	30	40	50	56.82
X2: soybean meal (g/L)	12.68	20	30	40	46.82
X3: initial inoculum OD_{600}	0.03	0.10	0.20	0.30	0.37

2.3.3. Statistical analysis and modeling

The obtained data from the central composite design with regard to BUPM98 delta-endotoxin production were subjected to the analysis of variance (ANOVA) to check the errors and the significance of each parameter (Table 3). Delta-endotoxin production (Y) was taken as a response parameter. The data were then subjected to a multiple regression analysis to obtain an empirical model that could relate the response measured to the independent variables (Table 4). The behavior of the system was explained by the following quadratic equation:

$$\hat{Y} = b_0 + b_1 * X_1 + b_2 * X_2 + b_3 X_3 + b_{1\text{-}1} * X_1^2 + b_{2\text{-}2} * X_2^2 + b_{3\text{-}3} * X_3^2 + b_{1\text{-}2} * X_1 * X_2 + b_{1\text{-}3} * X_1 * X_3 + b_{2\text{-}3} * X_2 * X_3$$

Where Ŷ refers to the predicted response, X_1, X_2 and X_3 are the studied coded factors (Table 2), b_0 is the intercept, b_1, b_2 and b_3 are the linear coefficients, $b_{1\text{-}1}$, $b_{2\text{-}2}$ and $b_{3\text{-}3}$ are the squared coefficients and $b_{1\text{-}2}$, $b_{1\text{-}3}$ and $b_{2\text{-}3}$ are the interaction coefficients.

The model coefficients were estimated by the multi-linear regression method using the Nemrod-W statistical software package by LPRAI Marseille, France. The statistical significance of the model was evaluated on the basis of Fisher's F test with unequal variance ($p < 0.05$) (Nair and Panda, 1997). The response surface graphs representing the system behavior were plotted after having conducted the regression analyses on the experimental data (Ghribi et al., 2012). The *iso*-response contour plot, qualified as a two-dimensional graphical representation, depicted the individual and cumulative effects of the parameters as well as the possible correlations existing between them. Comparison was carried out using a factorial ANOVA with subsequent post hoc test (Ryan, Einot, Gabriel, & Welsch).

2.4. Delta-endotoxin quantification

After a 72 h incubation, 1 ml of the culture was used to measure the total amount of proteins. After centrifugation, the crystal-spore pellets were washed twice with NaCl (1 M) and twice with bi-distilled water. The samples were then solubilized with NaOH (50 mM, pH 12.5) for 2 h at 30 °C in a rotary shaker (200 rpm). The delta-endotoxin concentration was determined by the Bradford method (Bradford, 1976) using bovine

Table 2
Three variables central composite design and the experimental and predicted responses for BUPM98 delta-endotoxin production.

Run order	X1: SFI flour (g/L)	X2: soybean meal (g/L)	X3: initial inoculum OD_{600}	Experimental response (mg/L)	Predicted response (mg/L)
1	30.00	20.00	0.10	1155.70	1018.77
2	50.00	20.00	0.10	1076.20	887.85
3	30.00	40.00	0.10	756.30	670.44
4	50.00	40.00	0.10	365.30	402.40
5	30.00	20.00	0.30	880.85	737.96
6	50.00	20.00	0.30	622.50	602.56
7	30.00	40.00	0.30	516.60	599.16
8	50.00	40.00	0.30	295.50	326.64
9	23.18	30.00	0.20	694.15	811.47
10	56.82	30.00	0.20	439.95	472.22
11	40.00	13.18	0.20	907.20	1146.41
12	40.00	46.82	0.20	711.10	621.48
13	40.00	30.00	0.03	631.60	802.99
14	40.00	30.00	0.37	524.95	503.15
15	40.00	30.00	0.20	1979.18	1889.72
16	40.00	30.00	0.20	1938.20	1889.72
17	40.00	30.00	0.20	1956.10	1889.72
18	40.00	30.00	0.20	1711.10	1889.72

Table 3
ANOVA analysis for response surface quadratic model (Y).

Variation Source	Sum of squares	Df	Mean square	Rapport	Significance
Regression	5.2113E + 0006	9	5.7904E + 0005	18.667	0.00019***
Residual	2.4815E + 0005	8	3.1019E + 0004		
Regression	2.0165E + 0005	5	4.0330E + 0004	2.602	0.23
Validity	4.6500E + 0004	3	1.5500E + 0004		
Total	5.4595E + 0006	17			

*** $P < 0.0001$ (very significant).

serum albumin (BSA) as a standard (Monroy et al., 2021). A negative control was carried out, with the acrystalliferous strain 4Q7 to consider the dissolved proteins from cell debris, spores' envelope, and insoluble or particulate materials. Ultimately, the delta-endotoxin concentration was determined as described by Zghal et al. (2018).

