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Optimization of mosquitocidal toxins production by *Lysinibacillus sphaericus* under solid state fermentation using statistical experimental design

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ABSTRACT   Taguchi's experimental design of surface response methodology was applied to optimize the culture medium conditions for *Lysinibacillus sphaericus* (Ls) mosquitocidal toxins production under solid state fermentation (SSF). The predicted results of this design revealed that the optimum culture medium conditions for the maximum mosquitocidal activity against second instar *Culex pipiens* larvae were: 3.08 ± 0.05% substrate concentration, 33 ± 1.5% moisture content, 7.8 ± 0.1 initial pH, 1.35 ± 0.15% (5.7 × 10⁷ CFU) inoculum size and 5.9 ± 0.2 days incubation period. Sporulation titer of *Lysinibacillus sphaericus* (Ls 14N1) and mortality percentage of second instar *Culex pipiens* larvae of the fermented culture under these conditions were 2.8×10^{10} CFU/g fermented culture and 97.5 ± 1%, respectively. The results of practical validation of the design were incomparable with the mathematical results. Sporulation titer was 2.7×10^{10} CFU/g fermented culture; LC_{50} was 2.8×10^{-5} final fermented culture dilution and toxin protein concentration was 2.24 mg/g fermented culture.
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Introduction

Bacterial insecticides have been tested for the control of vector mosquitoes for more than two decades (Lacey 2007). Among them, *Lysinibacillus sphaericus* (Ls) showed high toxicity towards mosquito larvae. Mosquitocidal strains of Ls synthesize crystal proteins during sporulation. They are pathogenic upon ingestion by susceptible insect larvae and they are eco-friendly bioinsecticides (Luna-Finkler and Finkler 2012). Mosquitocidal toxins production of Ls has been reported under both submerged and solid state fermentation (SSF). In SSF the microbial growth and their products formation are on solid particle in near absence of water (Pandey 2003). The advantages of SSF over submerged fermentation was higher product yields, better product characteristics, lower capital and operating costs and simpler design reactor (Mussatto et al. 2012). The optimization of process conditions under SSF is generally done by varying one factor at a time approach. However, this strategy is laborious and time consuming, especially for a large number of variables and

often do not consider interactions among variables. Individual and interactive effects enable each reaction parameter to be optimized in coherence with others for achieving maximum product yield. Alternatively, statistical design of experiments can be used. It is a collection of mathematical and statistical analysis that is useful for determining the factors that influence the response and/or their optimum levels (Sunitha and Lee 1999). Statistical experimental design has been applied for optimization of cultural conditions for the production of microbial metabolites in many fermentation processes (Li et al. 2002). Considering the lack of reports, investigating statistical optimization of cultural conditions for commercial

Table 1. Factors used in Taguchi model with low and high levels.

Factor symbol	Factor name	Low actual	High actual
A	Substrate concentration (%)	3	15
B	Moisture content (%)	10	40
C	Initial pH	6.5	8.5
D	Inoculum size (%)	1(4 × 10⁷ CFU)	10(4 × 10⁸ CFU)
E	Incubation period (days)	3	11

production of mosquitocidal toxins of Ls under SSF, Taguchi's experimental design of surface response methodology was applied for this purpose. Various parameters as the effects of substrate concentrations, 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

Lysinibacillus sphaericus 14N1 (Ls 14N1) was previously isolated from Egypt (El-Bendary et al. 2003). Inoculum was prepared by inoculating nutrient broth medium (5 g/l peptone, 3 g/l beef extract) with the bacterial culture and incubated for 24 h at 30 °C under shaking at 150 rpm.

Substrate and SSF

Previous results of our group study have shown that a mixture of linen and wheat germ meals (each 4.5% of solid materials) was promising ingredients for Ls 14N1 toxin production under SSF (El-Bendary et al. 2016). Fine sand, which was 50 grams (carrier material, less than 0.35 mm in diameter) and substrates (linen and wheat germ meals, 4.5% each) were taken in 250 ml Erlenmeyer flasks, moistened with tap water (10-40% of solid materials) and autoclaved. These flasks were inoculated with the tested culture and incubated at 30 °C under static conditions. Each fermentation test was in triplicate and repeated twice.

