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Research Article

Vegetative insecticidal protein (Vip3A) production by *Bacillus thuringiensis* Bt294 and its efficacy against Lepidopteran pests (*Spodoptera exigua*)

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ABSTRACT

A vegetative insecticidal protein, Vip3A, is highly active against lepidopteran pests, which are the most important pests in most tropical countries. An important aspect of the successful commercial production of this bacterial insecticide is the development of bacterial culture media that maximize the titres of this protein and cost reduction. This study aimed to investigate and optimize Vip3A production by *Bacillus thuringiensis* Bt294 using statistical methods and 3-step sequential approaches. The experimental design showed that the production of Vip3A was maximized to 300 mg/L when the bacterium was cultivated in medium composed of 5.05 g/L glycerol, 49.17 g/L soytone, 30.05 g/L casein hydrolysate, 1.99 g/L $CaCl_2.2H_2O$, 7.5 mg/L CuSO4, 15 mg/L $MnSO_4.H_2O$, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 5 g/L yeast extract, 2.5 mg/L $NiCl_2$.6H2O and 3 mL/L vitamin solution. *B. thuringiensis* Bt294 Vip3A toxin was highly toxic to *Spodoptera exigua* with LC50 values of 187.1 ng/cm^2 at 7 days. This result demonstrated that a high titre of Vip3A produced by *B. thuringiensis* Bt294 will be useful as a biological control agent. This optimization will allow production to be scaled up for commercial production in the future.

1. Introduction

In addition to chemical insecticides, bioinsecticides are important components of agricultural production that control insect pests and enhance crop productivity. However, the use of chemical insecticides has now been found to cause toxic environmental contamination (soil, natural water reservoirs, air and crops), and the consumption of these agricultural products is harmful to humans. The use of bioinsecticides is an alternative approach to reduce the high use of chemical insecticides. The entomopathogenic bacteria *Bacillus thuringiensis* have been widely used as biological controls in the microbial pesticide market since they are a source of insecticidal proteins. These bacteria produce a wide variety of insecticidal proteins, i.e., crystal (Cry) proteins, cytolytic (Cyt) proteins and secreted insecticidal proteins, including vegetative insecticidal protein (Vip), that are active against the larvae of very diverse insects [1]. The most widely used bioinsecticides in the biological control of agricultural pests are based on insecticidal Cry proteins. However, Cry proteins are not very efficient in controlling some of the important insect pests, including some lepidopteran pests. Moreover, some pests have developed resistance against some Cry toxins.

Vegetative pesticidal proteins are produced by *B. thuringiensis* during the vegetative growth phase as well as during sporulation and are secreted into the culture medium [2]. Currently, based on amino acid sequence identity, vegetative pesticidal proteins are classified into four families, i.e. Vpb1, Vpa2, Vip3 and Vpb4 [1]. The binary toxin Vpb1/Vpa2 are toxic to some coleopteran and hemipteran insects, while proteins in the Vip3 family are described as toxic against a wide variety of lepidopteran insects [3]. Proteins in the Vip4 family have not yet been reported to target insects [4].

Proteins in the Vip3 family have been shown to have insecticidal activity against a broad spectrum of economically important lepidopteran pests, such as tobacco cutworm (*Spodoptera litura*) [5], beet

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Table 1
Vip3A production by *B. thuringiensis* Bt294 using the Plackett-Burman experimental design.

Treatments	Factors[a]											Vip3A concentration (mg/L)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	
1	40	30	10	5	1	42	2.5	2.5	14	7.5	1	120.0 ± 26.5
2	10	30	30	2	1	42	7.5	2.5	14	2.5	3	3.5 ± 2.1
3	40	10	30	5	0.1	42	7.5	7.5	14	2.5	1	3.5 ± 2.1
4	10	30	10	5	1	14	7.5	7.5	42	2.5	1	4.0 ± 1.4
5	10	10	30	2	1	42	2.5	7.5	42	7.5	1	225.0 ± 50.0
6	10	10	10	5	0.1	42	7.5	2.5	42	7.5	3	1.6 ± 1.9
7	40	10	10	2	1	14	7.5	7.5	14	7.5	3	12.5 ± 3.5
8	40	30	10	2	0.1	42	2.5	7.5	42	2.5	3	137.5 ± 17.7
9	40	30	30	2	0.1	14	7.5	2.5	42	7.5	1	1.8 ± 1.1
10	10	30	30	5	0.1	14	2.5	7.5	14	7.5	3	225.0 ± 50.0
11	40	10	30	5	1	14	2.5	2.5	42	2.5	3	1.3 ± 1.8
12	10	10	10	2	0.1	14	2.5	2.5	14	2.5	1	1.1 ± 0.2
13	10	10	30	2	0.1	14	7.5	7.5	42	2.5	3	6.3 ± 5.3
14	40	10	10	5	0.1	14	2.5	7.5	42	7.5	1	166.7 ± 28.9
15	10	30	10	2	1	14	2.5	2.5	42	7.5	3	200.0 ± 0.0
16	40	10	30	2	0.1	42	2.5	2.5	14	7.5	3	100.0 ± 0.0
17	40	30	10	5	0.1	14	7.5	2.5	14	2.5	3	0.8 ± 1.1
18	40	30	30	2	1	14	2.5	7.5	14	2.5	1	225.0 ± 35.4
19	10	30	30	5	0.1	42	2.5	2.5	42	2.5	1	3.0 ± 2.8
20	10	10	30	5	1	14	7.5	2.5	14	7.5	1	7.5 ± 4.3
21	10	10	10	5	1	42	2.5	7.5	14	2.5	3	120.0 ± 26.5
22	40	10	10	2	1	42	7.5	2.5	42	2.5	1	3.5 ± 2.1
23	10	30	10	2	0.1	42	7.5	7.5	14	7.5	1	3.5 ± 2.1
24	40	30	30	5	1	42	7.5	7.5	42	7.5	3	4.0 ± 1.4
25	25	20	20	3.5	0.55	28	5	5	28	5	2	225.0 ± 50.0

