Brazilian Journal of Microbiology 44, 3, 927-933 (2013)
ISSN 1678-4405

Research Paper

Application of statistical experimental design for optimisation of bioinsecticides production by sporeless *Bacillus thuringiensis* strain on cheap medium

Saoussen Ben Khedher[1], Samir Jaoua[1,2], Nabil Zouari[1,2]

[1]Team of Biopesticides, Centre of Biotechnology of Sfax, University of Sfax, Sfax, Tunisia.
[2]Biological and Environmental Sciences Department, College of Arts and Sciences, Qatar University, Doha, Qatar.

Submitted: October 04, 2011; Approved: November 13, 2012.

Abstract

In order to overproduce bioinsecticides production by a sporeless *Bacillus thuringiensis* strain, an optimal composition of a cheap medium was defined using a response surface methodology. In a first step, a Plackett-Burman design used to evaluate the effects of eight medium components on delta-endotoxin production showed that starch, soya bean and sodium chloride exhibited significant effects on bioinsecticides production. In a second step, these parameters were selected for further optimisation by central composite design. The obtained results revealed that the optimum culture medium for delta-endotoxin production consists of 30 g L^{-1} starch, 30 g L^{-1} soya bean and 9 g L^{-1} sodium chloride. When compared to the basal production medium, an improvement in delta-endotoxin production up to 50% was noted. Moreover, relative toxin yield of sporeless *Bacillus thuringiensis* S22 was improved markedly by using optimised cheap medium (148.5 mg delta-endotoxins per g starch) when compared to the yield obtained in the basal medium (94.46 mg delta-endotoxins per g starch). Therefore, the use of optimised culture cheap medium appeared to be a good alternative for a low cost production of sporeless *Bacillus thuringiensis* bioinsecticides at industrial scale which is of great importance in practical point of view.

Key words: sporeless *Bacillus thuringiensis*, delta-endotoxins, cheap medium, optimisation, response surface methodology.

Introduction

During the last decades, the efficiency of *Bacillus thuringiensis* (*B. thuringiensis*) in pest management has extended its use in agriculture, forestry and urban sectors (Navon, 2000). The larvicidal activity of these bioinsecticides is based on parasporal crystals produced by the bacterial cells during the sporulation phase (Aronson, 1993). In order to enhance the efficiency of *B. thuringiensis*, overproducing sporeless mutants have been isolated by random mutagenesis (Ben Khedher *et al.*, 2011b). These sporeless strains are more environmentally friendly and have many advantages such as a high delta-endotoxin production, a protection of encapsulated crystal from the harmful effect of UV-radiations and no viable spores would be present in their formulated products. For achieving an industrial production on a large scale of such bioinscticides, it is necessary to improve the production process, especially by designing a suitable culture medium. The use of low cost carbon and nitrogen sources is an attractive alternative because of the ability of *B. thuringiensis* to use complex substrates (Kanekar *et al.*, 2002). Several locally available cheap materials such as gruel, fish meal (Zouari *et al.*, 1998), corn steep liquor, coconut waste, rice bran and molasses were reported for *B. thuringiensis* production (Saalma *et al.*, 1983; Desai and Shethna, 1991; Lee and Seleena, 1991; Kumar *et al.*, 2000). In order to improve delta-endotoxin production by sporeless *B. thuringiensis*, commercial grades of starch and soya bean were used as carbon and nitrogen sources respectively, for medium optimisation. Since, in a previous reported work (Ben Khedher *et al.*, 2011b) overproducing sporeless (asporogenic and

Send correspondence to N. Zouari. Team of Biopesticides, Centre of Biotechnology of Sfax, University of Sfax, P.O. Box 1177, 3018 Sfax, Tunisia. E-mail: nabil.zouari@cbs.rnrt.tn.

oligosporogenic) mutants were shown to overproduce delta-endotoxins compared to sporulating wild strain, an asporogenic *B. thuringiensis* strain S22 was used in this work. In the present study, a Plackett-Burman design was carried out to screen the cultural parameters that may affect delta-endotoxin production, followed by a central composite design (CCD) involved in the optimisation of significant ones, needed towards the optimal production of delta-endotoxin by sporeless *B. thuringiensis*, in a low cost medium.