Experimental design

A complete Taguchi's factorial design based on five levels, five factors and 25 runs (L25) were used to optimize the effect of substrate concentrations (A), moisture content (B), initial pH (C), inoculum size (D) and incubation period (E) for maximum spore and toxins production by Ls 14N1 (Table 1). Experimental design was performed using Design-Expert software (ver. 7.0.0; Stat-Ease Inc., Minneapolis, USA). Analysis of variance (ANOVA) was used to estimate the statistical parameters for optimization of culture conditions. All the experiments were done in triplicates and repeated twice.

A total of two response variables were measured; sporulation of the culture and toxicity against *Culex 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 lack of fit of the model. Coefficients with a p-value 0.05 were considered significant.

Spore count and crystal protein

For extraction of cells/spores/toxins, 1 gram of solid state fermented culture was added to 100 ml of sterile distilled water. SSF samples were shaken at 150 rpm for 1 h. Tenfold serial dilutions of each sample were prepared. Enumeration of spores was made by heating the dilutions (10^{-5}, 10^{-6} and 10^{-7}) at 80 °C for 12 min. They were then cooled and the spore titer was counted by plating onto nutrient agar plates (three replicates per dilution).

Soluble crude toxin protein was extracted from spore/crystal complex of the tested organisms according to Poncet et al. (1997) with some modification. After, extraction of spore/toxin complex from solid state fermented cultures as mentioned in the above section. These extracts were centrifuged and the pellets were washed with distilled water. The pellets were suspended in 0.05 N NaOH solution followed by incubation for 30 min at 37 °C with shaking. The mixture was centrifuged at 13000 rpm for 30 min. The supernatant containing the crude soluble crystal protein was used for determination of toxin protein concentration in triplicates according to Ohnistti and Barr (1978), using bovine serum albumin (BSA) as a standard.

Bioassay

Bioassay of mosquitocidal activity of fermented culture produced under SSF was adopted from Ampofo (1995) with some modifications. Toxicity was determined with laboratory reared second instar larvae of *Culex pipiens*. From the final product 1 gram was mixed with dechlorinated tap water (100 ml) and shaken for 1 h. Serial dilutions were prepared and the dilutions of each fermentation products were placed into 100 ml beakers in triplicate along with 10 larvae of *Culex pipiens*. Control was run simultaneously using tap water only. About 10 mg of ground fish meal was added to each cup. The beakers were covered with muslin and kept at 26 ± 2 °C with 10 h light/14 h dark cycle. The mortality percentage was calculated by counting the number of living larvae after 48 h and adopting Abbott's formula (1925). Each bioassay was repeated two times in different days.

Results and Discussion

Optimum conditions for sporulation and toxin formation

As shown in Table 2, the level of toxicity is not related to the sporulation content of the fermented culture. The highest mosquitocidal activities were shown with treatment no 2, 11, 16, 19, 21, 22, 23, 24 and 25.

Table 2. Actual and predicted values of sporulation and mortality percentage according to Taguchi's model.

Run	Sporulation CFU × 10^10/g fermented culture			Mortality (%) at 5 × 10^-5 dilution		
	Actual value	Predicted value	Residuals	Actual value	Predicted value	Residuals
1	1.7	1.7	0.06	33.33	33.33	0.00
2	1.9	1.9	0.30	76.67	76.67	0.00
3	2.1	2.1	-0.86	60.00	60.00	0.00
4	2.5	2.5	-0.02	0.00	0.00	0.00
5	2.6	2.6	0.06	0.00	0.00	0.00
6	1.9	1.9	-0.47	16.67	16.67	0.00
7	2.1	2.1	-0.16	56.67	56.67	0.00
8	1.9	1.9	0.57	40.00	40.00	0.00
9	2.1	2.1	0.24	16.67	16.67	0.00
10	2.4	2.4	0.24	16.67	16.67	0.00
11	2.4	2.4	0.12	83.33	83.33	0.00
12	2.2	2.2	-0.61	36.67	36.67	0.00
13	2.3	2.3	0.06	60.00	60.00	0.00
14	1.5	1.5	0.00	0.00	0.00	0.00
15	2.2	2.2	-0.28	0.00	0.00	0.00
16	2.2	2.2	1.33	76.67	76.67	0.00
17	2.0	2.0	-0.08	13.33	13.33	0.00
18	2.4	2.4	0.12	36.67	36.67	0.00
19	2.4	2.4	0.86	80.00	80.00	0.00
20	2.4	2.4	-0.56	40.00	40.00	0.00
21	2.3	2.3	0.02	80.00	80.00	0.00
22	2.2	2.2	0.13	86.67	86.67	0.00
23	2.2	2.3	-0.34	86.67	86.67	0.00
24	2.1	2.1	-0.40	76.67	76.67	0.00
25	2.5	2.5	-0.30	76.67	76.67	0.00