Base medium: 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, pH 7.2.
[a]X_1, glycerol (g/L); X_2, soytone (g/L); X_3, casein hydrolysate (g/L); X_4, yeast extract (g/L); X_5, $CaCl_2.2H_2O$ (g/L); X_6, $ZnSO_4.7H_2O$ (mg/L); X_7, $CuSO_4$ (mg/L); X_8, $NiCl_2.6H_2O$ (mg/L); X_9, $FeSO_4.7H_2O$ (mg/L); X_{10}, $MnSO_4$. H_2O (mg/L); X_{11}, vitamin solution (Blackmore) (mL/L).

armyworm (*Spodoptera exigua*) [6], carob moth (*Ectomyelois ceratoniae*) [7], cotton leafworm (*Spodoptera littoralis*) [8], and cotton bollworm (*Helicoverpa armigera*) [9]. Research on Vip3A production by *B. thuringiensis,* including research with recombinant bacteria, has been conducted for a long time. Recently, Hmani et al. [8] reported the use of nitrous acid and UV (classical mutagenesis) to increase Vip3A production in *B. thuringiensis* BUPM65 and detected improvements in the production and/or efficiency of *B. thuringiensis* Vip3Aa16 toxins. However, previous studies have shown a low concentration of the Vip3 toxin, which limits its use as a biocontrol agent. In addition, few reports on the optimization of Vip3A production by wild-type *B. thuringiensis* has been published. To address these problems related to Vip3A production, this study investigated the enhancement of Vip3A protein production by *B. thuringiensis* Bt294, a Vip3A-producing bacterium, using response surface methodology (RSM). In addition, the toxin activity of Vip3A from *B. thuringiensis* Bt294 against *Spodoptera exigua*, which is the most important pest in most tropical countries, was evaluated. This Vip3A protein production by *B. thuringiensis* Bt294 might be useful for sustainable organic crops in many countries.

2. Materials and methods

2.1. Microorganism

Bacillus thuringiensis Bt294 (accession number: MK955482) was isolated from paddy soil collected in Kalasin province, Thailand. The culture was stored in Luria Bertani (LB) medium with 20% glycerol at −80 °C.

2.2. Optimization of Vip3A production by B. thuringiensis Bt294

The bacterial inoculum was prepared in 250 mL Erlenmeyer flasks containing 50 mL of LB broth and incubated on a rotary shaker at 200 rpm at 30 °C for 16–18 h. The bacterial inoculum was transferred into 50 mL of production medium with an initial OD600 of 0.10–0.15.

2.3. General factorial design

The effects of carbon and nitrogen sources on Vip3A production by *B. thuringiensis* Bt294 were estimated based on the protein yield of the bacterium. Various carbon sources (glucose, glycerol, lactose, sucrose and xylose) and nitrogen sources (yeast extract, soytone, casein hydrolysate, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, skimmed milk, KNO_3, monosodium glutamate and peptone) were used at the concentrations of 5 and 36 g/L, respectively. The base medium consisted of 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4 and 0.2 g/L $MgSO_4.7H_2O$. The pH of the medium was adjusted to 7.2, and the medium was cultured while shaking at 200 rpm on an orbital shaker (Innova™ 4900, New Brunswick Scientific, NJ, USA) at 30 °C for 4 days.

The concentration of Vip3A protein was measured using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The culture supernatant was mixed with SDS-PAGE sample buffer (consisting of 0.6 mL of 1 M Tris buffer (pH 6.8), 5 mL of 50% glycerol, 2 mL of 10% SDS, 1 mL of 1% bromophenol blue, 50 μL of β-mercaptoethanol, 0.9 mL of water) and boiled at 100 °C for 10 min. After that, the sample was loaded and separated by SDS-PAGE (ATTO AE-6530 system, Tokyo, Japan) using a continuous gel that included a 12% resolving gel and 4% stacking gel. The gel was stained using Coomassie blue R250 stain [10]. The protein concentration of Vip3A was estimated by a densitometer with Gene Directory software (Syngene, Cambridge, UK) after SDS-PAGE separation. Bovine serum albumin (BSA) was used as a standard.

For Western blot analysis, Vip3A was separated by SDS-PAGE and transferred to a nitrocellulose blot. The nitrocellulose blots were treated with a polyclonal antibody targeting Vip3A (diluted 1/10,000) and then with alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (diluted 1/8000). Bromochloroindolyl-phosphate and nitroblue tetrazolium were used as substrates for the alkaline phosphatase reaction.

2.4. Plackett-Burman (PB) design

The PB design [11] was used to identify the factors that influence

Table 2
Vip3A production by *B. thuringiensis* Bt294 using a central composite design.