Materials and Methods

Strain

The sporeless *B. thuringiensis* subsp *kurstaki* strain S22 is used as a representative strain for the study (Ben Khedher *et al.*, 2011b).

Inocula preparation

The inocula were prepared as reported by Ghribi *et al.* (2004). The culture broth was used to inoculate the studied media to start with an initial cell density of 1.95 x 10^7 cfu mL^{-1}.

Culture medium

Commercial grade starch was kindly provided from a local agro-industry (G.I.A. Slama, Bouargoug, Tunisia). Commercial soya bean, containing 46% proteins, was obtained from a local mill of animal meals (ALCO Affes Group, Sfax, Tunisia). The modified complex medium (NaCl was added) previously described by Ghribi *et al.* (2007) was used with the following composition (g L^{-1}): starch, 30; soya bean, 25; KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4$, 0.3; $MnSO_4$, 0.01 and $FeSO_4$, 0.01. Medium pH was adjusted to 7.0 before sterilization at 121 °C for 20 min. In 250 mL flask, 0.4 g of $CaCO_3$ was added for maintaining of pH stability. The 250 mL flask containing 20 mL of culture medium was incubated for 96 h at 30 °C in a rotary shaker set at 200 rev min^{-1}.

Screening of important medium components

A Plackett-Burman design with 12 experiments was carried out for screening the effect of eight potential parameters. Each row of the matrix represented a trial and each column represented an independent factor whose levels were varied. Each variable was evaluated at two levels, a high (1) and a low (-1) level (Table 1). The levels attributed to each variable were determined based on results of preliminary study (data not shown). For the present study, the selected variables included starch, soya bean, NaCl, KH_2PO_4, K_2HPO_4, $MgSO_4$, $MnSO_4$ and $FeSO_4$.

Optimisation of significant variables by a CCD

A CCD for the three selected variables (starch, soya bean and NaCl) was carried out in order to optimise delta-endotoxin production and to determine the optimum levels of the investigated parameters. Each variable was analysed at five levels coded as $-\alpha$,-1, 0, +1 and $+\alpha$ (Table 2). The second order model associated to the three variables CCD is:

$$\eta = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

In the above equation, η is the theoretical response model, β_0, β_1, ..., β_{12} represent the model coefficients and X_j (j = 1 to 3) are the coded parameters selected for the CCD. The NemrodW software (Mathieu *et al.*, 2000) was used for experimental design and data analysis.

Table 1 - Coded and real values of variables in screening experiments.

Variable code	Variables	Level of variables (g L^{-1})	
		-1	+1
X_1	K_2HPO_4	0.5	1.5
X_2	KH_2PO_4	0.5	1.5
X_3	Starch	20	40
X_4	Soya bean	15	35
X_5	NaCl	3	11
X_6	$MgSO_4$	0.2	0.4
X_7	$FeSO_4$	0.008	0.012
X_8	$MnSO_4$	0.008	0.012

Delta-endotoxin determination

Delta-endotoxin concentration was determined in the solubilised crystal preparation from each culture medium as described by Zouari *et al.* (1998). The values presented are the average of two separate experiments for each cultural condition.

Results and Discussion

Screening of medium parameters affecting delta-endotoxin production

The objective of Plackett-Burman design (Table 3) was to screen, among eight independent variables, the factors with main effects on toxin production of a sporeless *B. thuringiensis* strain S22 (Myers and Montgomery, 1995; Khuri and Cornell, 1996). The importance of eight medium components, namely, starch, soya bean, K_2HPO_4, KH_2PO_4,

Table 2 - Experimental range of the three variables studied using CCD in terms of actual and coded factors.