Table 3. Analysis of variance (ANOVA) for sporulation model.

Source	Sum of squares	Df	Mean square	F-value	P-value Prob>F
Model	46706.98	23	2030.74	11.892	<0.0001
A-substrate conc.	998.09	1	998.09	5.845	0.0192
B-moisture content	735.03	1	735.03	4.304	0.0431
C-initial pH	22.83	1	22.83	0.134	0.7162
D-inoculum size	991.26	1	991.26	5.805	0.0196
E-incubation period	1234.16	1	1234.16	7.227	0.0097
AB	132.03	1	132.03	0.773	0.3834
AC	15.27	1	15.27	0.089	0.7662
AD	62.29	1	62.29	0.365	0.5486
AE	444.26	1	444.26	2.602	0.1129
BC	65.83	1	65.83	0.385	0.5375
BD	178.83	1	178.83	1.047	0.3110
BE	1093.82	1	1093.82	6.405	0.0145
CD	321.90	1	321.90	1.885	0.1758
CE	3589.27	1	3589.27	21.019	<0.0001
DE	193.40	1	193.40	1.133	0.2922
ABC	1261.70	1	1261.70	7.389	0.0089
ABE	4001.48	1	4001.48	23.433	<0.0001
ACD	1146.22	1	1146.22	6.712	0.0125
ACE	2129.12	1	2129.12	12.468	0.0009
ADE	1207.82	1	1207.82	7.073	0.0104
BCD	1899.30	1	1899.30	11.122	0.0016
BCE	1995.42	1	1995.42	11.685	0.0012
BDE	1247.01	1	1247.01	7.302	0.0093
Residual	8709.02	51	170.77		
Lack of fit	15.69	1	15.69	0.090	0.7651
Pure error	8693.33	50	173.87		
Cor total	55416.00	74			

Values of p-value (Prob>F) less than 0.05 indicates model terms are significant.

The analysis of variance (ANOVA) (Table 3) shows the sporulation model (F-value) and (Prob>F) 11.89, less than 0.05 repectively, implies the model is significant. Although the initial pH is not a significant factor, its interaction with other factors is highly significant. The "Lack of Fit F-value" of 0.09, implies the Lack of Fit is not significant relative to the pure error.

The model R^2 is 0.843, which indicates a good model. The "Pred R^2" of 0.655 is in reasonable agreement with the "Adj R^2" of 0.772. "Adeq Precision", which measures the signal to noise ratio, is 14.872 indicates an adequate signal.

In conclusion, this model can be used to navigate the design space. Substrate concentration, moisture content, initial pH, inoculum size and incubation period contribute differently in sporulation process; 2.96, 2.18, 0.07, 2.94 and 3.66%, respectively. Although, the most contributions were attributed to factor-factor interactions; AE, BE, CE, ABC, ABE, ACD, ACE, ADE, BCD, BCE and BDE, which contribute with 1.32, 3.25, 10.66, 3.75, 11.88, 3.40, 6.32, 3.59, 5.64, 5.93 and 3.70, The other factor-factor interactions contribute only with less than 1%.