2.5. Spores count

Viable spore counts from the culture were calculated by colony counting after killing the vegetative cells by heating the culture sample at 80 °C for 10 min. The samples were serially diluted and the suitable dilutions were plated on LB nutrient agar. The plates were incubated overnight at 30 °C; then, the developed colonies were counted to analyze the colony-forming units (CFU) (Zghal et al., 2018).

2.6. Insecticidal activity against Aedes aegypti (Bora-Bora) and Anopheles gambiae ss (kis) larvae

Almost, the larvicidal activity experiments were conducted in secured insectarium platform from MIVEGEC, Montpellier, France, using the spore-crystal mixture produced from BUPM98 in the newly developed medium. The aqueous suspension formulation VectoBac® 12AS based on the *B. thuringiensis israelensis* strain AM65-52, was used as positive control. The larval bioassays were performed using a standard protocol described by the World Health Organization (OMS, 1970). Each bioassay was repeated three times using late third- and early fourth-instar larvae of susceptible reference strains: *Ae. aegypti* Bora-Bora from French Polynesia *and An. gambiae s.s* Kis, from Kisumu in Kenya. For each bioassay, 25 larvae were transferred to cups containing 99 mL of distilled water. For each test, we used four cups per concentration (100 larvae) and five to eight concentrations of each insecticide in a range that causes 0 to 100 % mortality. One mL of each insecticide, at the desired concentration, was added to the cup. Control treatments, using 1 mL of distilled water, were performed for each test. Bioassays were maintained at 27 (±1) °C for all tests. Larval mortality was recorded after 24 h of exposure. The dose–mortality relationships were analyzed by the log-probit regression using the R studio software (version 2023.03.0 Build 386 © 2009–2023) and the script BioRssay 6.2 (Milesi et al., 2013) for the determination of LC_{50} and LC_{95} values (Karunarathne et al., 2022). This software uses the iterative method of maximum likelihood to fit a regression between the logarithm of concentration and the probit of mortality. The goodness-of-fit was estimated by a weighted chi-squared test. It also estimated the lethal concentrations and the slope of the regression lines with their confidence intervals ($P = 0.05$).

Some experiments were conducted in the Laboratory of Biopesticides, in the Centre of Biotechnology of Sfax, Sfax, Tunisia, using the late third- and early fourth-instar larvae of susceptible reference strain, *Ae. aegypti* Bora-Bora obtained from MIVEGEC, Montpellier, France.

3. Results

3.1. SFI flour proximate composition

The SFI flour chemical composition and metal quantification are performed and resumed in Table 5. Air-dried cladode flour has a moisture content of 7.23 % (± 0.51) with a low pH value (4.7). The dominant component of this flour is the crude fibers (30 %). Also, it presents an important total organic carbon (TOC) rate of 18.92 % with a remarkable rate of carbohydrates and a moderated amount of reducing sugar (Table 5). Ash is an important component of SFI flour (24 %). The mineral quantification study showed that K^+ is the dominant mineral followed by Ca^{2+} and Mg^{2+} with the detection of other minerals in traces such as Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+}. However, a low fat, protein and total nitrogen (Nt) contents were observed (4.30 %, 3.18 % and 0.51 %, respectively). Based on the identified SFI composition, this substrate can be optimized to support the growth of *B. thuringiensis* cells and enhance the production of protein crystals.

3.2. Substitution of starch by SFI flour

In order to develop a cost-effective medium for BUPM98 delta-endotoxin production, the SFI flour was screened as a carbon source substrate instead of starch used in the complex medium (Ghribi et al., 2007). The results (Table 6) show that the delta-endotoxin production achieves approximately the same value obtained with the complex medium.

Table 4
Estimate regression coefficients for delta-endotoxin production (Y) using data in coded units.

Number	Estimate Coefficient	A. inflation	Ecart-Type	t. experimental	Significance
b_0	1889.729		87.931	21.49	< 0.0001***
b_1	−100.862	1.00	47.658	−2.12	0.067
b_2	−156.064	1.00	47.658	−3.27	0.011*
b_3	− 89.143	1.00	47.658	−1.87	0.098
$b_{1\text{-}1}$	− 441.188	1.08	49.520	−8.91	< 0.0001***
$b_{2\text{-}2}$	− 355.594	1.08	49.520	−7.18	< 0.0001***
$b_{3\text{-}3}$	−437.220	1.08	49.520	−8.83	< 0.0001***
$b_{1\text{-}2}$	− 34.281	1.00	62.268	−0.55	0.597
$b_{1\text{-}3}$	− 1.119	1.00	62.268	−0.02	0.986
$b_{2\text{-}3}$	52.381	1.00	62.268	−0.84	0.425

b_1 corresponds to X_1 (SFI flour g/L); b_2 corresponds to X_2 (Soybean meal g/L); b_3 corresponds to X_3 (initial inoculum OD_{600}); $b_{1\text{-}1}$, $b_{2\text{-}2}$, and $b_{3\text{-}3}$ represent the second-order effects of X_1, X_2, and X_3, respectively; $b_{1\text{-}2}$, $b_{1\text{-}3}$, and $b_{2\text{-}3}$ represent the interaction effects.
*** $P < 0.0001$ (very significant).
* $P < 0.05$ (Significant).