Variables	Level of variables (g L^{-1})				
	$-\alpha$	-1	0	1	$+\alpha$
X_1: Starch	13.18	20	30	40	46.81
X_2: Soya bean	8.18	15	25	35	41.81
X_3: NaCl	0.27	3	7	11	13.72

Table 3 - The 12 experiments Plackett-Burman design matrix and the corresponding delta-endotoxins production values.

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Delta-endotoxins ($mg.L^{-1}$)
1	1	1	-1	1	1	1	-1	-1	2820
2	-1	1	1	-1	1	1	1	-1	2792
3	1	-1	1	1	-1	1	1	1	3268
4	-1	1	-1	1	1	-1	1	1	2688
5	-1	-1	1	-1	1	1	-1	1	2800
6	-1	-1	-1	1	-1	1	1	-1	2482
7	1	-1	-1	-1	1	-1	1	1	2122
8	1	1	-1	-1	-1	1	-1	1	1660
9	1	1	1	-1	-1	-1	1	-1	2620
10	-1	1	1	1	-1	-1	-1	1	3200
11	1	-1	1	1	1	-1	-1	-1	4056
12	-1	-1	-1	-1	-1	-1	-1	-1	1812

NaCl, $MgSO_4$, $FeSO_4$ and $MnSO_4$ for delta-endotoxin production is shown in Table 4. Results of Table 4 illustrated the statistical significance of the model coefficients, determined by Student's *t*-test. Starch, soya bean and NaCl appeared to be the major variables that positively affected delta-endotoxin production. Our results agree with reports that both carbon and nitrogen sources are the main components that affect the synthesis rate of delta-endotoxins (Farrera *et al.*, 1998). Moreover, our findings confirmed that adaptation of *B. thuringiensis* cells to NaCl was beneficial in cheap complex production media (Ghribi *et al.*, 2005). The other components were considered as least important factors. However, Tokcaer *et al.* (2006) reported that K_2HPO_4 and $MnSO_4$ levels were critical for effective synthesis of crystal toxin specifically favoured biosynthesis. Nevertheless, according to the coefficient estimate analysis (Table 4), KH_2PO_4, K_2HPO_4, $MgSO_4$, $MnSO_4$ and $FeSO_4$ have no significant effect on delta-endotoxin synthesis, although the yield of toxin production is known to be greatly influenced by trace metals and other minerals.

Table 4 - Coefficient estimates corresponding to the Plackett-Burman design.

Name	Coefficient	Error	t. exp.	Significance %
b_0	2693.3	23.149	116.35	< 0.01 ***
b_1	64.3	23.149	2.78	6.9
b_2	-63.3	23.149	-2.74	7.2
b_3	429.3	23.149	18.55	0.0342***
b_4	392.3	23.149	16.95	0.0447***
b_5	186.3	23.149	8.05	0.4**
b_6	-56.3	23.149	-2.43	9.3
b_7	-31.3	23.149	-1.35	26.9
b_8	-70.3	23.149	-3.04	5.6

$R^2 = 0.996$; $R_A^2 = 0.985$; **indicates significant at the level 99%; *** indicates significant at the level 99.9%.

Therefore, the three most significant components (starch, soya bean and NaCl) were selected for further optimisation by response surface analysis.

Response surface analysis

On the basis of our findings, starch, soya bean and NaCl, the most influenced factors in low cost medium (Table 2), were optimised using a CCD design. In this regard, a set of 24 experiments including, six center points and four check-points, (runs numbers 21 to 24) in order to check the validity of the fitted model, were carried out. The experimental and predicted responses for delta-endotoxin production are reported in Table 5. All experiments were carried out in duplicate. A multiple regression analysis of the data was carried out with the statistical analysis (Table 6). According to Table 6, the regression effect was statistically highly significant [(P > F) < 0.01] at 97.7% of confidence level. The model also showed insignificant lack of fit [(P > F) = 11.7]. The fit of the model was also expressed by the coefficient of regression R^2, which was found to be 0.977, indicating that 97.7% of the variability in the response (delta-endooxin production) could be explained by the model. Other parameters of ANOVA for response surface quadratic model were also studied. The R_{Pred}^2 of 0.928 is in reasonable agreement with the R_A^2 of 0.963. These results reinforced that the response equation provided a suitable model for the CCD experiment.