Sporulation final equation in terms of actual factors

Sporulation = 6812.97 - 468.03 * A - 108.44 * B - 1096.38 * C + 954.20 * D - 1144.83 * E + 8.64 * A * B + 80.74 * A * C - 38.03 * A * D + 44.95 * A * E - 23.31 * B * C - 27.65 * B * D + 14.45 * B * E - 118.88 * C * D + 186.79 * C * E - 10.49 * D * E - 1.85 * A * B * C + 0.86 * A * B * E + 4.48 * A * C * D - 9.05 * A * C * E + 0.80 * A * D * E + 3.55 * B * C * D - 3.31 * B * C * E + 0.20 * B * D* E.

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

Table 4 shows the mortality model F-value of 82.64, which implies that the model is significant. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case B, C, D, E, AE, BC, BD, BE, CE, DE, ABC, ABD, ABE, ACD, ACE, ADE, BCD, BCE and BDE are significant model terms.

Table 4. Analysis of variance (ANOVA) for mortality model.

Source	Sum of squares	Df	Mean square	F-value	P-value Prob>F
Model	71400.00	24	2975.00	82.64	<0.0001
A- substrate conc.	41.17	1	41.17	1.14	0.2900
B - moisture content	1018.26	1	1018.26	28.28	<0.0001
C - initial pH	150.54	1	150.54	4.18	0.0461
D - inoculum size	1024.93	1	1024.93	28.47	<0.0001
E - incubation period	4589.80	1	4589.80	127.49	<0.0001
AB	96.72	1	96.72	2.69	0.1075
AC	141.50	1	141.50	3.93	0.0529
AD	20.11	1	20.11	0.56	0.4583
AE	341.28	1	341.28	9.48	0.0034
BC	380.14	1	380.14	10.56	0.0021
BD	1106.16	1	1106.16	30.73	<0.0001
BE	1153.63	1	1153.63	32.05	<0.0001
CD	85.24	1	85.24	2.37	0.1302
CE	1654.92	1	1654.92	45.97	<0.0001
DE	394.73	1	394.73	10.96	0.0017
ABC	897.30	1	897.30	24.93	<0.0001
ABD	1920.99	1	1920.99	53.36	<0.0001
ABE	2423.60	1	2423.60	67.32	<0.0001
ACD	1207.76	1	1207.76	33.55	<0.0001
ACE	3329.41	1	3329.41	92.48	<0.0001
ADE	1211.09	1	1211.09	33.64	<0.0001
BCD	2515.36	1	2515.36	69.87	<0.0001
BCE	2996.78	1	2996.78	83.24	<0.0001
BDE	1846.37	1	1846.37	51.29	<0.0001
Pure error	1800.00	50	36.00		
Cor total	73200.00	74			

Values of p-value (Prob>F) less than 0.05 indicates model terms are significant.

The mortality model R^2 of 0.97541 indicates the data are in a good correspondance. The "Pred R-Squared" of 0.9447 is in reasonable agreement with the "Adj R-Squared" of 0.9636. "Adeq Precision" of this model is 25.019, which indicates an adequate signal. In conclusion, this model can be used to navigate the design space. Substrate concentration, moisture content, initial pH, inoculum size and incubation period contribute differently in produced toxicity 0.13, 3.15, 0.47, 3.17 and 14.19%, respectively. ACE, BCE, ABE, BCD, ABD, BDE and CE (as a factor-factor interaction) showed the highest contribution to mortality as follows: 10.29, 9.26, 7.49, 7.78, 5.94, 5.71 and 5.12%. The other factor-factor interactions contribute with less than 4%.

Toxicity final equation in terms of actual factors

Mortality = 16422.74 - 553.70 * A - 337.86 * B - 2741.28 * C + 2418.12 * D - 3023.19 * E - 2.32 * A * B + 127.36 * A * C - 93.97 * A * D + 76.11 * A * E + 66.70 * B * C - 53.68 * B * D + 57.70   * B * E - 315.65 * C * D + 492.63 * C * E - 30.79 * D * E - 1.59 * A * B * C - 0.63 * A * B * D + 2.37 * A * B * E + 12.69 * A * C * D - 18.79 * A * C * E + 1.92 * A * D * E + 7.58 * B * C * D - 11.27 * B * C * E + 0.70 * B * D * E.

