

Research Article



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Improvement of delta-endotoxin production from local *Bacillus thuringiensis* Se13 using Taguchi's orthogonal array methodology

Lokal *Bacillus thuringiensis* Se13'ün Delta-endotoxin Üretiminin Taguchi Ortogonal Dizi Metodu Kullanılarak Arttırılması

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Abstract

Objective: The insecticidal activity of *Bacillus thuringiensis* directly depends on the yield of delta-endotoxins. In this study, various nutritional and cultural parameters influencing delta-endotoxin synthesis by a local isolate of *B. thuringiensis* Se13 were investigated using Taguchi methods.

Methods: In the first experiment, four factors, incubation period, incubation temperature, initial pH and medium, each at four levels, were selected and an orthogonal array layout of L_{16} was carried out. In the second experiment, Taguchi's orthogonal array method of L_{27} was used to evaluate the effects of the different concentration of medium components. Taguchi's signal-noise ratio and variance analysis were applied to determine the effect of the factors. After each experiment, verification studies were carried out using determined optimum conditions.

Results: The optimum conditions for incubation period, incubation temperature, initial pH, and medium determined as 72 h, 30°C, pH 9, and M4 medium, respectively. In the second experiment, soybean flour (5%), glucose (5%), KH_2PO_4 (0.3%), K_2HPO_4 (0.1%), MgSO_4 (0.4%) were determined as the optimum conditions. The delta-endotoxin yield was elevated to 1559.25 $\mu\text{g mL}^{-1}$ when the factors were adjusted to optimum level.

Conclusion: Optimization using the Taguchi method appeared to be a good choice for the overproduction of delta-endotoxin.

Keywords: Delta-endotoxin; *Bacillus thuringiensis*; Taguchi method; Optimization.

Özet

Amaç: *Bacillus thuringiensis*'in insektisidal aktivitesi delta-endotoksin miktarı ile doğrudan ilgilidir. Bu çalışmada, lokal bir izolat olan *B. thuringiensis* Se13'ün delta-endotoksin sentezini etkileyen çeşitli besinsel ve kültürel parametreler Taguchi metodu kullanılarak araştırıldı.

Gereç ve Yöntem: İlk deneyde, inkübasyon süresi, inkübasyon sıcaklığı, besiyeri ve besiyerinin başlangıç pH'ını kapsayan dört faktörün dört farklı seviyesi seçildi ve uygulamada L_{16} ortogonal dizisi kullanıldı. İkinci deneyde ise besiyeri içeriğindeki maddelerin farklı konsantrasyonlarının toksin üretimine etkisi Taguchi L_{27} ortogonal dizisi kullanılarak test edildi. Faktörlerin etkisini belirlemek için sinyal-gürültü oranı ve varyans analizi uygulandı. Her deneyden sonra optimum koşullar kullanılarak doğrulama çalışmaları gerçekleştirildi.

Bulgular: İnkübasyon süresi, inkübasyon sıcaklığı, başlangıç pH'sı ve besiyeri için optimum koşullar, 72 saat, 30°C, pH 9 ve M4 besiyeri olarak tespit edildi. İkinci deney sonucunda, M4'ün optimum içeriği de 5% soya unu, 5% glukoz, 0.3% KH_2PO_4 , 0.1% K_2HPO_4 , 0.4% MgSO_4 olarak belirlendi. Belirlenen optimum koşulların kullanılmasıyla endotoksin miktarı 1559.25 $\mu\text{g mL}^{-1}$ 'ye yükseldi.

Sonuç: Sonuç olarak, Taguchi metodu ile optimizasyonun yüksek miktarda delta-endotoksin üretimi için iyi bir seçenek olduğu ortaya çıktı.

Anahtar Kelimeler: Delta-endotoksin; *Bacillus thuringiensis*; Taguchi Metodu; Optimizasyon.

Introduction

After the World War II, the development of chemical insecticides and their usage in management of insect pests have resulted in significant growth in agricultural production. However, uncontrolled overuse of chemical insecticides has led to the development of insect resistance, extinction

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of natural enemies and predominance of the secondary pest [1, 2]. Also, serious environmental and health issues began to be recognized by the presence of chemical residues in food, water, and air [3–6].

