Volume 61(2):135-140, 2017
Acta Biologica Szegediensis
http://www2.sci.u-szeged.hu/ABS

ARTICLE

Optimization of mosquitocidal toxins production by *Bacillus thuringiensis* under solid state fermentation using Taguchi orthogonal array

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ABSTRACT Optimization of the culture medium conditions for *Bacillus thuringiensis* var. *israelensis* mosquitocidal toxins production under solid state fermentation using Taguchi experimental design of surface response methodology was studied. The obtained results revealed that the optimum culture medium conditions for the maximum mosquitocidal activity against second instar *Culex pipiens* larvae were 6% substrate concentration, 40% initial moisture content, 2% inoculum size and initial pH 6.5 for 7 days incubation period. The obtained sporulation titer and larval mortality % were 2.2 × 10^{10} CFU/g final product and 90%, respectively. LC_{50} of this product was 3.2 ppm.
Acta Biol Szeged 61(2):135-140 (2017)

KEY WORDS

Bacillus thuringiensis
Culex pipiens
mosquitocidal toxins, solid state fermentation
surface response methodology

Introduction

Bacillus thuringiensis var. *israelensis* (Bti) has been used in mosquito vector control programs since two decades. Bti forms crystal protein endotoxin during sporulation and it is pathogenic upon ingestion by mosquito larvae (Poopathi and Archana 2012).

Mosquitocidal toxins production by Bti has been reported under both submerged and solid state fermentation (SSF). Advantages of SSF are: (1) low production cost, (2) saving water and energy, (3) low capital investments, (4) low waste effluent, (5) stability of the product, (6) concentrated products, and (7) some microorganisms can form endospores only by growing on a solid substrate (Holker and Lenz 2005).

The conventional growth optimization method namely, one factor at a time, is time-consuming, requires high experimental data sets and is unable to study the interactions between factors. Alternatively, statistical experimental design allows multiple control variables, is faster and cost-effective as compared to traditional univariate approach. It is a collection of mathematical and statistical analysis useful for determining the factors that influence the response or to define their optimum levels (Sunitha et al. 1999). Statistical experimental design has efficiently been applied for optimization of cultural conditions to produce microbial metabolites in many fermentation processes (Li et al. 2002). There are few reports about optimization of toxin production by Bti using statistical experimental design in submerged fermentation (Moreira et al. 2007; Ben Khedher et al. 2011, 2013; Hoa et al. 2014).

This study aimed to optimize the culturing conditions for commercial production of mosquitocidal toxins of Bti under solid state fermentation using Taguchi experimental design of surface response methodology. Substrate concentration, moisture content (%), initial pH, inoculum size and incubation period were evaluated for maximum mosquitocidal activity against second instar larvae of *Culex pipiens*.

Materials and Methods

Microorganism and inoculum preparation

Bti was obtained from Prof. Dr. Fergus G. Priest (Heriot-Watt University, UK). Inoculum was prepared by inoculating nutrient broth medium (5 g/l peptone and 3 g/l beef extract) with the bacterial culture and incubated for 24 h at 30 °C under shaking at 150 rpm.

SSF conditions and substrates used

Previous results of our group have shown that a mixture of

Submitted June 5, 2017; Accepted September 18, 2017
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Table 1. Taguchi orthogonal array design based on five factors/ five levels.

Run	By-product concentra-tion (%)	Moisture content (%)	pH	Inoculum size (%)	Incubation period (days)
1	15	40	8	4	5
2	12	20	8.5	4	3
3	9	20	8	1	7
4	3	40	8.5	10	11
5	15	25	7	1	11
6	9	10	7.5	10	5
7	3	30	8	8	9
8	12	25	6.5	8	5
9	12	40	7.5	1	9
10	12	10	8	2	11
11	15	10	8.5	8	7
12	6	40	6.5	2	7
13	15	20	6.5	10	9
14	9	30	6.5	4	11
15	9	40	7	8	3
16	3	25	7.5	4	7
17	9	25	8.5	2	9
18	6	20	7.5	8	11
19	3	20	7	2	5
20	6	10	7	4	9
21	6	25	8	10	3
22	15	30	7.5	2	3
23	3	10	6.5	1	3
24	6	30	8.5	1	5
25	12	30	7	10	7

Table 2. Summary of Taguchi orthogonal array design.