Table 5
The proximate composition of spineless *Opuntia ficus-indica* cladodes flour (SFI).

	SFI flour
Chemical composition (%)	
Total sugars	13.50 ± 0.0
Reducing sugars	4.10 ± 0.0
Glucose	2.50 ± 0.1
Proteins	3.18
Fat	4.30 ± 0.09
Crude fiber	30.45 ± 0.94
Ash	24.80 ± 0.0
Total carbon	18.92
Total organic carbon	18.92
Nitrogen total	0.51
Mineral elements %	
K^{+}	2.1
Ca^{2+}	1.422
Mg^{2+}	0.873
Na^{+}	0.002
Mn^{2+}	0.0008
Zn^{2+}	0.001
Fe^{2+}	0.0005
Cu^{2+}	0.0001
Pb^{2+}	<0.0002

Table 6
BUPM98 delta-endotoxin production in SFI based medium.

Medium	Delta-endotoxins (mg/L)	CFU (10^7 spores/mL)
Complex medium	1358.13 ± 23.67	37.56 ± 2.02
SFI based medium	1239.66 ± 43.75	38 ± 2.82

CFU: Colony-Forming Unit.

3.3. Optimization of BUPM98 biopesticide production using response surface methodology

The central composite experimental design was applied to reveal the optimum conditions for *B. thuringiensis israelensis* BUPM98 production through the determination of the optimum levels of the selected factors related to the SFI flour (X1), soybean meal (X2), and initial inoculum OD_{600} (X3). As shown in Table 2, there was a considerable variation in the delta-endotoxin production that strongly depended on the levels of the three independent variables in the medium. The delta-endotoxin concentrations varied from 295.5 mg/mL (run 8) to 1979.2 mg/mL (run 15).

The cubic response surface model was used to analyze the results. A regression equation (Y) reflecting the empirical relationships between production yield and test variables was obtained in coded units. The regression-estimated coefficients were obtained by the multi-linear regression method (Table 4). The *p*-values indicated that the first order of X_2 and the second order of X_1, X_2 and X_3 were significant ($p < 0.01$). However, none of the interaction effects was significant ($p > 0.05$). The analysis of variance (ANOVA) of the response surface quadratic model for delta-endotoxin production is shown in Table 3. ANOVA allows us to verify the validity of the model. The results showed that the *p*-values were significant ($p < 0.01$) for the regression model. Besides, the lack of fit is not significant for the response ($p > 0.05$). Consequently, the model could predict the optimal delta-endotoxin production and define the optimal variable values. The determination coefficient (R^2) for the delta-endotoxin production response is 0.96 indicating that 96 % of the variability in the response could be explained by the model which reflects a high correlation between the experimental and predicted values. The adjusted determination coefficient value (Adj $R^2 = 0.90$) was also close to the predicted R^2.

The second order polynomial regression equation for the delta-endotoxin concentration response (Y) is as follows (Eq. 2):

$$Y1 = 1889.7–156.1\ X_2 - 441.2\ (X_1^2) - 355.6\ (X_2^2) - 437.2\ (X_3^2)$$

The 3D response surface curves and their respective contour plots generated by the NEMROD software are displayed in Fig. 1. This figure provides information about the interaction between different paired factors and allows an easy prediction and interpretation of the optimum experimental conditions. As shown in Fig. 1A, by fixing the initial inoculum OD_{600} to its average value ($OD_{600} = 0.2$), the isoreponse curves and the response surface show that the SFI flour does not have a major effect on production in the presence of soybean meal concentrations ranging from 15 to 23 g/L. Above 23 g/L of soybean meal, the SFI flour (X1) exerts a positive effect on the synthesis of delta-endotoxins. The optimal production of delta-endotoxins (1890 mg/L) is achieved for SFI flour and soybean meal concentrations varying from 35 to 40 g/L and from 23 g/L to 27 g/L, respectively. A gradual decrease in the delta-endotoxins amount was observed, particularly when increasing the carbon and nitrogen concentrations beyond 40 g/L and 27 g/L, respectively. An increase of the SFI flour concentration value above 40 g/L was accompanied by a decrease in the production yield.

According to Fig. 1(B and C), increasing the initial inoculum OD_{600} from 0.15 to 0.2 resulted in an increase in the delta-endotoxin production. In addition, at a low soybean meal concentration and an initial inoculum OD_{600} below 0.15, toxins synthesis was greatly reduced, while the optimum production was obtained by using a concentration of soybean meal around 27 g/L and a moderate initial inoculum OD_{600} of 0.2. Interestingly, increasing the *B. thuringiensis israelensis* strain - BUPM98 initial inoculum OD_{600} above 0.2, at an optimal soybean meal concentration of 27 g/L, had a negative effect on the delta-endotoxin production (Fig. 1B). The Fig. 1C shows an optimum of the delta-endotoxin production with an initial inoculum OD_{600} of 0.19 in the presence of an SFI flour concentration ranging between 35 g/L and 40 g/L. Above the value 0.2 of the initial inoculum OD_{600}, the delta-endotoxins production decreased.