To neutralize these problems, microbial insecticide is one of the most promising alternatives over chemical insecticides, which offers less or no harm to non-target fauna and flora. *Bacillus thuringiensis* is insect pathogen that has been used to control insect pests for several decades [7, 8]. The insecticidal activity of this pathogen is based on delta endotoxins produced by the bacterial cells during the sporulation. For improving the efficiency of *B. thuringiensis*, the yield of the delta-endotoxin must be optimized. These toxin proteins have many characteristics of secondary metabolites and their quantity is affected by cultural conditions such as incubation period, incubation temperature, initial pH, concentrations of medium components, etc. [9–11]. So that, optimization of the culture conditions is still one of the most important issue.

Conventional experimental design methods for optimization are too complicated and difficult to be used. In addition, these methods need many experiments when the number of application parameters increases. However, statistical design of experimental methods provides an easier and equally efficient approach to optimize several operational variables. The frequently used methods are: factorial design, central composite design (CCD), Taguchi methods, response surface methodology (RSM), Plackett-Burmana and Box-Behnken (BBD) design, and artificial neural network (ANN) [12]. Taguchi's optimization technique reduces cost and improves quality. The advantages of Taguchi method over the other methods are that several factors can be simultaneously optimized, and more quantitative information can be extracted from fewer experimental trials [13]. Taguchi experimental design method, a useful tool for designing high quality system was developed by Taguchi. This method uses a design of orthogonal arrays to conduct a set of experiments and tells us how different parameters affect the yield in a small number of experiments instead of testing all the possible combinations [14]. The results of the Taguchi method are then transformed into a signal-to-noise (S/N) ratio. Taguchi uses the S/N ratio to measure the quality characteristics deviating from the desired values. Based on the analysis of the S/N ratio, the optimal levels of the process factors are determined. Furthermore, a statistical analysis of variance (ANOVA) is performed to see which process factors are statistically significant. Finally, a confirmation experiment is conducted to validate the optimal process factors obtained from the Taguchi method [15].

Although Taguchi method initially was developed for improving the quality of goods manufactured, more recently, it has been also applied to engineering [16–18], biotechnology [12, 19, 20], marketing [21] and advertising [22]. Several reports are available about the application of Taguchi's method in the field of biotechnology. The effect of growth factors including carbon, nitrogen, and mineral concentrations, physical, chemical and other fermentation factors have been investigated and reported in various studies which have discussed the optimum conditions of these factors required for optimization of primer and seconder metabolites. Shukla and Goyal [23] developed an efficient fermentation process for improving dextranase production from *Weissella confusa* Cab3 by Taguchi's orthogonal array methodology. Muhammad et al. [24] applied Taguchi's experimental design for optimizing the production of thermostable polypeptide antibiotic from *Geobacillus pallidus* SAT4. El-Bendary et al. [19] studied cultural parameters influencing mosquitocidal toxin using Taguchi experimental design. Chenthamarakshan et al. [25] used Taguchi method to optimize extracellular laccase enzyme production in solid state fermentation from the fungi *Marasmiellus palmivorus* LA1.

Considering this information, we focused to investigate the statistical optimization of cultural conditions for production of delta-endotoxins of *B. thuringiensis* strain Se13. Various parameters as the effects of incubation period, incubation temperature, initial pH, medium, and concentrations of medium components were evaluated for maximum delta-endotoxin production using Taguchi's orthogonal array method.

Materials and methods

Microorganism and culture maintenance

The strain Se13 of *B. thuringiensis* was obtained from Karadeniz Technical University, Department of Biology, and Laboratory of Microbiology. This strain isolated from *Spodoptera exigua* cadaver (Lepidoptera: Noctuidae) in our previous study and were found to contain cry1 and cry2 endotoxins which are effective on lepidopterans. Also, it had efficiency on the larvae of the *S. exigua* which is a polyphagous pest (unpublished data).

Stock culture of the isolate strain was stored in 30% glycerol at -80°C . Before each experiment, the bacterium was subcultured from frozen stock onto a tryptic soy agar medium (TSA) and a loopful of bacteria was used to

inoculate a 250 mL Erlenmeyer flask containing 50 mL of sterilized tryptic soya broth (TSB) medium. The flask was incubated in a rotary shaker at 250 rpm at 30°C for 12 h. A 1% (v/v) inoculum from this flask was then used to inoculate 500 mL Erlenmeyer flasks containing 100 mL of sterilized medium.