Treatments	Factors[a]						Vip3A concentration (mg/L)
	X_1	X_2	X_3	X_4	X_5	X_6	
1	10	50	40	2	7.5	7.5	8.3 ± 2.9
2	10	50	40	1	15	7.5	6.7 ± 2.9
3	10	50	30	2	7.5	15	233.3 ± 57.7
4	10	30	40	1	15	15	266.7 ± 57.7
5	5	50	30	2	15	15	333.3 ± 57.7
6	10	30	40	2	7.5	15	266.7 ± 57.7
7	5	50	40	1	7.5	7.5	266.7 ± 57.7
8	10	50	30	1	15	15	83.3 ± 28.9
9	10	30	30	2	7.5	7.5	216.7 ± 76.4
10	5	30	40	1	7.5	15	183.3 ± 28.9
11	5	50	30	1	7.5	15	150.0 ± 0.0
12	10	30	30	1	15	7.5	200.0 ± 0.0
13	5	30	30	2	15	7.5	150.0 ± 0.0
14	5	30	40	2	15	15	125.0 ± 0.0
15	5	50	40	2	15	7.5	125.0 ± 35.4
16	5	30	30	1	7.5	7.5	166.7 ± 28.9
17	3.34	40	35	1.5	11.25	11.25	166.7 ± 28.9
18	11.66	40	35	1.5	11.25	11.25	17.5 ± 10.6
19	7.5	23.36	35	1.5	11.25	11.25	158.3 ± 14.4
20	7.5	56.64	35	1.5	11.25	11.25	37.5 ± 17.7
21	7.5	40	26.68	1.5	11.25	11.25	166.7 ± 28.9
22	7.5	40	43.32	1.5	11.25	11.25	133.3 ± 28.9
23	7.5	40	35	0.67	11.25	11.25	166.7 ± 28.9
24	7.5	40	35	2.33	11.25	11.25	233.3 ± 28.9
25	7.5	40	35	1.5	5	11.25	283.3 ± 28.9
26	7.5	40	35	1.5	17.5	11.25	266.7 ± 28.9
27	7.5	40	35	1.5	11.25	5	250.0 ± 50.0
28	7.5	40	35	1.5	11.25	17.5	266.7 ± 57.7
29	7.5	40	35	1.5	11.25	11.25	175.0 ± 43.3

Base medium: 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 5 g/L yeast extract, 2.5 mg/L $NiCl_2.6H_2O$, 3 mL/L vitamin solution (Blackmore), pH 7.2.
[a]X_1, glycerol (g/L); X_2, soytone (g/L); X_3, casein hydrolysate (g/L); X_4, $CaCl_2.2H_2O$ (g/L); X_5, $CuSO_4$ (mg/L); X_6, $MnSO_4.H_2O$ (mg/L).

Vip3A production by *B. thuringiensis* Bt294. A total of 11 factors, i.e., glycerol, soytone, casein hydrolysate, yeast extract, $CaCl_2.2H_2O$, $ZnSO_4.7H_2O$, $CuSO_4$, $NiCl_2.6H_2O$, $FeSO_4.7H2O$, $MnSO_4$. H_2O and vitamin solution (Blackmore), were evaluated. The base medium component and growth conditions were as described above. The Plackett-Burman experimental design with three center points (25 treatments) for Vip3A production by *B. thuringiensis* Bt294 is shown in Table 1. The center point was run to evaluate the curvature and the linearity of the variables. The culture conditions were as follows: shaking at a speed of 200 rpm on an orbital shaker at 30 °C for 5 days. The experiment was performed in triplicate.

2.5. Central composite design (CCD)

After screening the influential factors by PB design, the optimal value of influencing factors was optimized using CCD [12] to enhance Vip3A production. The experimental design (29 treatments) with the factor name, code level and actual level of the factors are shown in Table 2. The culture conditions were as follows: shaking at 200 rpm on an orbital shaker at 30 °C for 5 days. The experiment was performed in triplicate. The adequacy of the quadratic model was determined using the coefficient of determination (R2) and analysis of variance (ANOVA). Design Expert software version 10.0 (State-Ease, US) was used to draw contour plots and to explain the interactions of the variables with Vip3A yield. The predicted best condition for Vip3A production by *B. thuringiensis* Bt294 was identified. The bacterium was grown in Terrific Broth (TB) enriched with 4 mL/L glycerol as a control [13].

After optimizing Vip3A production in shaking flask cultures, a 3-L working volume of optimal medium was used to evaluate Vip3A production in a 5-L fermenter (Biostat B Plus, Sartorius Stedim, Germany). The inoculum used in this experiment was 5% of the working volume. The dissolved oxygen and pH were not controlled during fermentation. The fermenter was operated at a temperature of 30 °C, agitation speed of 400 rpm, and aeration of 4.0 L/min (equivalent to 1.33 vvm).

2.6. Insect toxicity assay

Vip3A bioassays were performed using third instar larvae of *S. exigua* obtained from the NPV production pilot plant at Thailand Science Park, Thailand. The insect toxicity of Vip3A in culture filtrates of *B. thuringiensis* Bt294 was analyzed by surface contamination assays. The fermented medium that contain Vip3A was diluted by adding the optimal medium to adjust the concentration of Vip3A to a required concentration prior to apply on the diet surface in a 24-well tissue culture plate. Six different concentrations of Vip3A (50, 100, 200, 400 and 1000 ng/cm^2) were poured on the surface of the artificial diet (consisting of 10 g/L yeast extract, 1.5 g/L sorbic acid, 2.5 g/L ascorbic acid, 20 mL/L multivitamin stock, 120 g/L ground mung bean and 12.5 g/L agar). After the protein solution was completely absorbed, the larvae were added to each well and incubated at room temperature (25 ± 2 °C). *S. exigua* mortality was recorded for 7 days after incubation. Forty eight *S. exigua* larvae were treated with each concentration, and 3 independent experiments were performed. LC50 and LT50 was assessed using Probit analysis [14]. The optimal medium was used as a negative control.