Therefore, the model was probably adequate for prediction within the range of variables employed. The model can be shown as follows:

$$\hat{y}\ (\text{mg L}^{-1}) = 4206.374 + 183.616X_1 + 724.487X_2 + 272137X_3 - 192132X_1^2 - 60103X_2^2 - 190884X_3^2 + 46974X_1X_2 + 25861X_1X_3 + 73511X_2X_3 \quad (2)$$

where $\hat{y}$ is the response that is delta-endotoxin production, X_1, X_2 and X_3 are the coded values of starch, soya bean and

Table 5 - Three variables CCD design with experimental and predicted values of delta-endotoxin production by *B. thuringiensis* S22.

Runs	X_1: Starch	X_2: Soya bean	X_3: NaCl	Experimental response (mg L^{-1})	Predicted response (mg L^{-1})
1	-1	-1	-1	2158	2188.435
2	1	-1	-1	2418	2409.996
3	-1	1	-1	3338	3396.438
4	1	1	-1	3700	3805.896
5	-1	-1	1	2610	2533.965
6	1	-1	1	2866	2858.969
7	-1	1	1	3903	4036.012
8	1	1	1	4580	4548.913
9	-1.68	0	0	3499	3354.132
10	1.68	0	0	3851	3971.741
11	0	-1.68	0	1180	1287.949
12	0	1.68	0	3900	3724.833
13	0	0	-1.68	3279	3208.789
14	0	0	1.68	4105	4124.148
15	0	0	0	4000	4206.374
16	0	0	0	4280	4206.374
17	0	0	0	4110	4206.374
18	0	0	0	4200	4206.374
19	0	0	0	4270	4206.374
20	0	0	0	4156	4206.374
21	-0.70	-0.40	-0.28	3400	3517.617
22	0.70	-0.40	-0.28	4132	3739.610
23	0	0.81	-0.28	4240	4285.424
24	0	0	0.86	4355	4298.890

Table 6 - Results of the analysis of variance.

Source	Sum of Squares	Degrees of freedom	Mean square	F-value	Significance
Model	15446100	9	1716230	66.6969	***
Residual	360247	14	25731.9		
Lack of fit	304554	9	33839.3	3.038	11.7%
Pure error	55693.3	5	11138.6		
Total	15806300	23			

R^2 = 0.977; R_A^2 = 0.963; R_{Pred}^2 = 0.928; *** indicates significant at the level 99.9%.

NaCl concentrations, respectively. The significance of the regression coefficients was tested by the Student's *t*-test. The regression coefficients and corresponding p-values for the model are presented in Table 7. The p-values were used as a tool to check the significance of each coefficient, which was necessary to understand the pattern of the mutual interactions between the three variables. Values of P less than 0.05 indicate model terms are significant. The results showed that the independent factors, starch (X_1), soya bean (X_2), NaCl (X_3) and their quadratic terms (X_1^2, X_2^2 and X_3^2) have significant effects on delta-endotoxin production. However, their interaction effects are found to be insignificant (Table 7).

The coded model was used to generate three-dimensional response surface curves presentations to understand the interaction of medium components and to determine the optimal concentration of each one conducting to the maximal delta-endotoxin production (Figure 1a-c).

Figure 1a illustrated the effect of starch (14-45 g L^{-1}) and soya bean (9-41 g L^{-1}) concentrations on delta-endotoxin production at 7 g L^{-1} NaCl. Starch concentration (X_1) has no significant effect on the considered response in the presence of 15-25 g L^{-1} soya bean. A positive effect of

Table 7 - Coefficient estimates corresponding to the central composite design.