Design of experiments

The first experiments were designed using Taguchi's orthogonal array method based on four levels and four factors, and 16 runs were used to optimize the effect of incubation period, incubation temperature, initial pH, and medium for maximum delta-endotoxin production from *B. thuringiensis* strain Se13. Table 1 indicates the selected factors and their levels for optimization of delta-endotoxin production by this bacterial strain. Shake flask experiments were performed according to the experimental design listed in Table 2. The compositions of the media used in the study were given in Table 3. All chemicals used in the study were purchased from Sigma-Aldrich (USA).

In the second experiments, Taguchi's orthogonal array method of L_{27} used to evaluate the effects of components of the M4 which was determined as the optimum medium in the first experiment. The levels of the factors were studied, and the layout of the L_{27} Taguchi's orthogonal array was shown in Tables 4 and 5, respectively.

Taguchi's signal-noise ratio and variance analysis (ANOVA) were applied to determine the effect of the factors. All assays were performed in triplicate runs, and results were evaluated using Minitab 17 software (Minitab Inc., USA).

Validation of the model

In order to validate the models, fermentations were carried out using the optimum conditions obtained by Taguchi method. The results were compared to estimated values obtained by the software.

Table 1: Factors and their levels used in the first experiment.

Factors	Level 1	Level 2	Level 3	Level 4
Incubation period (h)	48	72	96	120
Incubation temperature (°C)	25	30	34	37
Initial pH	6	7	8	9
Medium	M1	M2	M3	M4

Table 2: L_{16} orthogonal array of Taguchi experimental design and corresponding delta-endotoxin production by *B. thuringiensis* strain Se13.

Experiments	Factors and levels				δ -Endotoxin ($\mu\text{g mL}^{-1}$)
	Incubation period	Incubation temperature	Initial pH	Medium	
1	1	1	1	1	491.242
2	1	2	2	2	346.596
3	1	3	3	3	535.989
4	1	4	4	4	641.258
5	2	1	2	3	710.043
6	2	2	1	4	844.860
7	2	3	4	1	674.987
8	2	4	3	2	504.868
9	3	1	3	4	772.569
10	3	2	4	3	779.386
11	3	3	1	2	340.050
12	3	4	2	1	329.689
13	4	1	4	2	584.684
14	4	2	3	1	762.890
15	4	3	2	4	708.128
16	4	4	1	3	569.580

Table 3: Composition of the media used in the first experiment.

Media			
M1	M2	M3	M4
Dextrose (%1.5)	Starch (%1.5)	Glucose (%4)	Soybean flour (%2.5)
Cottonseed flour (%1)	Yeast (%1)	Yeast (%1.5)	Glucose (%2.5)
Peptone (%0.2)	Corn steep liquor (%0.2)	Corn steep liquor (0.4)	K_2HPO_4 (%0.3)
Yeast (%0.2)	CaCO_3 (%0.8)	$(\text{NH}_4)_2\text{SO}_4$ (%0.4)	KH_2PO_4 (%0.3)
CaCO_3 (%0.1)	NaPO_2H_2 (%0.4)	MgSO_4 (%0.06)	MgSO_4 (%0.4)
MgSO_4 (%0.03)		FeSO_4 (%0.06)	
FeSO_4 (%0.002)		ZnSO_4 (%0.001)	
ZnSO_4 (%0.002)		MnSO_4 (%0.01)	
		CaSO_4 (%0.001)	

Determination of delta-endotoxins

Delta-endotoxin concentration was determined in the solubilized crystal preparation from each culture medium as illustrated by Zouari et al. [26]. At the end of the fermentation,

Table 4: Factors and their levels used in the second experiment.