3. Results

3.1. Optimization of Vip3A production by B. thuringiensis Bt294

3.1.1. Vip3A production by B. thuringiensis Bt294 using a general factorial design

Among the five carbon sources tested, glycerol was found to be the best carbon source for Vip3A production by *B. thuringiensis* Bt294 (Figure S1). In addition to SDS-PAGE analysis, the presence of Vip3A (a protein band at 88 kDa) in the culture supernatant was confirmed by Western blot analysis (Figure S2). Moreover, the analysis of the effects of glycerol in combination with nitrogen sources on Vip3A production by *B. thuringiensis* Bt294 indicated that soytone (Figure S1F) and casein

Table 3
Analysis of variance (ANOVA) of Vip3A production by *B. thuringiensis* Bt294 using a Plackett–Burman design.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Block	15,713.97	3	5237.99		
Model	3.585E+005	22	16,297.41	110.93	< 0.0001
X_1-Glycerol	2.734E+005	1	2.734E+005	1860.76	< 0.0001
X_2-Soytone	24,752.08	1	24,752.08	168.47	< 0.0001
X_3–Casein hydrolysate	2310.19	1	2310.19	15.72	0.0005
X_4-Yeast extract	709.17	1	709.17	4.83	0.0375
X_5–$CaCl_2.2H_2O$	2067.19	1	2067.19	14.07	0.0009
X_6-$ZnSO_4.7H_2O$	259.01	1	259.01	1.76	0.1963
X_7–CuSO4	6864.08	1	6864.08	46.72	< 0.0001
X_8–$NiCl_2.6H_2O$	649.01	1	649.01	4.42	0.0458
X_9-$FeSO_4.7H_2O$	99.19	1	99.19	0.68	0.4190
X_{10}–$MnSO_4.H_2O$	3825.26	1	3825.26	26.04	< 0.0001
X_{11}-Vitamin solution	86.67	1	86.67	0.59	0.4496
Curvature	0.000	0			
Residual	3673.04	25	146.92		
Lack of Fit	3635.54	23	158.07	8.43	0.1113
Pure Error	37.50	2	18.75		
Cor Total	3.779E+005	50			

$R^2 = 0.99$, Adjusted $R^2 = 0.98$

hydrolysate (Figure S1G) resulted in the highest Vip3A yield. In addition, yeast extract also favored the production of Vip3A by *B. thuringiensis* Bt294 (Figures S1A, S1E, S1I, S1M and S1Q). The other carbon and nitrogen sources did not favor Vip3A production by this bacterium. This result indicated that the most suitable carbon and nitrogen sources for Vip3A production by *B. thuringiensis* Bt294 were glycerol, soytone and casein hydrolysate. Hence, glycerol, soytone and casein hydrolysate, including yeast extract, were chosen for further studies.

3.1.2. *Vip3A production by B. thuringiensis Bt294 using a Plackett-Burman design*

The data listed in Table 1 show a wide variation in the protein concentration of Vip3A (mg/*L* ± SD), from 0.8 to 250 mg/L, in the 25 treatments. The *p*-values from analysis of variance (ANOVA) of 11 factors showed that glycerol, soytone, casein hydrolysate, yeast extract, $CaCl_2.2H_2O$, $CuSO_4$, $NiCl_2.6H_2O$ and $MnSO_4.H_2O$ had direct effects on Vip3A production ($p = 0.05$), whereas $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$ and vitamin solution Blackmore) showed a nonsignificant effect on Vip3A production by *B. thuringiensis* Bt294 (Table 3). The equation that relates the 11 variables and the analytical response is as follows:

$$Y = 60.12 - 75.47\times_1 + 22.71\times_2 + 6.94\times_3 + 3.84\times_4 + 6.56\times_5 - 2.32\times_6 + 11.96\times_7 + 3.68\times_8 - 1.44\times_9 + 8.93\times_{10} + 1.34\times_{11}$$

where Y is the Vip3A concentration (mg/L), X_1 is glycerol, X_2 is soytone, X_3 is casein hydrolysate, X_4 is yeast extract, X_5 is $CaCl_2.2H_2O$, X_6 is $ZnSO_4.7H_2O$, X_7 is $CuSO_4$, X_8 is $NiCl_2.6H_2O$, X_9 is $FeSO_4.7H_2O$, and X_{10} is $MnSO_4$. H_2O and X_{11} is vitamin solution (Blackmore).