Name	Coefficient	Error	t. exp.	Significance %
b_0	4206.374	56.07	75.02	< 0.01 ***
b_1	183.616	41.944	4.38	0.0631 ***
b_2	724.487	41.944	17.27	< 0.01 ***
b_3	272.137	41.943	6.49	< 0.01 ***
b_{11}	-192.132	40.824	-4.71	0.0337***
b_{22}	-601.030	40.824	-14.72	< 0.01 ***
b_{33}	-190.884	40.731	-4.69	0.035***
b_{12}	46.974	56.177	0.84	41.7
b_{13}	25.861	56.446	0.46	65.4
b_{23}	73.511	56.446	1.3	21.4

*** indicates significant at the level 99.9%.

starch is noticed in the presence of a concentration of soya bean higher than 25 g L^{-1}. Indeed, the surface curves showed that a high delta-endotoxin production (4470 mg L^{-1}) can be reached when using high concentrations of both starch and soya bean (over 30 g L^{-1}). However, delta-endotoxin production declined sharply thereafter, by increasing both starch and soya bean concentrations over 35 g L^{-1}. In fact, increasing starch concentration beyond 35 g L^{-1} led to a decline of delta-endotoxin production yield. This might be explained by the fact that toxin production is subject to catabolite repression which could be exhibited at high starch concentration (over 35 g L^{-1}). This metabolic limitation was previously described (Zouari *et al.*, 1998, 2002a) when using glucose or gruel as carbon sources (Zouari *et al.*, 2002b) in delta-endotoxin production. But, this effect seems to be less exhibited with starch. Consequently, the results of this study revealed that starch, known to have a repressive and/or inhibitory effect on toxin synthesis (Özkan *et al.*, 2003), supported a good delta-endotoxin production, since *B. thuringiensis* S22 strain has been shown to produce protease in substantially high yields (data not shown). So, this carbon source, used as low-cost and available substrate at an industrial scale, is readily used. This finding is promising since Içgen *et al.* (2002) reported that the use of maltose, starch and dextrin did not improve *B. thuringiensis* crystal titers. On the other hand, they also demonstrated that soya bean was among the best nitrogenous substrates which supported an optimal toxin production (Içgen *et al.*, 2002), which agree with our findings.

When using more than 35 g L^{-1} soya bean, a decrease in delta-endotoxin production was noticed, which could be attributed to a high biomass production as that could be expected (Zouari and Jaoua, 1999; Prabakaran and Balaraman, 2006) and therefore affected crystal production. On the other hand, this decrease of toxin production could be due to the repression or inhibition of secondary metabolism by nitrogen sources. In fact, the nitrogen catabolite regula-