Factor code	Name	Units	Factor level	
			Low	High
A	By-product	%	3	15
B	Moisture content	%	10	40
C	Initial pH	-	6.5	8.5
D	Inoculum size	%	1	10
E	Incubation period	days	3	11

Table 3. Taguchi's actual and predicted results of sporulation and larval mortality (%).

Run	Sporulation (CFU x 10^8/g)			Mortality (%) at 10 ppm		
	Mean actual value	Mean pre-dicted value	Residual	Mean actual value	Mean pre-dicted value	Residual
1	151.33	167.68	-16.35	0.00	-9.86	9.86
2	110.33	181.11	-70.78	50.00	54.32	-4.32
3	213.33	216.80	-3.47	60.00	63.16	-3.16
4	220.67	212.27	8.40	0.00	13.87	-13.87
5	225.33	233.29	-7.95	13.33	7.57	5.77
6	198.00	189.72	8.28	60.00	56.55	3.45
7	224.33	243.81	-19.48	86.67	49.72	36.94
8	192.67	217.17	-24.51	76.67	67.69	8.97
9	206.33	195.52	10.81	0.00	7.87	-7.87
10	188.33	188.58	-0.25	0.00	-3.76	3.76
11	193.33	196.06	-2.73	90.00	82.15	7.85
12	215.33	218.18	-2.85	90.00	92.39	-2.39
13	236.00	231.84	4.16	0.00	10.64	-10.64
14	193.33	189.69	3.64	50.00	42.34	7.66
15	225.67	213.99	11.68	86.67	87.61	-0.95
16	223.33	232.65	-9.31	76.67	64.03	12.64
17	203.00	195.38	7.62	0.00	15.65	-15.65
18	238.67	241.71	-3.05	0.00	12.25	-12.25
19	230.67	223.88	6.79	36.67	71.81	-35.14
20	229.67	218.63	11.04	0.00	4.19	-4.19
21	234.33	232.38	1.96	86.67	100.93	-14.26
22	238.67	225.61	13.05	60.00	65.42	-5.42
23	221.33	181.11	40.22	76.67	54.32	22.35
24	213.67	213.89	-0.22	56.67	40.60	16.06
25	244.00	235.46	8.54	80.00	76.54	3.46

sugar beet pulp and sesame meal at 1:1 ratio was promising ingredients for Bti toxin production under SSF (El-Bendary et al. 2016a). Fifty grams fine sand (carrier material) and substrates (sugar beet pulp and sesame meal) were taken in 250 ml Erlenmeyer flasks, moistened with tap water (11 ml) and autoclaved. These flasks were inoculated with the tested culture and incubated at 30 °C under static conditions. Each fermentation test was in triplicate.