The models are statistically significant at *p*-values lower than 0.0001 (Table 3). In addition, the obtained model showed a determination coefficient value (R^2) of 0.99 for Vip3A production (Table 3). The significant factors, glycerol, soytone, casein hydrolysate, $CaCl_2.2H_2O$, $CuSO_4$ and $MnSO_4.H_2O$, showed *p*-values lower than 0.001. These results indicated that these factors are key components that increase Vip3A production by *B. thuringiensis* Bt294. Fig. 1 show the concentration of Vip3A (mg/*L*±SD) against the upper, lower and middle level of influencing factors. This figure demonstrates that increased glycerol

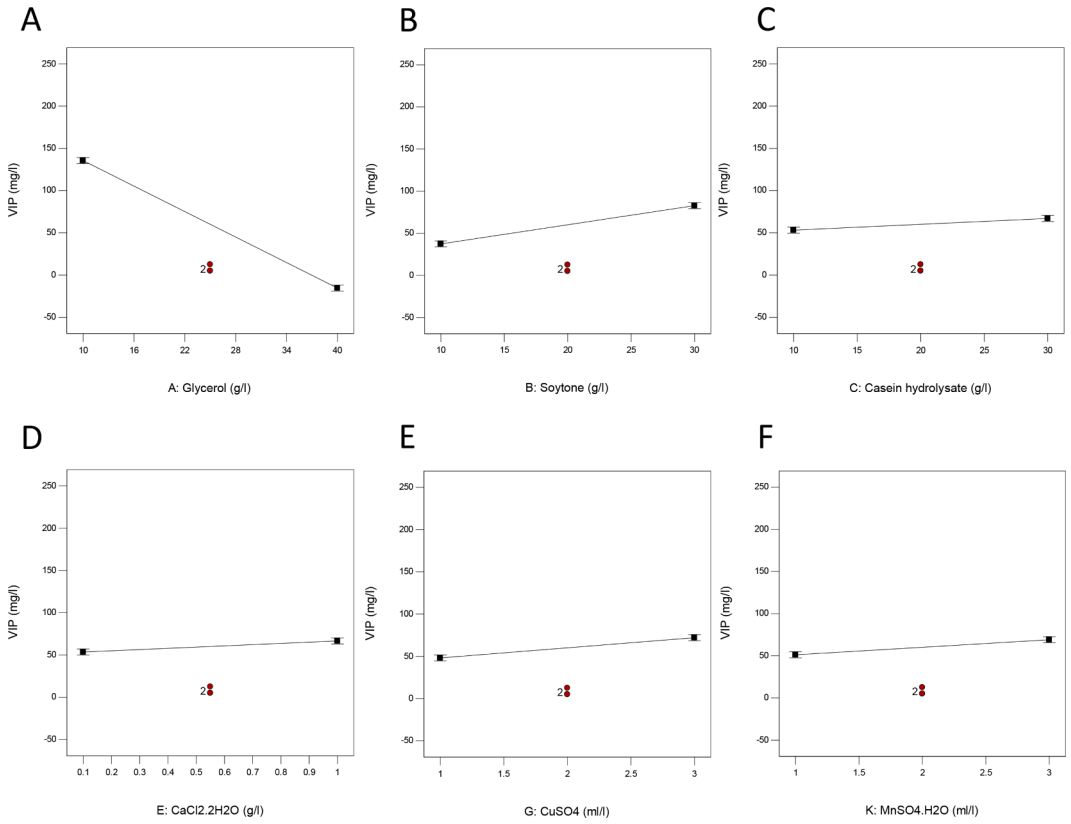

Fig. 1. Effects of glycerol (A), soytone (B), casein hydrolysate (C), $CaCl_2.2H_2O$ (D), $CuSO_4$ (E) and $MnSO_4.H_2O$ (F) on Vip3A production by *B. thuringiensis* Bt294 using a Plackett-Burman design.

Table 4
ANOVA of the response surface quadratic model of Vip3A production by *B. thuringiensis* Bt294 using a central composite design.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Model	6.311E+005	27	23,374.80	17.38	< 0.0001
X_1-Glycerol	33,376.04	1	33,376.04	24.81	< 0.0001
X_2-Soytone	21,901.04	1	21,901.04	16.28	0.0002
X_3–Casein hydrolysate	1666.67	1	1666.67	1.24	0.2702
X_4–$CaCl_2{\cdot}2H_2O$	6666.67	1	6666.67	4.96	0.0298
X_5–$CuSO_4$	416.67	1	416.67	0.31	0.5799
X_6–$MnSO_4.H_2O$	416.67	1	416.67	0.31	0.5799
$X_1{\times}_2$	1.414E+005	1	1.414E+005	105.11	< 0.0001
$X_1{\times}_3$	1354.69	1	1354.69	1.01	0.3197
$X_1{\times}_4$	712.66	1	712.66	0.53	0.4696
$X_1{\times}_5$	1658.30	1	1658.30	1.23	0.2714
$X_1{\times}_6$	21,042.19	1	21,042.19	15.64	0.0002
$X_2{\times}_3$	8570.79	1	8570.79	6.37	0.0143
$X_2{\times}_4$	11,875.52	1	11,875.52	8.83	0.0043
$X_2{\times}_5$	63.02	1	63.02	0.047	0.8294
$X_2{\times}_6$	750.98	1	750.98	0.56	0.4579
$X_3{\times}_4$	53,000.52	1	53,000.52	39.40	< 0.0001
$X_3{\times}_5$	7625.52	1	7625.52	5.67	0.0205
$X_3{\times}_6$	2176.71	1	2176.71	1.62	0.2083
$X_4{\times}_5$	11,991.07	1	11,991.07	8.91	0.0041
$X_4{\times}_6$	32,292.19	1	32,292.19	24.01	< 0.0001
$X_5{\times}_6$	4313.02	1	4313.02	3.21	0.0785
X_1^2	48,625.82	1	48,625.82	36.15	< 0.0001
X_2^2	42,528.32	1	42,528.32	31.62	< 0.0001
X_3^2	6185.25	1	6185.25	4.60	0.0361
X_4^2	1920.90	1	1920.90	1.43	0.2369
X_5^2	51,774.39	1	51,774.39	38.49	< 0.0001
X_6^2	34,862.50	1	34,862.50	25.92	< 0.0001
Residual	79,359.87	59	1345.08		
Lack of Fit	151.54	1	151.54	0.11	0.7403
Pure Error	79,208.33	58	1365.66		
Cor Total	7.105E+005	86			
R^2 0.89, adj R^2 0.84					