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

Using the obtained models with tested factors and factors range levels for numerical process optimization to obtain maximum sporulation titer and mortality percentage, revealed that substrate concentration (3.08 ± 0.05%), moisture content (33 ± 1.5%), initial pH (7.8 ± 0.1), inoculum size (1.35 ± 0.15, 5.4×10^7 CFU) and incubation period (5.9 ± 0.2 days) are the optimum conditions for maximum sporulation by *Lysinibacillus sphaericus* under solid state fermentation and mortality of the second instar *Culex pipiens* larvae. Under these conditions, the experimental sporulation and mortality percentage will be ($2.8 \pm 0.5 \times 10^{10}$ CFU/g) and (97.5 ± 1%), respectively.

Figures 1-2 show 3D surface views of the effect of different factors on sporulation titer and mosquitocidal activity by varying only two factor levels, while keeping other factor levels constant. These figures show, how sporulation titer and mosquitocidal activity are sensitive to the different factor levels and the factor-factor interactions. Figures indicate that the optimum conditions cannot be concluded based on a single factor at a time, since the interactions between factors have a significant effect on both productivity and activity.

Validation of the optimum conditions

Ls14N1 was cultivated using the predicted optimum conditions of Taguchi's factorial design. These conditions showed the highest predicted mosquitocidal activities. The sporulation content, toxin concentration and LC_{50} were 2.7×10^{10} CFU/g fermented culture, 2.24 mg/g fermented culture and 2.8×10^{-5}.

In previous study of our group, wheat germ meal and linen meal (1:1) at 9% concentration, pH 6.5-7.5, 20-30% moisture (20-30 ml/100g solid materials), 4-10% (1.6×10^8 - 4×10^8 CFU) inoculum and 5 days incubation were the best conditions for toxin production by Ls14N1 under SSF using the conventional one-factor-at-a-time method (El-Bendary et al. 2016).

The conventional one-at-a-time factorial design experiments, is time consuming, costly process, requires high experimental data sets and is unable to analyze the interactive effects among the tested variables (Chaari et al. 2012).

Therefore, statistical methods have been developed to reduce the cost and time of experiments and to determine any interacting factors in the final process response. One of the
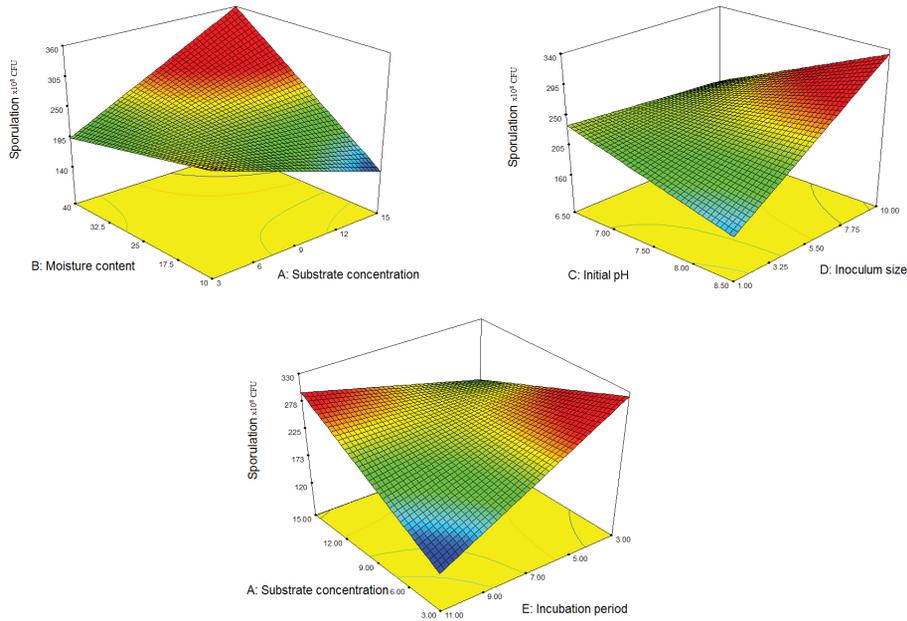
Figure 1. 3D surface views show the effect of different factor-factor interactions on sporulation titer.
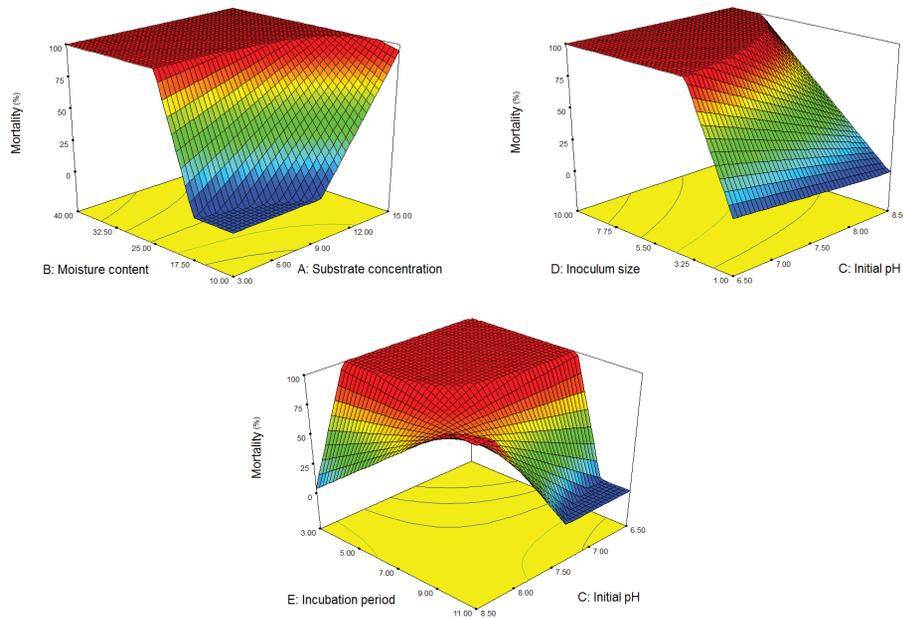
Figure 2. 3D surface views show the effect of different factor-factor interactions on mosquitocidal activity.