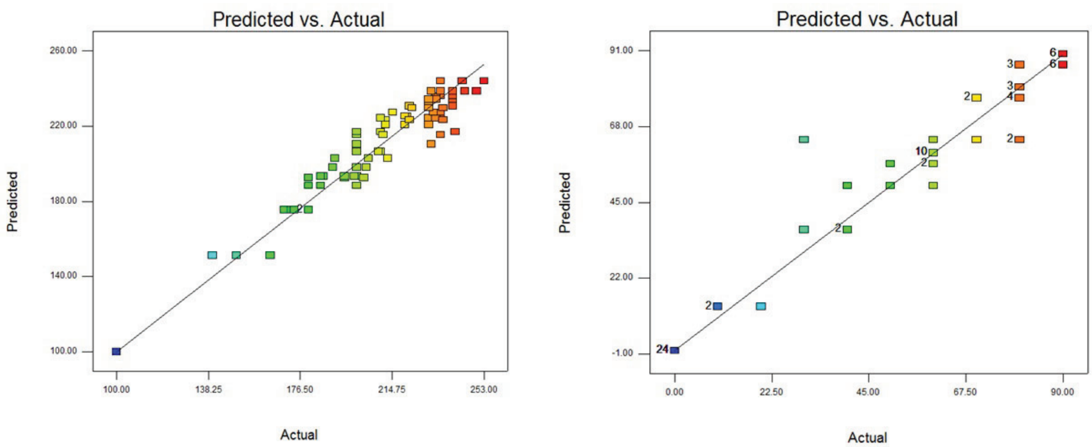
Experimental design

Taguchi orthogonal array based on five levels for five factors were used for maximum spore and toxins production by Bti under SSF (Tables 1, 2). These factors were substrate concentrations (A), moisture content (B), initial pH (C), inoculum size (D), and incubation period (E). Experimental design was performed using Design-Expert Software Version 7.0.0 (Stat-Ease, Minneapolis, MN, USA). Analysis of variance (ANOVA) was used to estimate the statistical parameters for optimization of culture conditions. All the experiments were done in triplicates.

Two response variables were measured: sporulation of the culture and toxicity against *C. pipiens* larvae. The quality of obtaining model was measured using the correlation coefficient of determination (R^2), the significance of each parameter through an F-test (calculated P-value) and the model lack of fit. Coefficients with a P-value<0.05 were considered significant.

Spore count

Endospores were counted by the plate count method. One gram of SSF product was suspended in 100 ml of sterile distilled water and shaken for one hour. Tenfold serial dilu-



(a) **(b)**

Figure 1. Actual versus predicted sporulation (a) and larval mortality % (b) results based on Taguchi's design.

tions of each sample were prepared and heated at 80 °C for 12 min. Dilutions were spread onto nutrient agar plates (three replicates per dilution) and incubated at 30 °C for 48 h.

Bioassay

Bioassay of mosquitocidal activity of fermented culture produced under SSF was adopted from Ampofo (1995) with some modifications. Toxicity was determined using second instar larvae of *C. pipiens*. One gram of fermented culture was mixed with tap water (100 ml) and shaken for one hour. Serial dilutions were prepared and placed into 100 ml beakers in triplicates along with 10 larvae of *C. pipiens* and kept at 26 ± 2 ºC with 10 h light/14 h dark cycle. The mortality percentage was calculated after 48 h.

Results

In previous study of our group, sugar beet pulp and sesame meal (1:1) at 6% concentration, pH 7-8, moisture content 20-30%, inoculums size 4-10% and 7 days incubation were the best conditions for toxin production by Bti under SSF using the conventional one-factor-at-a-time method (El-Bendary et al. 2016a).

According to Taguchi's design, good correlations among the actual and predicted results (Table 3) can be noticed for both sporulation and mosquitocidal activity due to low residuals. The relations among actual and predicted results were graphed in Figure 1 a, b. The analysis of variance (ANOVA) of the sporulation results (Table 4) obtained from Taguchi's design revealed that the model with F-value of 20.17 is significant and the model terms: E, AB, AC, BC, CD, ABD, ACE, BCD and BCE are significant as well. The F-value of 0.000554 implies that the model lack of fit is not significant relative to the pure error. Regression analysis of the model indicated that correlation coefficient (R^2) is 0.900942 and the adjusted R^2 of 0.856269 is in reasonable agreement with the predicted R^2 of 0.785149. The model coefficient of variation (C.V.) of (4.97) indicated a greater reliability of the experiments performed. The model adequate precision of 24.294, in addition to the previously mentioned parameters, indicates that the model can be used to navigate the design space (Fig. 2).