Factors	Level 1	Level 2	Level 3
Soybean flour (%)	1	2.5	5
Glucose (%)	1	2.5	5
KH_2PO_4 (%)	0.1	0.3	0.5
K_2HPO_4 (%)	0.1	0.3	0.5
MgSO_4 (%)	0.2	0.4	0.8

1 mL of each sample was centrifuged at $13,000 \times g$ for 10 min at a temperature of 4°C . The pellets were washed thrice with 1 M cold NaCl and then thrice with cold sterile distilled water. After, the pellets were dissolved with 50 mM NaOH and incubated 2 h at 30°C , the suspensions were centrifuged at $13,000 \times g$ for 10 min at 4°C . The supernatant including insecticidal crystal protein was used to determine the delta-endotoxins concentration according to Bradford method [27], using bovine serum albumin as a standard.

Results

The Taguchi orthogonal array is a convenient and useful choice for optimization of biotechnological processes. So, the effects of culture conditions, incubation period, incubation temperature, initial pH and medium differences, on the delta-endotoxin production by *B. thuringiensis* strain Se13 were tested via Taguchi experimental design in 16 runs. The experimental results showed significant variation in the yield of endotoxin, and its production was found to be extensively dependent on the culture conditions. The yield of delta-endotoxin ranges from 346.60 to $844.86 \mu\text{g mL}^{-1}$ (Table 2). The signal-noise ratio was used to determine optimum levels of the tested factors. The S/N ratio should have a maximum value to obtain optimum delta-endotoxin production, according to the Taguchi method. So, the larger is better approach was used. Although the highest yield of endotoxin was obtained from run 6 with a combination of 72 h for incubation

Table 5: L_{27} orthogonal array of Taguchi experimental design and corresponding δ -endotoxin production by *B. thuringiensis* Se13.

Experiments	Factors and levels					δ -Endotoxin ($\mu\text{g/mL}$)
	Soybean flour	Glucose	KH_2PO_4	K_2HPO_4	MgSO_4	
1	1	1	1	1	1	697.54
2	1	1	1	1	2	773.16
3	1	1	1	1	3	749.81
4	1	2	2	2	1	762.11
5	1	2	2	2	2	945.15
6	1	2	2	2	3	925.36
7	1	3	3	3	1	756.75
8	1	3	3	3	2	817.18
9	1	3	3	3	3	902.51
10	2	1	2	3	1	934.44
11	2	1	2	3	2	998.45
12	2	1	2	3	3	978.56
13	2	2	3	1	1	1151.29
14	2	2	3	1	2	1311.29
15	2	2	3	1	3	1302.56
16	2	3	1	2	1	1263.56
17	2	3	1	2	2	1325.65
18	2	3	1	2	3	1261.60
19	3	1	3	2	1	942.98
20	3	1	3	2	2	1020.06
21	3	1	3	2	3	1061.94
22	3	2	1	3	1	1196.48
23	3	2	1	3	2	1256.48
24	3	2	1	3	3	1188.12
25	3	3	2	1	1	1467.26
26	3	3	2	1	2	1638.75
27	3	3	2	1	3	1471.56

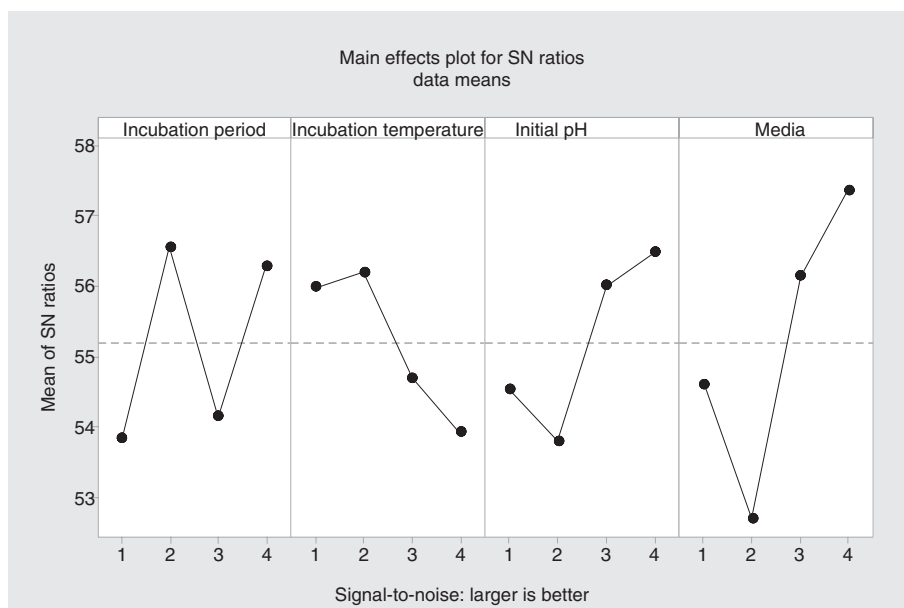


Figure 1: Effects of cultural conditions on the S/N ratios for the production of delta-endotoxin.