The representation of the optimal path calculated from the response surface analysis is shown in Fig. 2. The right part of the curve refers to the maximization of the response whereas the left part shows its minimization. The distance (r) from the center of the model is indicated in abscissa. Fig. 2A shows that the optimal response totally depends on the distance r. This figure demonstrates that the production reaches its maximum value at the center of the domain (r = 0). While Fig. 2B displays the coordinates of each factor as coded variables. It shows that when the delta-endotoxin production is close to the maximum, it is more sensitive to variations in soybean meal (X2) concentration and initial inoculum OD_{600} (X3) than to the SFI flour (X1) concentration.

The maximum production was achieved using 40 g/L SFI flour, 23 g/L soybean meal and an initial inoculum OD_{600} of 0.2 (OM-SFI). Under these optimal conditions (OM-SFI), the predicted delta-endotoxin production is around 1890 mg/L. The predicted value was validated experimentally using cultures that were performed in triplicates. The statistical analysis confirms that the experimental production value of 1959 ± 3 mg/L agreed with the predicted one. Under these conditions, the CFU count was 70 10^7 spores/mL. Our results confirm the efficiency of the model. Through the optimization process using Response Surface Methodology, an improvement of 58 % was achieved in the OM-SFI medium.

3.4. Improvement of delta-endotoxin production in 1L shake flasks with baffles

The determination of spore concentration and delta-endotoxins production were studied in a 1 L shake flasks with baffles, using the optimum medium OM-SFI. The sporulation rate increased approximately threefold (206.8 x 10^7 spores/mL) the reported value in the 500 ml shake flasks with baffles (70 10^7 spores/mL). In addition, the delta-endotoxin production is about 2405.5 ± 45 mg/L. Thus, an additional improvement of 22.85 % was observed. The obtained product was called