highly successful methods, as described by Box and Wilson (1951) is the response surface methodology (RSM). RSM is a collection of mathematical and statistical method, which studies the effects of various parameters at the same time. It is faster, saving time, cost effective and successfully used in

the optimization of media conditions and process parameters for microbial growth (Han et al. 2014).

In this study, there is no correlation between level of toxicity and sporulation titer. Although, Ls crystals proteins are produced during sporulation and the expression of crystal

protein genes is functionally related to sporulation-specific events, the independence of toxicity level and extent of sporulation in Ls has been reported by several authors (El-Bendary et al. 2008; Foda et al. 2015; Karim et al. 1993; Yousten et al. 1984; Yousten and Wallis 1987).

Some reports about efficiently applying, the statistical experimental design for optimization of the cultural conditions for production of endotoxins of *Bacillus thuringiensis* under submerged fermentation were published (Moreira et al. 2007; Ben Khedher et al. 2011; Ben Khedher et al. 2013; Hoa et al. 2014).

Conclusions

Optimization of the microbial cultivation medium and conditions are critical, since they affect overall process economics. In this study, statistical experimental design (Taguchi's experimental design) was applied in order to optimize the mosquitocidal toxins production by Ls 14N1 under SSF. Five factors, namely; substrate concentration, moisture content, initial pH, inoculums size, and incubation periods were optimized for these commercially important bacteria. The design unveiled the optimum values of the different factors; substrate concentration, moisture content, initial pH, inoculums size and incubation period were $3.08 \pm 0.05\%$, $33 \pm 1.5\%$, 7.8 ± 0.1, $1.35 \pm 0.15\%$ (5.4×10^7CFU) and 5.9 ± 0.2 days, respectively to obtain high sporulation titer (2.8×10^{10} CFU/g fermented culture) and the maximum mosquitocidal activity ($97.5 \pm 1\%$). Practical validation of the predicted optimum conditions showed sporulation titer of 2.7×10^{10} CFU/g fermented culture) LC_{50} was 2.7×10^{-5} final fermented culture dilution and 2.24 mg/g crude toxin protein.

Acknowledgement

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