period, 30°C for incubation temperature, pH6 for initial pH and M4 for medium, optimum conditions determined as 72 h, 30°C, pH9 and M4 medium (Figure 1).

In addition, analysis of variance (ANOVA) was used to determine the how much variation of each factor has contributed and, the results were shown in Table 6. While medium with 46.79% has shown highest positive impact on the delta endotoxin production among the tested factors, pH with 13.81% showed the least impact. The ANOVA of the delta-endotoxin production has the model F-value of 18.22, indicated that the model is significant. The model obtained from ANOVA displayed that the multiple correlation coefficient of R^2 is 0.9865 i.e. the model can explain 98.65% variation in the response. Also, adequate precision value of the model was determined as 14.038. So, this model may be used to navigate the design space. The model showed standard deviation, mean, coefficient of variance (CV) and predicted residual

sum of square (PRESS) values of 43.04, 599.8, 7.18, and $1581E+005$, respectively. Point prediction for achieving the highest delta-endotoxin production based on the levels of the factors was shown in Table 7. Validation experiment was performed under optimum conditions, and the values found to be $971.2 \mu\text{g mL}^{-1}$. It displayed that the experimental value was compatible with the predicted value.

In a second step, the effect of M4 medium components were tested using Taguchi's orthogonal array method of L_{27} . The yield of delta-endotoxin ranges from 697.54 to $1638.75 \mu\text{g mL}^{-1}$ (Table 5). The results of the Taguchi orthogonal array experiments were evaluated using S/N ratio and ANOVA and, the results were shown in Figure 2 and Table 8, respectively. The optimum conditions of delta-endotoxin production were obtained from run 26 with a combination of soybean flour (5%), glucose (5%), K_2HPO_4 (0.3%), KH_2PO_4 (0.1%) and, MgSO_4 (0.4%). Contributions

Table 6: Analysis of variance for the first experiment.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-value	p-Value
Incubation period	3	85691	20.87%	85691	28564	15.42	0.025
Incubation temperature	3	70523	17.18%	70523	23508	12.69	0.033
Initial pH	3	56704	13.81%	56704	18901	10.20	0.044
Medium	3	192091	46.79%	192091	64030	34.57	0.008
Error	3	5557	1.35%	5557	1852		
Total	15	410566	100.00%				

$S = 43.03$ $R\text{-Sq} = 98.65\%$ $R\text{-Sq}(\text{adj}) = 93.23\%$.

Table 7: Point prediction for the first optimization process.

Prediction	Standard error of mean	95% Confidence interval low	95% Confidence interval high	Optimum conditions
979.50	38.7	856.04	1102.96	72 h, 30°C, pH 9, M4

of the medium ingredients on the delta-endotoxin production were shown in Table 8. While soybean flour with 57.95% has shown highest positive impact on the delta-endotoxin production among the tested factors, KH_2PO_4 with 2.44% showed the least impact. The ANOVA of the delta endotoxin production has the model F-value of 76.14 indicated that the model was significant. The model obtained from ANOVA displayed that the multiple correlation coefficient of R^2 was 0.9794 i.e. the model can explain 97.94% variation in the response. Also, adequate precision value of the model was determined as 30.032. So, this model may be used to navigate the design space. The model showed standard deviation, mean, coefficient of variance (CV) and predicted residual sum of square (PRESS) values of 46.29, 1077.77, 165 4.29 and 97610.61, respectively. Point prediction for achieving the highest delta-endotoxin production based on the levels of the factors was shown in Table 9. The result of validation experiment was found to be 1559.25, and it was corresponded well with the predicted value of 1568.74 by the model.