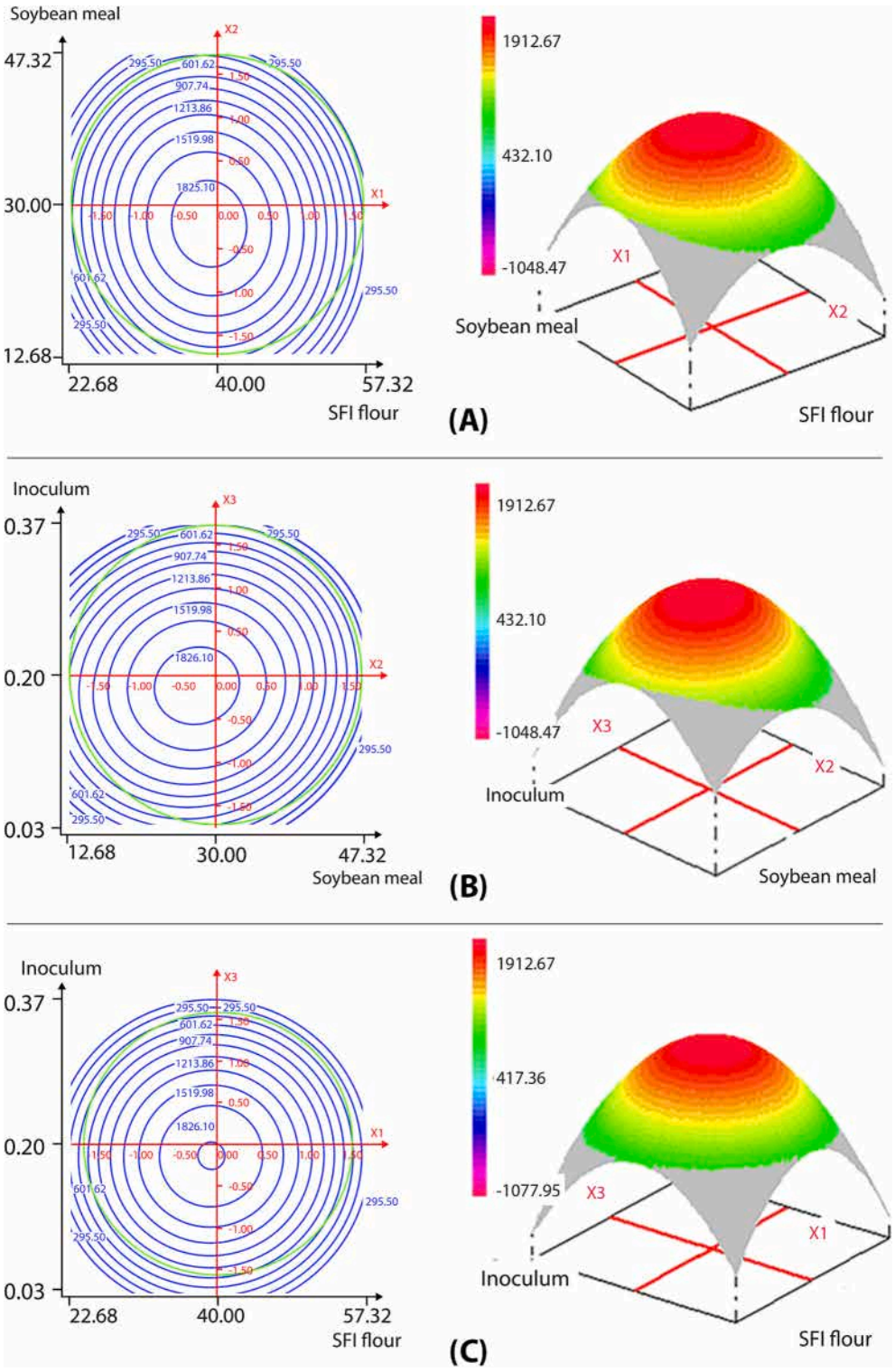

Fig. 1. Effects of the SFI flour ƒ (g/Lƒ), the soya bean ƒ(g/Lƒ), and the initial optical density (OD) of inoculum on the delta-endotoxin production at 600 nm: response surface plot (left) and its interaction contour plot (right). (A): Variation in delta-endotoxin production as a function of SFI flour (X1) and soybean meal (X2), with a fixed initial inoculum OD_{600} (X3) of 0.2. (B): Variation in delta-endotoxin production as a function of soybean meal (X2) and initial inoculum OD_{600} (X3), with a fixed SFI flour (X1) of 40 g/L. (C): Variation in delta-endotoxin production as a function of SFI flour (X1) and initial inoculum OD_{600} (X3), with a fixed soybean meal (X2) of 23 g/L.

fermentation product from the optimized SFI based media: FPOM-SFI.

3.5. Larvicidal activity of BUPM98 fermentation product from the SFI based media

The larvicidal activity of BUPM98 fermentation product FPOM-SFI produced in the shake flasks with baffles, was evaluated against *Ae.*

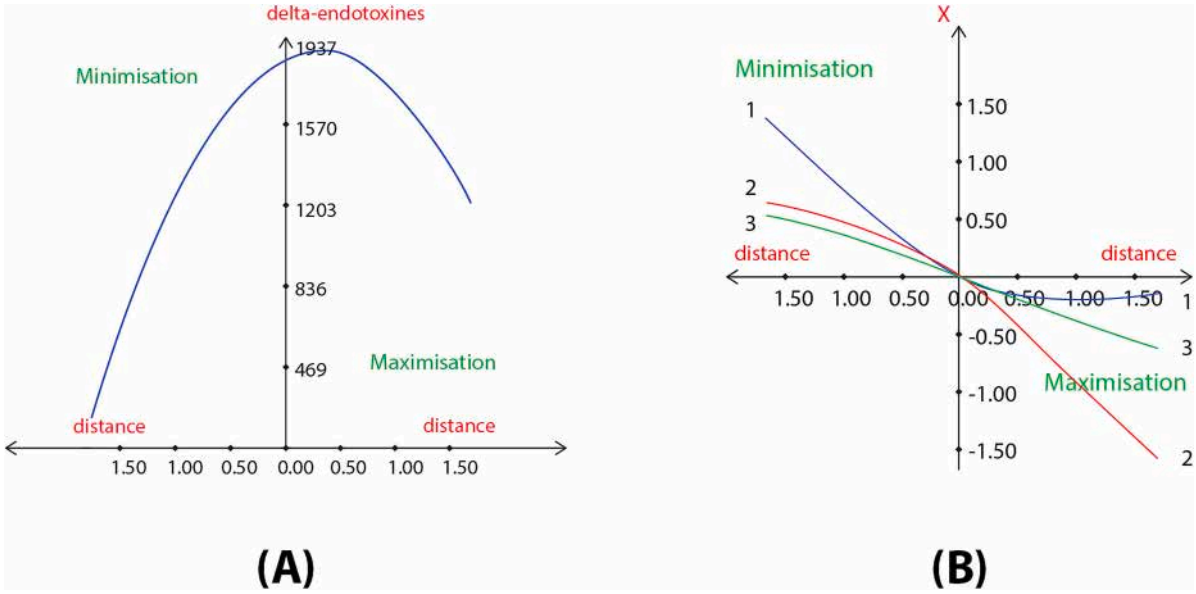

Fig. 2. Ridge analysis. (A) Optimal response plot: predicted values of the maximum and minimum responses versus the distance from the center of the design space, (B) optimum coordinate plot: coordinates of the optimum points versus the distance from the center of the design space. X: Coded values of variables; 1: SFI flour; 2: Soybean; 3: initial inoculum OD_{600}.

aegypti (Bora-Bora) using the spore-crystal mixture produced in the reference medium as control. For each insecticide, the three replicates were not statistically different as indicated by similar LC_{50} and LC_{95} values (data not shown) and the overlap of confidence intervals. For the negative control, no mortality was recorded. The obtained results of LC_{50} values are displayed in Table 7. The starch substitution by the SFI flour showed an improvement of the BUPM98 larvicidal activity (LC_{50} = 0.00267 mg/L). Therefore, the LC_{50} decreased by 15 % using FPOM-SFI.