concentrations had a negative effect on Vip3A production, whereas increased soytone, casein hydrolysate, $CaCl_2.2H_2O$, $CuSO_4$ and $MnSO_4.H_2O$ concentrations had a positive effect. Thus, six factors, namely, glycerol, soytone, casein hydrolysate, $CaCl_2.2H_2O$, $CuSO_4$ and $MnSO_4.H_2O$, were retained for further optimization using CCD. Yeast extract and vitamins were fixed at high levels, and $NiCl_2.6H_2O$ was fixed at a low level.

3.1.3. *Vip3A production by B. thuringiensis Bt294 using a central composite design*

A wide range of Vip3A production (6.7–333.3 mg/L) was revealed by the CCD experimental design (Table 2). The experimental results were analyzed and are shown in Table 4. The ANOVA results of the regression model demonstrated that the model is highly significant ($p < 0.0001$), and the lack-of-fit value ($p = 0.7403$) showed that the model fit well in the experimental design. The coefficient of determination ($R^2 = 0.89$) indicated that the obtained model could explain up to 89% of the variability in Vip3A concentration. In addition, the adjusted determination coefficient value (adjusted $R^2 = 0.84$) also supports the significance of the model. For Vip3A production by *B. thuringiensis* Bt294, X_1, X_2, X_4, $X_1{\times}_2$, $X_1{\times}_6$, $X_2{\times}_3$, $X_2{\times}_4$, $X_3{\times}_4$, $X_3{\times}_5$, $X_4{\times}_5$, $X_4{\times}_6$, X_1^2, X_2^2, X_3^2, X_5^2 and X_6^2 are significant model terms. The quadratic model equation for Vip3A concentration (Y) was:

$$\text{Vip3A} = -2342.16 + 113.62{\times}_1 + 48.69{\times}_2 + 90.18{\times}_3 + 287.70{\times}_4 - 22.16{\times}_5 - 71.31{\times}_6 - 2.17{\times}_1{\times}_2 - 0.43{\times}_1{\times}_3 + 6.08{\times}_1{\times}_4 + 1.24{\times}_1{\times}_5 + 2.23{\times}_1{\times}_6 - 0.53{\times}_2{\times}_3 + 3.15{\times}_2{\times}_4 - 0.03{\times}_2{\times}_5 + 0.21{\times}_2{\times}_6 - 13.29{\times}_3{\times}_4 - 0.67{\times}_3{\times}_5 - 0.71{\times}_3{\times}_6 - 16.62{\times}_4{\times}_5 + 13.83{\times}_4{\times}_6 + 0.67{\times}_5{\times}_6 - 5.20{\times}_1^2 - 0.30{\times}_2^2 - 0.46{\times}_3^2 + 25.83{\times}_4^2 + 2.38{\times}_5^2 + 1.96{\times}_6^2$$

where Y is the Vip3A concentration (mg/L), X_1 is glycerol (g/L), X_2 is soytone (g/L), X_3 is casein hydrolysate (g/L), X4 is $CaCl_2.2H_2O$ (g/L), X_5 is $CuSO_4$ (mg/L), and X_6 is $MnSO_4.H_2O$ (mg/L).

To determine the optimal concentration of each factor to maximize Vip3A production by *B. thuringiensis* Bt294, contour plots were drawn and are shown in Fig. 2. The contour plots further validated the experimental model when the factors were at the following concentrations: 5.05 g/L glycerol, 49.17 g/L soytone, 30.05 g/L casein hydrolysate, 1.99 g/L $CaCl_2.2H_2O$, 7.5 mg/L $CuSO_4$, and 15 mg/L $MnSO_4.H_2O$, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 5 g/L yeast extract, 2.5 mg/L $NiCl_2.6H_2O$ and 3 mL/L vitamin solution (Blackmore). Three independent experiments were carried out to validate the predicted value (383 mg/L of Vip3A). Under these conditions, the Vip3A concentration was 300 mg/L, which was close to the value predicted by the regression model. In comparison with TB medium (75 mg/L Vip3A), the optimal medium identified in this study resulted in an increase in Vip3A production by a factor of four.

When the production of Vip3A was evaluated in a 5-L fermenter, the maximum Vip3A production of 300 mg/L was obtained (Fig. 3). This result indicated that the optimal medium components can be applied for Vip3A production in a larger production volume. This optimized condition will be used in additional scale-up steps with other fermentation processes, such as fed-batch fermentation, to increase the titer of Vip3A protein.

3.2. *Insect toxicity assay of Vip3A from B. thuringiensis Bt294*

Vip3A proteins exhibit insecticidal activity against a wide variety of lepidopterans; interestingly, certain species are less susceptible to some Cry proteins. For example, the genus *Agrotis* (e.g., *Agrotis ipsilon*) is known to be tolerant to Cry proteins. In addition, the genus *Spodoptera* (e.g., *S. exigua* and *S. frugiperda*) showed low susceptibility to Cry proteins [5]. Thus, in this study, the toxicity of Vip3A in the culture supernatant of *B. thuringiensis* Bt294 was assessed with the lepidopteran insect pest *S. exigua*. Vip3A was highly active against third instar larvae of *S. exigua*, with LC_{50} values of 187.1 ng/cm^2 at 7 days (Table 5).