Final equation for sporulation process based on Taguchi's model

Sporulation (CFU x 10^8/g) = -9096.649386 + 289.2259698 * A + 202.3358255 * B + 1532.900751 * C - 1312.765921 * D + 1334.384945 * E + 8.071716958 * A * B - 69.37795286 * A * C + 45.43127539 * A * D - 30.55467821 * A * E - 37.4764861 * B * C + 22.17990073 * B * D - 29.85177773 * B * E + 172.4304541 * C * D - 211.3568499 * C * E + 9.754873376 * D * E + 0.32065505 * A * B * D -1.012333282 * A * B * E - 6.120312005 * A * C * D + 7.592903649 * A * C * E - 0.485031566 * A * D * E -3.313657279 * B * C * D + 5.331671129 * B * C * E - 0.198329236 * B * D * E

Where, A: by-product concentration (%), B: initial moisture content (%), C: initial pH, D: inoculum size (%) and E: incubation period (days).

The analysis of variance (ANOVA) of mortality percentage model (Table 5) revealed that its F-value of 79.66 implies the model is significant and the model terms: B, C, D, E, AB, AC, AD, AE, BC, BD, BE, CD, CE, DE, ABC, ABD, ABE,

Table 4. ANOVA of sporulation results based on Taguchi's design.

Source	Sum of squares	Df	Mean square	F-value	P-value Prob>F*
Model	50944.28	23	2214.969	20.16749	<0.0001
A: By-product	82.83164	1	82.83164	0.75419	0.3892
B: Moisture content	430.4247	1	430.4247	3.919056	0.0532
C: Initial pH	440.5467	1	440.5467	4.011218	0.0505
D: Inoculum size	370.9611	1	370.9611	3.377634	0.0719
E: Incubation period	1289.989	1	1289.989	11.74547	0.0012
AB	785.0195	1	785.0195	7.147674	0.0101
AC	680.381	1	680.381	6.194931	0.0161
AD	414.5053	1	414.5053	3.774108	0.0576
AE	46.11053	1	46.11053	0.419841	0.5199
BC	635.311	1	635.311	5.784564	0.0198
BD	324.4477	1	324.4477	2.954125	0.0917
BE	16.81807	1	16.81807	0.15313	0.6972
CD	824.1224	1	824.1224	7.503709	0.0085
CE	90.50257	1	90.50257	0.824034	0.3683
DE	8.305885	1	8.305885	0.075626	0.7844
ABD	501.5207	1	501.5207	4.566391	0.0374
ABE	425.6583	1	425.6583	3.875657	0.0544
ACD	276.8605	1	276.8605	2.52084	0.1185
ACE	591.507	1	591.507	5.385725	0.0243
ADE	79.13797	1	79.13797	0.720558	0.3999
BCD	495.5149	1	495.5149	4.511709	0.0385
BCE	641.5471	1	641.5471	5.841345	0.0193
BDE	160.5758	1	160.5758	1.462058	0.2322
Residual	5601.262	51	109.8287		
Lack of fit	0.062038	1	0.062038	0.000554	0.9813
Pure error	5601.2	50	112.024		
Cor total	56545.55	74			

*Value of "Prob>F" less than 0.05 indicates model term is significant.

Table 5. ANOVA of larval mortality (%) results based on Taguchi's design.