3.6. Promising larvicidal potential of FPOM-SFI against Aedes aegypti (Bora-Bora) and Anopheles gambiae ss (kis): A Comparative study with VectoBac® 12AS

The larvicidal activity of FPOM-SFI based on BUPM98 against *Ae. aegypti* (Bora-Bora) and *An. gambiae ss* (kis) larvae was compared to the commercial formulation VectoBac® 12AS. The Log-probit analysis performed for each replicate made with *Ae. aegypti* (Bora-Bora) and *An. gambiae ss* (kis) larvae that the dose–response relationship fitted well to a straight line ($P > 0.05$) (Table in Supplementary data). Both mosquito species exhibited homogeneous responses to the biopesticides. The three replicates were not statistically different for each mosquito species and each insecticide as indicated by the similar LC_{50} and LC_{95} values and the overlap of confidence intervals. For the negative control, no mortality was recorded. Compared to VectoBac® 12AS, the obtained results revealed that FPOM-SFI based on BUPM98 exhibited the highest larvicidal activity (Fig. 3). This spore–crystal mixture was more than three-fold active toward *Ae. aegypti* (Bora-Bora) (0.0039 mg/L) and *An. gambiae ss* (kis) (0.0041 mg/L) larvae than VectoBac® 12AS. This result highlights the efficiency of BUPM98 strain's FPOM-SFI.

Table 7
Lethal concentrations in mg/L (LC_{50} and LC_{95}) of 3rd to 4th instar larvae of Ae. aegypti (Bora-Bora) exposed to the fermentation products developed in the SFI based medium (FPOM-SFI) and the reference medium (FP-RM).

Fermentation product	LC_{50} mg/L (CI 95 %)	LC_{95} mg/L (CI 95 %)
FP-RM	**0.0030 (0.0026–0.0034)**	**0.0085 (0.0071–0.0107)**
FPOM-SFI	**0.0026 (0.0023–0.003)**	**0.0061 (0.0053–0.0073)**

LC_{50}: lethal concentration for 50% of larvae; LC_{95}: lethal concentration for 95% of larvae; CI: confidence interval.

4. Discussion

The choice of growth medium or raw materials is crucial for the commercial production of biopesticides. To promote cost-effective biopesticide production, the use of less expensive raw materials is highly recommended (Choe et al., 2022). Incorporating agricultural by-products into fermentation media for the production of *B. thuringiensis*-based biopesticides could provide a reliable and affordable method for utilizing this bioinsecticide in pest management. As mentioned by Gounina-Allouane et al. (2022), various agricultural raw materials and agro-industrial wastes were used as an alternative culture media for the spores and delta-endotoxins production by different *B. thuringiensis* strains. So, in this study, an innovative carbon source was used for the production of a Tunisian *B. thuringiensis israelensis* (BUPM98) based biopesticide. It is the spineless *Opuntia ficus-indica* cladodes flour (SFI flour), which is highly available in Tunisia and it can be used as a cheap carbon source for toxin production. Based on the physico-chemical characterization results, the SFI flour could be a good alternative substrate for *B. thuringiensis israelensis* bioinsecticide production. In fact, the high TOC rate supported the growth of BUPM98 strain and stimulated the delta-endotoxins synthesis. This effect was further influenced by the abundant carbohydrates and a moderate amount of reducing sugars, which are easily assimilated by microorganisms and played a significant role in delta-endotoxin production (Navarro-Mtz et al., 2019; Elsayed et al., 2014). In addition, the metal composition is in agreement with the obtained results by EL-Mostafa et al., (2014). In fact, it showed that K^+ is the major mineral in this flour followed by Ca^{2+} and $Mg^{2+}$and some oligo-elements like Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+}. All these elements could enclose the nutritional needs of *B. thuringiensis* biopesticide production. Although, K^+ is a main factor for the production of crystals protein by *B. thuringiensis* (Wakisaka et al., 1982). However, the SFI flour has a low protein and total nitrogen (Nt) contents. This suggests that this plant material is insufficient to be a nitrogen source for bacterial growth. In summary, SFI flour as free biosource could replace the starch for *B. thuringiensis* cells growth and delta-endotoxin production, as an innovative carbon source with the presence of minerals and some oligo-elements.