4. Discussion

B. thuringiensis Bt294 was used as a Vip3A production factory. The optimized production culture was developed to obtain the highest yield of Vip3A. The first step to obtain the optimized medium is to choose the proper carbon and nitrogen source. The results from general factorial design showed that the glycerol gave the highest yield of Vip3A. From previous studied demonstrated advantage of glycerol was that it is not a carbon catabolite repressor. In addition, glycerol exhibited a lower carbon catabolite repression effect on delta-endotoxin synthesis in *B. thuringiensis* subsp. kurstaki strain BNS3 than glucose [15]. However, *B. thuringiensis* strain 81 showed low amounts of toxin when grown on glycerol [16]. This result indicated that optimal carbon sources for cell growth and toxin production were dependent on the bacterial strain. Regarding nitrogen sources, similar to carbon sources, various nitrogen sources, such as peptone [16] and corn steep liquor [17], promoted cell growth and toxin production by *B. thuringiensis*.

After obtain the proper carbon and nitrogen source, the others factor such as vitamin, trace elements were chosen to perform the PB design to identify the influencing factors for VipA production. The results showed that, there were six factors including glycerol, soytone, casein hydrolysate, $CaCl_2.2H_2O$, $CuSO_4$ and $MnSO_4.H_2O$ that effected the Vip3A production from *B. thuringiensis* Bt294. Apart from these factors, Song et al. [18] and Boukedi et al. [19] reported that post-induction

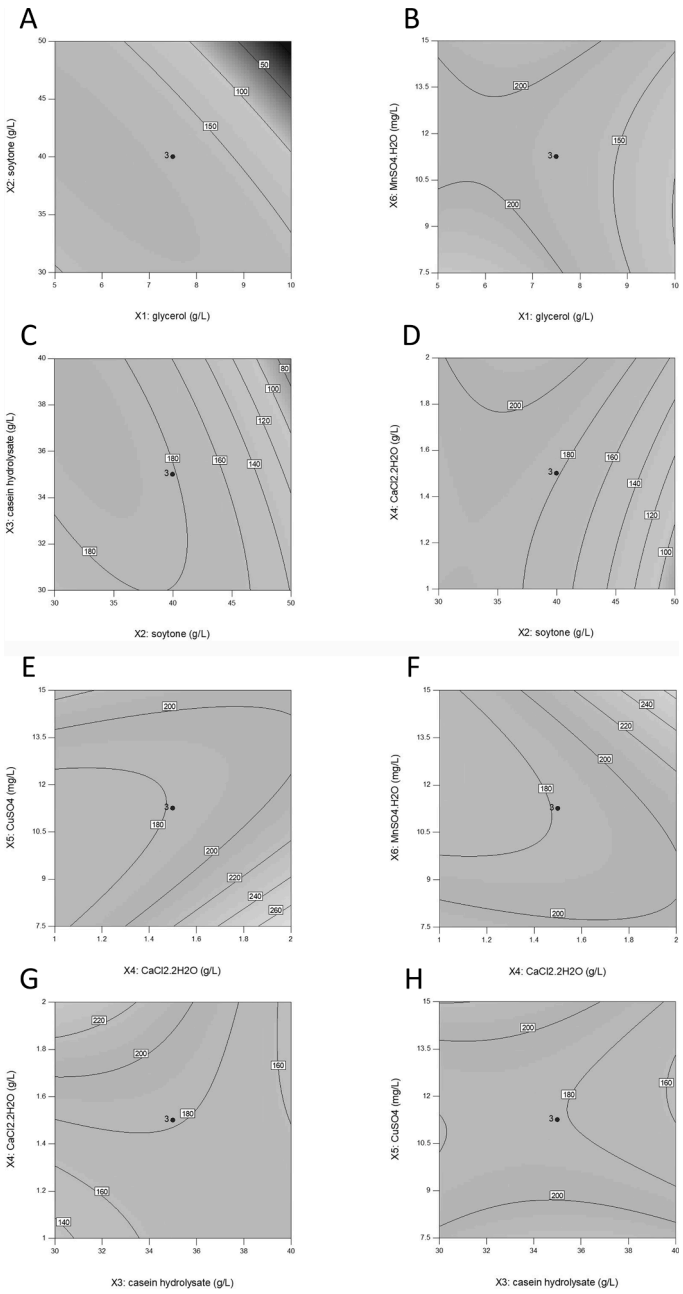

Fig. 2. Contour plots of interaction terms that significantly influence Vip3A production by *B. thuringiensis* Bt294 using a central composite design, showing the interactive effects of glycerol/ soytone (A) and glycerol/ $MnSO_4$. H_2O (B), soytone/ casein hydrolysate (C), soytone/ $CaCl_2.2H_2O$ (D), casein hydrolysate/ $CaCl_2.2H_2O$ (E), casein hydrolysate/ $CuSO_4$ (F), $CaCl_2.2H_2O$/ $CuSO_4$ (G), and $CaCl_2.2H_2O$/ $MnSO_4.H_2O$ (H).

temperature and post-induction time had effects on the expression level of Vip3A target protein.