Source	Sum of squares	Df	Mean square	F-value	P-value Prob>F*
Model	93805.33	24	3908.556	79.65806	<0.0001
A: By-product	28.62968	1	28.62968	0.583485	0.4485
B: Moisture content	1009.783	1	1009.783	20.57981	<0.0001
C: Initial pH	2014.93	1	2014.93	41.06515	<0.0001
D: Inoculum size	1060.997	1	1060.997	21.62358	<0.0001
E: Incubation period	462.4049	1	462.4049	9.424013	0.0035
AB	2320.415	1	2320.415	47.29106	<0.0001
AC	2649.103	1	2649.103	53.98987	<0.0001
AD	3070.268	1	3070.268	62.57339	<0.0001
AE	2861.235	1	2861.235	58.31322	<0.0001
BC	2227.916	1	2227.916	45.40589	<0.0001
BD	1173.85	1	1173.85	23.92358	<0.0001
BE	3337.242	1	3337.242	68.01444	<0.0001
CD	2423.493	1	2423.493	49.39183	<0.0001
CE	702.1798	1	702.1798	14.31073	0.0004
DE	3290.32	1	3290.32	67.05815	<0.0001
ABC	2653.042	1	2653.042	54.07014	<0.0001
ABD	398.7633	1	398.7633	8.12697	0.0063
ABE	419.122	1	419.122	8.541889	0.0052
ACD	636.0368	1	636.0368	12.96271	0.0007
ACE	145.887	1	145.887	2.97324	0.0908
ADE	1081.454	1	1081.454	22.04049	<0.0001
BCD	36.99493	1	36.99493	0.753973	0.3894
BCE	35.85313	1	35.85313	0.730702	0.3967
BDE	628.9335	1	628.9335	12.81794	0.0008
Pure error	2453.333	50	49.06667		
Cor total	96258.67	74			

*Value of "Prob>F" less than 0.05 indicates model term is significant.

ACD, ADE, and BDE are significant as well. The results regression analysis indicates that the correlation coefficient (R^2) of the model is 0.974513 and the adjusted R^2 is 0.962279. The model adequate precision of 22.25409 indicates that the model has an adequate signal. Conclusively, the model can be used to navigate the design space (Fig. 3).

Final equation for mortality percentage based on Taguchi's model

Larval mortality (%) = -3370.208937 - 588.272023 * A + 31.1997336 * B + 771.2283616 * C - 1433.963373 * D + 50.25025007 * E + 31.73485427 * A * B + 34.56531874 * A * C + 89.90219504 * A * D + 51.25275965 * A * E - 16.49680701 * B * C + 4.840581588 * B * D -0.902106539 * B * E + 173.1436901 * C * D - 22.72783355 * C * E + 15.45709935 * D * E - 2.729875158 * A * B * C + 0.291181964 * A * B * D - 1.012706094 * A * B * E - 9.344204356 * A * C * D - 4.049384547 * A * C * E - 1.847729114 * A * D * E - 0.937834283 * B * C * D + 1.26341667 * B * C * E - 0.413341674 * B * D * E

Table 6. Optimum conditions and validation of the model.

By-product concentration (%)	Moisture content (%)	pH	Inoculum size (%)	Incubation period (days)	Sporulation (CFU x 10^8/g)		Mortality (%) at 10 ppm	
					Predicted	Actual	Predicted	Actual
6	40	6.5	2	7	218	215	92	90 ± 0

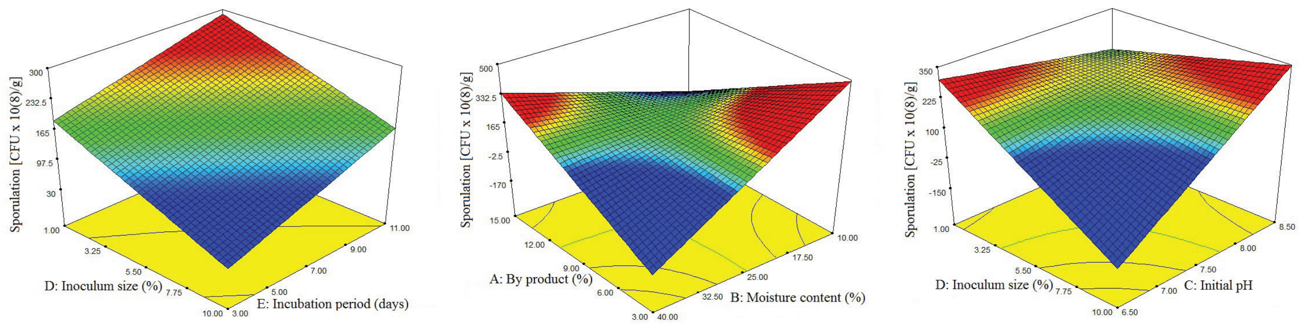


Figure 2. 3D response surface plots of the effect of various factors on sporulation.