Discussion

Using microorganisms for pest management is the best alternative to conventional pesticides. *Bacillus thuringiensis* producing delta endotoxin proteins during sporulation is the mostly used bacterium for this purpose. These proteins are harmless to non-target organisms, are completely biodegradable and cause no toxic residual products to accumulate in the environment. The yield of these delta endotoxins varies depending on the nutritional and cultural conditions. In this study, Taguchi orthogonal array method was successfully applied to test the relative importance of culture conditions and medium components on delta-endotoxin production of *B. thuringiensis* strain Se13. In the process, the effects of incubation period, incubation temperature, initial pH and, concentration of medium ingredients were investigated.

Incubation period is significantly important factor for delta-endotoxin production. Delta-endotoxins were produced during sporulation phase of *B. thuringiensis*. Sporulation starts after 48 h of growth and, spores and crystals are released from the cells, complete sporulation is achieved at 54 h, and by 72 h the spores and crystals are found to have been released. Seventy-two hours was determined as the best time for maximum delta-endotoxin production in our study (Figure 1). A decrease in the amount of endotoxin was observed after 72 h due to the fact that the endotoxins are degraded by the proteases [28].

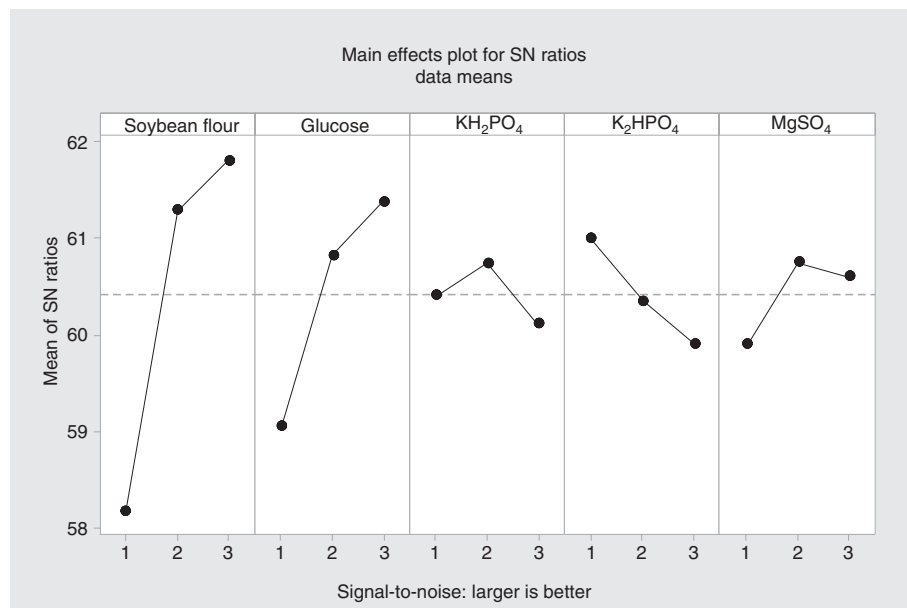
**Figure 2:** Effects of medium ingredients on the S/N ratios for the production of delta-endotoxin.

Table 8: Analysis of variance (ANOVA) for second experiment and contributions of the medium ingredients.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-value	p-Value
Model	10	1631000		1631000	163100	75.98	0.0001 ^a
Soybean flour	2	965147	57.95%	965147	482600	224.81	0.0001 ^a
Glucose	2	438604	26.34%	438604	219300	102.17	0.0001 ^a
KH ₂ PO ₄	2	40645	2.44%	40645	20322	9.47	0.0019 ^a
K ₂ HPO ₄	2	136904	8.22%	136904	68452	31.89	0.0001 ^a
MgSO ₄	2	49739	2.99%	49739	24869	11.59	0.0008 ^a
Error	16	34345	2.06%	34345	2146		
Total	26	16655383	100%				

S = 46.33 R-Sq = 97.94% R-Sq(adj) = 96.65%.

^aSignificant terms.