To improve the production yield of BUPM98 strain based bioinsecticide, the response surface methodology was adopted to define its optimal concentration in the SFI based medium. The maximum

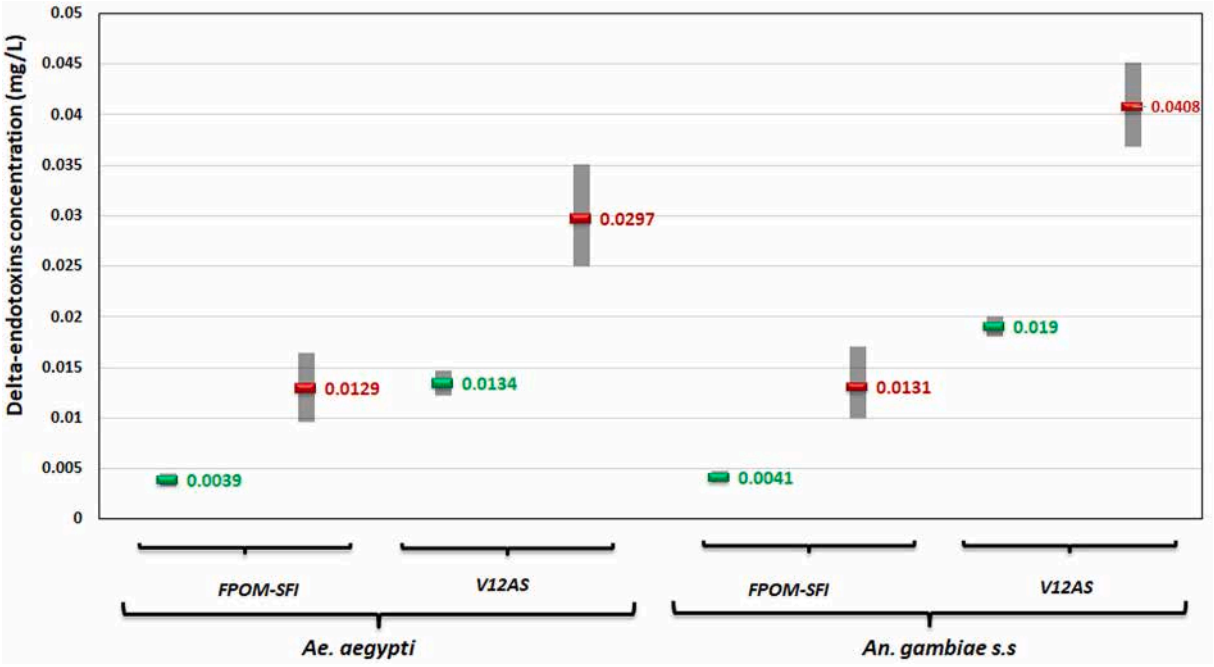

Fig. 3. The LC_{50} and LC_{95} values (mg/L) of BUPM98 fermentation product from the optimized medium (FOM-SFI) and Vectobac®12AS against 3rd to 4th instar of *Ae. aegypti* and *An. gambiae s.s* larvae; The bars in gray represent the 95 % confidence interval of LC_{50} values, the red dashes and the associated values in red show the calculated LC_{95} values for each insecticide and the green dashes and the associated values in green show the calculated LC_{50} values for each insecticide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

production yield can be achieved using 40 g/L SFI flour, 23 g/L soybean meal and an initial inoculum OD_{600} of 0.2. Moreover, the optimization of the culture medium composition, achieved through this model is a good alternative to improve the delta-endotoxin production by 58 % compared to the initial condition (Table 2). This is in agreement with several studies which proved the efficient use of the response surface methodology in the improvement of delta-endotoxin and spore production yield (Duarte Neto et al., 2020). However, an increase of the SFI flour concentration value above 40 g/L was accompanied by a decrease in the production yield. This decrease may be the consequence of the catabolic repression that was previously described for the *B. thuringiensis* fermentation (Masri and Ariff, 2020). In fact, several operons involved in delta-endotoxin synthesis could be repressed in the presence of high carbon source concentrations (Magasanik, 1987). Besides, at the optimum SFI flour and soybean meal concentrations, an increase of the initial inoculum OD_{600} above 0.2 resulted in decreased toxin production. In fact, according to Ennouri et al. (2013), a high initial cell concentration in the production medium may result in a rapid consumption of oxygen and other nutrients.

Using flasks with baffles, an additional increase was noted in the sporulation rate and delta-endotoxin production (22.85 %). This is in agreement with Avignone-Rossa et al. (1992) study which showed that spore counts and delta-endotoxin concentration of *B. thuringiensis israelensis* were proportional to the oxygen concentration supply. For the cultivation of aerobic organisms, the use of baffled flasks is one of several methods employed to improve aeration and ensure a constant supply of oxygen (Tunac, 1989). Numerous studies have demonstrated that dissolved oxygen levels significantly influence cell growth and δ-endotoxin synthesis during *B. thuringiensis* culture (da Silva et al., 2021; Malairuang et al., 2023). Our results are in agreement with Ben Khedher et al. (2014) study, which confirmed that a high level of aeration can enhance glucose consumption and cellular interaction with environmental compounds, thereby enhancing delta-endotoxin production.