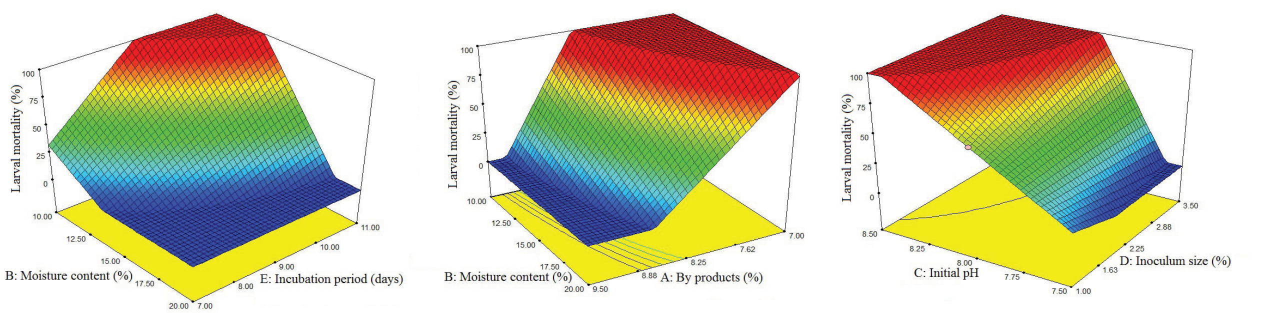


Figure 3. 3D response surface plots of the effect of various factors on larval mortality (%).

Where, A: by-product concentration (%), B: initial moisture content (%), C: initial pH, D: inoculum size (%) and E: incubation period (days).

Optimization and validation of the model

The optimum conditions for maximum mosquitocidal activity against second instar *C. pipiens* larvae were theoretically predicted from the model and then practically applied in triplicates and reported as (mean ± standard deviation) as shown in Table 6. Data shows that the model is 100% valid and the conditions can be used for production of *Bacillus thuringiensis* var. *israelensis*.

Discussion

In previous study using conventional one-at-a-time factorial design experiments, the optimum conditions for the maximum toxicity of Bti were 9% of sugar beet pulp-sesame meal (1:1) at pH 7-8, 20-30% moisture, 4-10% inoculum and 7 days incubation (El-Bendary et al. 2016a). In this study, Taguchi experimental design of surface response methodology was studied and the optimum conditions for the maximum mosquitocidal activity was 6% sugar beet pulp-sesame meal (1:1) at pH 6.5, 40% moisture, 2% inoculum size and 7 days incubation period. The difference between these two methods is statistical analysis shows the interactive effects among the variables tested, needs low experimental data sets and reduces time and cost.

Some reports about efficient application of the statistical experimental design for optimization of the cultural conditions for production of endotoxins of *Bacillus thuringiensis* under submerged fermentation were published by Moreira et al. (2007), Ben Khedher et al. (2011, 2013), and Hoa et al. (2014). It was reported that mosquitocidal toxins of *Lysinibacillus sphaericus* was successfully produced under SSF through applying response surface methodology design (El-Bendary et al. 2016b).

Conclusions

Optimization of the microbial cultivation medium and conditions are critical since they affect overall process economics. In this study, statistical experimental design (Taguchi orthogonal array) was applied in order, to optimize the mosquitocidal toxins production by Bti under SSF. Five factors, namely substrate concentration, moisture content, initial pH, inoculums size, and incubation periods were optimized for this commercially important bacterium. The predicted results of this design were confirmed by practical experiments. According to these results, the optimum conditions were 6% substrate concentration, 40% moisture content, initial pH 6.5, inoculum size 2% and 7 days incubation period to obtain high sporulation titer (2.2×10^{10} CFU/g) and the maximum mosquitocidal activity of about 90%.

Acknowledgement

The authors would like to thank the National Research Centre of Egypt for its professional and financial support provided for this study.

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