At the last step, the six influencing factors were used to perform CCD design to obtain the optimized medium for Vip3A production from *B. thuringiensis* Bt294. The optimized medium from this experiment gave the maximum Vip3A as 300 mg/L in 5-L fermenter. In a previous study, Song et al. [18] enhanced Vip3A production up to 2.3 times before optimization in recombinant *Escherichia coli* BL21-pCzn1-Vip. Then, in 2015, Boukedi et al. [19] improved Vip3A production by optimizing the culture conditions in recombinant *E. coli* MOSBlue cells using a PB design and response surface methodology (CCD), representing an approximately 21-fold increase compared to the starting conditions (8 mg/L). This study demonstrated and suggested that the optimization of media components by RSM is required for to increase production to meet the demand, particularly in field tests. The concentration of Vip3Aa16 produced (170 mg/L) by recombinant *E. coli* MOSBlue was still lower than that reported in the present study by *B. thuringiensis* Bt294 (300 mg Vip3A/L). Vip3 protein production declined after 48 hrs of cultivation might be due to the cleavage of this protein by protease produced by the bacterium resulted in 66 and 19 kDa of peptide bands [20]. Thus, the results indicated that *B. thuringiensis* strain Bt294 is a potent Vip3A-producing bacterium.

After obtain the high concentration of Vip3A, the insect toxicity assay was performed to validate the quality of Vip3A protein. Vip3A from this experiment was highly active against third instar larvae of *S. exigua*, with LC50 values of 187.1 ng/cm^2 at 7 days. In previous reports, the same toxin showed a higher LC_{50} value than the value observed in this study. For example, Chakroun et al. [21] and Palma et al. [4] showed that the LC_{50} values of Vip3Aa16 and Vip3Aa45a against *S. exigua* were 2600 and 119.7 ng/cm^2 at 7 days, respectively.

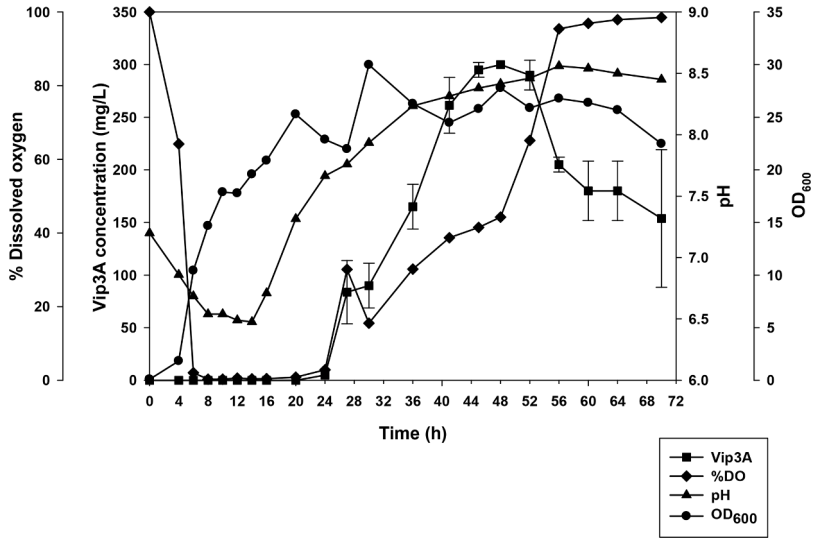

Fig. 3. Vip3A production by *B. thuringiensis* Bt294 in a 5-L batch fermenter.

Table 5
Analysis of the toxicity of culture supernatant containing Vip3A of *B. thuringiensis* Bt294 against *S. exigua*.

Vip3A concentration (ng/cm^2)	% Mortality	LC_{50} (ng/cm^2)
1000	100.0	187.1 (167.3–208.2)
400	83.2	
200	62.8	
100	36.8	
50	9.1	
0	0.0	

Baranek et al. [22] also showed that Vip3Aa58 and Vip3Aa59 were active against *S. exigua*, with LC50 values of 160 and 190 ng/cm^2 at 10 days, respectively.

5. Conclusions

Optimization of medium components is an essential step in increasing product yield and in scaling up microbial fermentation. The present study successfully and significantly optimized Vip3A production in *B. thuringiensis* strain Bt294, and this study improved Vip3A production by up to 4-fold compared to unoptimized conditions. The overall results suggested that *B. thuringiensis* Bt294 is one of the bacterial resources suitable for a microbial cell factory of Vip3A production. This bacterial Vip3A toxin also has potential as a biological control agent. This result supports the use of *B. thuringiensis* Bt294 Vip3A toxin as a biocontrol agent against lepidopteran pests (*S. exigua*) without further concentration steps. This optimization will be useful information for scaling up Vip3A production for large scales for field tests and commercial production.

Declaration of Competing Interest

The authors declare no conflicts of interest. Experimental investigation, methodology, data curation, formal analysis and writing-original draft preparation were done by Pumin Nutaratat, investigation and methodology were done by Borworn Werapan, Netnapa Phosrithong, Chutchanun Trakulnaleamsai, Amporn Rungrod and Kwanruthai Malairuang, supervision was done by Mongkon Utamatho, Sumarin Soonsanga, Boonhiang Promdonkoy, and conceptualization, funding acquisition, project administration, resources, supervision, writing-review and editing was accomplished by Wai Prathumpai. The funding source of the research work is already stated in the manuscript and there is no financial/personal interest that affected the objectives of the studies. All authors have read and agreed to the published version of the manuscript.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2023.e00812.

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