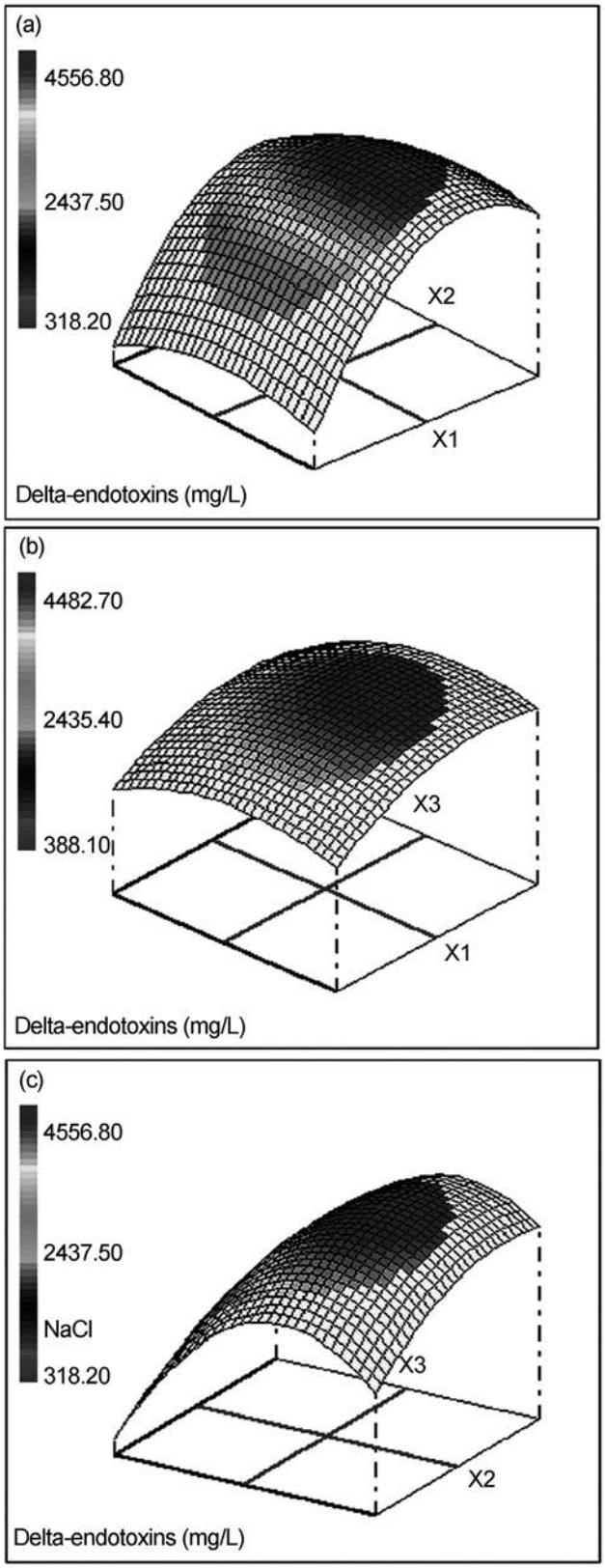

Figure 1 - Response surface plot of delta-endotoxin production showing the mutual interaction between (a) starch (X_1) and soya bean (X_2) concentrations at constant value of NaCl (7 g L^{-1}), (b) Starch (X_1) and NaCl (X_3) concentrations with soya bean fixed at 25 g L^{-1}, (c) Soya bean (X_2) and NaCl (X_3) concentrations at constant starch value (30 g L^{-1}).

tion has been frequently reported and well illustrated (Demain, 1995).

Therefore, delta-endotoxin production heavily depends on the availability of both carbon and nitrogen sources in the medium. Both exhibited regulatory effects on toxin synthesis. So, when using starch and soya bean at adequate concentrations, an overproduction of delta-endotoxins could be achieved. As shown in Figure 1b, delta-endotoxin production was enhanced especially by increasing starch concentration and using moderate NaCl concentration (9 g L^{-1}), at soya bean concentration of 25 g L^{-1}. This result suggested that NaCl supply led to an improvement of delta-endotoxin production (Ghribi *et al.*, 2005; Ben Khedher *et al.*, 2011a), particularly when used at 9 g L^{-1} and declined thereafter. In fact, NaCl addition plays the role of inducer of cell growth and consequently of delta-endotoxin production by sporeless *B. thuringiensis* mutants (Ben Khedher *et al.*, 2011a). It also could involve the synthesis of osmoprotectants, solutes, and/or amino acids which are known to protect cellular constituents in bacteria (Ruzal *et al.*, 1994). However, NaCl concentration over than 10 g L^{-1} can reduce the operational stability of the cells and affect its capacity to grow and produce crystal proteins (Amezega *et al.*, 1995). Figure 1c showed the effect of varying soya bean concentration from 8 to 42 g L^{-1} and NaCl concentration from 0.3 to 11 g L^{-1} on delta-endotoxin production at a starch concentration of 30 g L^{-1}. A positive effect of NaCl concentration is clear in the presence of high concentrations of soya bean (beyond 25 g L^{-1}).