The larvicidal activity of BUPM98 fermentation product FPOM-SFI against *Ae. aegypti* (Bora-Bora) and *An. gambiae ss* (kis) larvae showed a reduction of the LC_{50} value (15 %) comparing to the reference medium. These results agree with the hypothesis that the variation in toxicity might be related to the different components of the culture medium. In fact, in the literature, the LC_{50} values of *B. thuringiensis israelensis* products against dipterans range from 0.00075 to 8.2 mg/L (Duarte Neto et al., 2020). For example, when sugarcane and soybean bagasse were used as a culture medium instead of coconut cake (Poopathi and Archana, 2012), the LC_{50} values of *B. thuringiensis israelensis* IPS82 strain against *Ae. aegypti*, *C. quinquefasciatus*, and *An. stephensi* were higher (Poopathi and Archana, 2013).

Interestingly, the FPOM-SFI based on BUPM98 demonstrates significantly higher larvicidal effectiveness than the widely used commercial formulation VectoBac® in larval control of *Ae. aegypti* and *An. gambiae ss*. In fact, the LC_{50} values of FPOM-SFI decreased by more than three times. So, the efficiency of BUPM98 toxins could be attributed to the original composition of this medium rich in mineral elements (SFI flour and sea water). Indeed, it has been shown that a complex of mineral salts has a positive effect on the insecticidal activity of the studied bacteria (Drehval et al., 2003). In addition, Ghribi et al. (2007) demonstrated that the use of sea water in *B. thuringiensis* cultural medium strongly reduced the proteolytic activities concomitantly produced with delta-endotoxin which could improve the bio-larvicides stability and consequently their toxicity.

Recent advances in bioinsecticide formulation have highlighted the critical role of optimizing culture media to enhance toxin efficacy while reducing production costs, making biopesticides more accessible for large-scale applications (Duarte Neto et al., 2020; Mo-on et al., 2022; dos Santos et al., 2024). In this context, our study introduces a novel and affordable alternative based on SFI flour, a locally abundant carbon source. This innovative approach not only aligns with sustainable biocontrol strategies but also demonstrates superior larvicidal effectiveness compared to the commercial formulation VectoBac®. Given the growing interest in eco-friendly and economically viable bioinsecticides, the use of SFI flour as a fermentation substrate represents a promising direction for enhancing *B. thuringiensis*-based biopesticide production, particularly in regions where resource availability and production costs

are key constraints. Further investigations into its large-scale optimization and applicability could pave the way for its integration into future bioinsecticide development.

5. Conclusion

This research has successfully developed a novel, cost-effective medium using locally available and reproducible SFI flour as a carbon source for *B. thuringiensis israelensis* production. Using Response Surface Methodology, the optimized medium (OM-SFI) was formulated with SFI flour, soybean meal, and diluted seawater as carbon, nitrogen, and mineral sources, respectively. OM-SFI improved delta-endotoxin production by 58 % compared to the reference medium. The resulting biopesticide (FPOM-SFI) showed superior larvicidal activity against *Ae. aegypti* (Bora-Bora) and *An. gambiae*, with LC_{50} values more than three times lower than VectoBac® 12AS. These results highlight SFI flour as a promising, sustainable alternative for biopesticide production. Thus, FPOM-SFI could be a potent alternative to VectoBac® 12AS for larval control of major mosquito vector species. The optimization of medium composition and culture parameters not only enhances *B. thuringiensis israelensis* biolarvicide production but also offers significant economic benefits. The OM-SFI medium presents a viable solution for large-scale, low-cost production of effective mosquito biopesticides in Tunisia. Given the widespread burden of mosquito-borne diseases across Africa, this fermentation product has the potential for broader application within vector control efforts throughout the continent. Further pilot studies are recommended to validate the scalability of this process for widespread implementation.

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CRediT authorship contribution statement

Fatma Benjeddou: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Ines Mnif:** Writing – review & editing, Validation, Software, Methodology, Formal analysis. **Marie Rossignol:** Visualization, Software, Methodology, Formal analysis, Conceptualization. **Dhouha Ghribi:** Methodology. **Slim Tounsi:** Funding acquisition. **Fabrice Chandre:** Writing – review & editing, Visualization, Supervision, Resources. **Raida Zribi Zghal:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Ethics approval

This work has not been published previously, it is not under consideration for publication elsewhere. Its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the copyright holder. The study results are accurately and truly reported. The data from the study results can be produced. The submitted manuscript is an original work and has not plagiarized another work. The paper has not been submitted concurrently to another journal nor is it published elsewhere.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2025.105768.

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