The normal temperature for toxin production of *B. thuringiensis* is 30°C, but it can show variability. Özkan et al. [29] determined that synthesis of Cry4Ba toxin from *B. thuringiensis israelensis* HD500 was the optimal when the organism was grown at 25°C whereas Cry11Aa synthesis was optimal at 30°C. On the other hand, Yousten et al. [30] and Lacey [31] found that spore and toxin productions were adversely affected at 35°C. At the present work, optimum temperature for the delta-endotoxin production was determined as 30°C (Figure 1).

The changes of pH can affect the delta-endotoxin production. Morris et al. [32] found that starting pHs 7.0 and 8.0 were suitable for toxin production from the *B. thuringiensis* HD133. Zou et al. [33] indicated that although the bacteria could grow in weak acid medium, it blocked the metabolic pathway which directly affected the yield of delta-endotoxin. In neutral (7.0) or slightly alkaline (9.0) medium, both physical growth and delta-endotoxin production reached the maximum. On the contrary of these, İçgen et al. [34] found that crystal

protein synthesis was more efficient at a narrow pH range of 5.5–6.5. In our study, initial pH was a significant factor and the optimum initial pH was determined as 9.0 using Taguchi's experimental design (Figure 1). On the other hand, initial pH for mosquitocidal toxins production by *Lysinibacillus sphaericus* using Taguchi's experimental design was not a significant factor [19].

Optimization of medium is one of the effective approaches to promote delta-endotoxin production. Firstly, four different media were tested for maximum endotoxin production using the Taguchi assay design, and the most effective medium was identified as M4 medium (Figure 2). It contains soybean flour (2.5%) as nitrogen source, glucose (2.5%) as carbon source, K₂HPO₄ (0.3%), KH₂PO₄ (0.3%), MgSO₄ (0.4%) as mineral elements. When the effect of different concentrations of the medium ingredients on delta-endotoxin production was examined using the Taguchi method, and optimum concentrations of the components were determined as 5% soybean flour, 5% glucose, 0.3% K₂HPO₄, 0.1% KH₂PO₄ and 0.4% MgSO₄ (Figure 2).

The delta-endotoxin proteins which compose approximately 30% of total protein of *B. thuringiensis* are synthesized from amino acids derived from the complex nutrients. İçgen et al. [35] were used soybean flour, peptone, corn steep liquor, and casamino acid as a nitrogen source. They found that all of them had no negative effect on the delta-endotoxin synthesis. However, peptone was the best choice for optimum toxin production. Ben Khedher et al. [36] and Ennouri et al. [37] displayed that soybean had positive affect on delta-endotoxin production. Our results agree with reports that nitrogen and carbon sources are the main components that affect the synthesis of delta-endotoxin [38].

Different concentrations of inorganic phosphate and trace metals are known to affects the yield of

Table 9: Point prediction for the second optimization process.

Prediction	Standard error of mean	95% Confidence interval low	95% Confidence interval high	Optimum conditions
1568.74	29.57	1506.05	1631.43	Soybean flour (5%) Glucose (5%) KH ₂ PO ₄ (0.3%) K ₂ HPO ₄ (0.1%) MgSO ₄ (0.4%)

delta-endotoxin. Tokcaer et al. [39] indicated that K_2HPO_4 levels were critical for effective synthesis of crystal toxin. Özkan et al. [29] also, reported that the highest yield of both Cry4Ba and Cry11Aa were obtained at 50–100 mM concentration of K_2HPO_4 . We also determined that K_2HPO_4 , KH_2PO_4 , and $MgSO_4$ levels were important factors for optimum delta-endotoxin production (Table 8). On the other hand, Khedher et al. [36] showed that K_2HPO_4 , KH_2PO_4 , and $MgSO_4$ had no significant effect on delta-endotoxin synthesis using Plackett-Burman design.

Conclusion

Cultural conditions and nutritional requirements show differences for each strain of *B. thuringiensis*. So, Taguchi's experimental design was used for optimization of delta-endotoxin production from a local isolate of *B. thuringiensis* strain Se13 in this study. In view of the results obtained in the study showed that incubation period, incubation temperature, initial pH, concentrations of the medium components significantly affected delta-endotoxin production. The delta-endotoxin yield was elevated to $1542.25 \mu\text{g mL}^{-1}$ when the factors were adjusted to best level for endotoxin production. This study sets an example for the application of the Taguchi method for development of biological processes.

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