In this attempt, a careful balance of substrates which are convenient and attractive because they are inexpensive must be provided to achieve an optimal delta-endotoxin production. Indeed, the surface curves showed that high delta-endotoxin production (4432 mg.L^{-1}) was achieved by using 30 g L^{-1} soya bean and 9 g.L^{-1} NaCl. Consequently, under the following conditions, 30 g.L^{-1} starch, 30 g L^{-1} soya bean and 9 g L^{-1} NaCl, a maximal delta-endotoxin production was predicted by the model, at of 4432 ± 56.2 mg L^{-1}.

Confirmation

In order to validate the predicted results, S22 strain was cultivated using optimised medium, in duplicate. The results clearly showed that the experimental response values (4455 mg L^{-1}) agree with those calculated (4432 ± 56.2 mg L^{-1}). Once again, this verification revealed a high degree of accuracy of the model under the investigated conditions. When compared to basal medium production (2834 mg L^{-1}), we noted an improvement of delta-endotoxin production reaching 57%, after optimisation of cheap medium composition. This toxin production improvement was also associated to yields improvement, calculated as the ratio of delta-endotoxin (mg L^{-1}) over assimilated starch (g L^{-1}), which reached 148.5 mg g^{-1} of starch in optimised medium, compared to 94.46 mg g^{-1} of starch in basal medium. Zouari *et al.* (2002b) reported that BNS3 toxin production was 3194 mg L^{-1} when used 42 g L^{-1} gruel and 20 g L^{-1} fish meal. Interestingly, delta-endotoxin production of S22 in our optimised medium exhibited 39.48% toxin production improvement, comparatively to BNS3 toxin production. So, considering the high production and stability of bioinsecticides based on sporeless *B. thuringiensis,* S22 strain is a promising strain for biotechnological applications, especially when used our optimised cheap medium.

Acknowledgments

This work was supported by grants from the Tunisian Ministry of Higher Education and Scientific Research.

References

Amezega MR, Davidson I, McLaggan D, Verheyul A, Abee T, Booth I (1995) The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. Microbiology 141:41-49.

Aronson AI (1993) The two faces of *Bacillus thuringiensis*: insecticidal proteins and post exponential survival. Mol Microbiol 7:489-96.

Ben Khedher S, Jaoua S, Zouari N (2011a) Improvement of Bioinsecticides Production by Sporeless *Bacillus thuringiensis* Strains in Response to Various Stresses in Low Cost Medium. Curr Microbiol 62:1467-1477.

Ben Khedher S, Zouari N, Messaddeq N, Schultz P, Jaoua S (2011b) Overproduction of delta-endotoxins by sporeless *Bacillus thuringiensis* mutants obtained by nitrous acid mutagenesis. Curr Microbiol 62:38-43.

Demain AL (1995) Microbial secondary metabolism: The new frontier. In: W. Kuhn, H.P. Fiedler (eds) Sekundär Metabolismus bei Microorganismen. Altempo Verlag, Tübingen, pp 11-35.

Desai SY, Shethna YI (1991) Production and formulation of *Bacillus thuringiensis* var. *israelensis* and *B. sphaericus* 1593. Indian J Med Res 93:318-323.

Farrera RR, Pérez-Guevara F, de la Torre M (1998) Carbon: nitrogen ratio interacts with initial concentration of total solids on insecticidal crystal protein and spore production in Bacillus thuringiensis HD-73. Appl Microbiol Biotechnol 49:758-765.

Ghribi D, Zouari N, Jaoua S (2004) Improvement of bioinsecticides production through mutagenesis of *Bacillus thuringiensis* by U.V. and nitrous acid affecting metabolic pathways and/or delta-endotoxin synthesis. J Appl Microbiol 97:338-346.

Ghribi D, Zouari N, Jaoua S (2005) Improvement of bioinsecticides production through adaptation of *Bacillus thuringiensis* cells to heat treatment and NaCl addition. J Appl Microbiol 98:823-831.

Ghribi D, Zouari N, Trigui W, Jaoua S (2007) Use of sea water as salts source in starch and soya bean based media, for the production of *Bacillus thuringiensis* bioinsecticides. Process Biochem 42:374-378.

Içgen Y, Içgen B, Özcengiz G (2002) Regulation of crystal protein biosynthesis by *Bacillus thuringiensis*: II. Effects of carbon and nitrogen sources. Res Microbiol 153:605-609.

Kanekar PP, Nilegaonkar SS, Sarnaik SS, Kelkar AS (2002) Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. Bioresour Technol 85:87-93.

Khuri AI, Cornell JA (1996) Response Surfaces, Designs and Analyses. Marcel Dekker, New York.

Kumar A, Sra K, Sangodkar UMX, Sharma VP (2000) Advances in the bio-control of mosquito vectors utilizing *Bacillus sphaericus* and *Bacillus thuringiensis* var. *israelensis*. Proc. Natl Acad Sci India LXX 1-20.

Lee HL, Seleena P (1991) Fermentation of a Malaysian *Bacillus thuringiensis* serotype H-14 isolate, a mosquito microbial control agent utilizing local wastes. Southeast Asian J Trop Med Public Health 22:108-112.

Mathieu D, Nony J, Phan-Tan-Luu R (2000) NEMROD-W software. LPRAI, Marseille.

Myers RH, Montgomery DC (1995) Response Surface Methodology: Process and Product Optimization Using Designed Experiments. Wiley, New York.

Navon A (2000) *Bacillus thuringiensis* insecticides in crop protection - reality and prospects. Crop Prot 19:669-676.

Özkan M, Dilek FB, Yetis Ü, Özcengiz G (2003) Nutritional and cultural parameters influencing antidipteran delta-endotoxin production. Res Microbiol 154:49-53.

Prabakaran G, Balaraman K (2006) Development of a cost-effective medium for the large-scale production of *Bacillus thuringiensis* var. israelensis Biol Control 36:288-292.

Ruzal SM, Alice AF, Sanchez-Rivas C (1994) Osmoresistance of spores from *Bacillus subtilis* and the effects of *ssp* mutations. Microbiology 140:2173-2177.

Saalma HS, Foda MS, Dulmage HT, Sharaby EL (1983) Novel fermentation medium for production of delta endotoxin from *Bacillus thuringiensis*. J Invertebr Pathol 41:8-19.

Tokcaer Z, Bayraktar E, Mehmetoglu U, Özcengiz G, Alaeddinoglu NG (2006) Response surface optimization of antidipteran delta-endotoxin production by *Bacillus thuringiensis* subsp. *israelensis* HD 500. Process Biochem 41:350-355.

Zouari N, Achour O, Jaoua S (2002a) Production of delta-endotoxin by *Bacillus thuringiensis* subsp. *kurstaki* and overcome of catabolite repression, by using highly concentrated gruel and fish meal media in 2 and 20 l fermenters. J Chem Technol Biotechnol 77:877-82.

Zouari N, Ben Sik Ali S, Jaoua S (2002b) Production of delta-endotoxin by several *Bacillus thuringiensis* strains exhibiting various entomocidal activities towards Lepidoptera and Diptera in gruel and fish-meal media. Enzyme Microb Technol 31:411-418.

Zouari, N.; Dhouib, A.; Ellouz, R.; Jaoua, S. (1998). Nutritional requirements of a strain of *Bacillus thuringiensis* subsp *kurstaki* and use of gruel hydrolysate, for the formulation of a new medium for delta-endotoxin production. Appl Biochem Biotechnol 69:41-52.

Zouari, N.; Jaoua, S. (1999). Production and characterization of metalloproteases synthesized concomitantly with δ-endotoxin by *Bacillus thuringiensis* subsp. *kurstaki* strain grown on gruel-based medium. Enzyme Microb Technol 25:364-371.