Nutritional Requirements of *Bacillus thuringiensis* During Different Phases of Growth, Sporulation and Germination Evaluated by Plackett-Burman Method

Sarrafzadeh, Mohammad Hossein*+

School of Chemical Engineering, College of Engineering, University of Tehran, P.O. Box 11155-4563 Tehran, I.R. IRAN

ABSTRACT: *The effects of different sources of carbon, nitrogen and some of trace elements on cultivation of Bacillus thuringiensis H14 were studied. Glucose, glycerol, sodium acetate, $(NH4)_2SO_4$, corn steep liquor, yeast extract, hydrolyzed casein, Ca^{2+}, Mg^{2+} and Mn^{2+} were the main parameters evaluated by Plackett-Burman statistical method. All elements have insignificant effect on germination while spore production was mainly affected by corn steep liquor. Growth was affected by corn steep liquor, $MnSO_4$ and sodium acetate. The following medium has been proposed based on the responses to growth and sporulation of B. thuringiensis H14: glucose, 5 g/L; glycerol, 1 g/L; sodium acetate,0 g/L; $(NH_4)_2SO_4$, 1 g/L; corn steep liquor, 2 mL/L; yeast extract, 1 g/L; hydrolyzed casein, 1 g/L; $CaCl_2$, 20 mg/L; $MgSO_4$, 50 mg/L; $MnSO_4$ 100 mg/L; $FeSO_4$, 0 mg/L. This medium resulted in a maximum spore count.*

KEY WORDS: *Fermentation, Culture medium, Bacillus thuringiensis, Statistical method.*

INTRODUCTION

The potential of *Bacillus thuringiensis* for producting of toxin and its use as a biological control agent has been well documented [1]. During sporulation, *B. thuringiensis* produces some delta-endotoxins, that are responsible for insecticidal activity of this microorganism [2]. Successful commercialization of delta-endotoxin production depends on the development of an optimal fermentation medium [3]. Accordingly, a number of studies have been carried out and have given improvement to culture conditions, in particular the carbon and nitrogen sources for increasing spore and/or crystal protein production [4-7]. Microbial productivity could be enhanced by manipulating the nutritional parameters, environmental conditions and strain improvement [8]. Development of an economical production medium requires selection of carbon, nitrogen, and trace element sources. At the cell level, each of sporulation, germination and vegetative growth phases could be considered as the multi-stage processes which are very different in their nature and their special requirements. In the present study, bacterial growth was initially carried out in a medium reported in literature [9]. Thereafter, different carbon, mineral and nitrogen sources were tested to analyze their effects on each of these growth phases. Glucose is the most utilized source of carbon, but Smith [10] has reported that glycerol resulted in higher toxicity. Acetate has also been suggested instead of carbohydrates in the culture of *Bacillus sphaericus* [11].

* *To whom correspondence should be addressed.*
+ *E-mail: sarrafzdh@ut.ac.ir*
1021-9986/12/4/131 6/$/2.60

Regarding the nitrogen sources in the culture of *B. thuringiensis*, the presence of an organic source has been mentioned as an indispensable factor that sometimes is the most expensive part of medium [12-14]. Yeast extract is the most cited of nitrogen source, however, some authors report that the utilization of Corn Steep Liquor (CSL) gives a better results of growth, sporulation and toxicity of entomopathogene bacteria [15, 16]. Hydrolyzed casein is another source of protein utilized in the culture of *B. thuringiensis*, but *Pearson et Ward* [17] mentioned it as an improper element in medium composition. The presence of some ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} in the culture of *B. thuringiensis* is essential [18, 19]. The presence of the ion Fe^{2+} has also been mentioned as a determinant factor for the growth of this bacterium [19]. These numbers of parameters reveal that a statistical optimization methodology should be applied. A statistical design enables easy selection of important parameters from a large number of factors and explains the interactions between important variables. The Plackett–Burman design as a highly fractionated factorial design for screening of the most significant process variables has been widely used in biotechnological researches [20-24]. This study aimed to indicate the effects of medium compositions on the growth, spore production and germination of *B. thuringiensis*. They were separately been investigated and the usefulness of the statistical experimental design for such study has been demonstrated.

EXPERIMENTAL SECTION

Microorganism and media

Bacillus thuringiensis H14 (Ecautec, Tahiti) was used [25]. A preculture flask containing 500 mL of medium was incubated at 30 °C on an orbital shaker for 9 h and used to inoculate each of culture flasks. The composition of the preculture medium was: glucose (10 g/L); ammonium sulfate (1.5 g/L); yeast extract (2 g/L); K_2HPO_4 (1.5 g/L); KH_2PO_4 (1.5 g/L); $CaCl_2$ (60 mg/L); $MgSO_4$ (500 mg/L); $MnSO_4$ (50 mg/L). The pH of medium was adjusted at 7 by NaOH 2 M and autoclaved at 120 °C for 10 min.

For culture medium optimization, the different sources of carbon, nitrogen and mineral salts taken from diverse literatures were used. Different factors such as glucose, glycerol, sodium acetate, $(NH_4)_2SO_4$, corn steep liquor, yeast extract, hydrolyzed casein, Ca^{2+}, Mg^{2+} and Mn^{2+} as mentioned in the experimental design were considered and their effects on the cell growth, spore production and germination were evaluated.

Experimental design and statistical analysis

The Plackett–Burman (PB) experimental design was used to evaluate the relative importance of various nutrients in each cell state. It assumes that there are no interactions between the different media constituents, X_i, in the range of variables under consideration. A linear approach is considered for screening:

$$Y= \beta_0 + \sum \beta_i X_i \quad (i=1, \ldots, k) \qquad (1)$$

Where Y is the estimated target function or response and β_i are the regression coefficients. The PB design is a fractional factorial design and the main effect of such a design may be simply calculated as the difference between the average of measurements made at the high level (+) of the factor and the average of measurements made at the low level (-). Contrast coefficients allow the determination of the effect of each constituent. A large contrast coefficient either positive or negative indicates that a factor has a large impact on target function; while a coefficient close to zero means that the factor has little or no effect. The P-value is the probability that the magnitude of a contrast coefficient is due to random process variability. A low P-value indicates a ''real'' or significant effect. The significance of each variable was determined by applying the Student's t-test. For each variable a high (+1) and a low (-1) concentration were applied (Table 1). The rows in Table 1 represent the 12 different trials and each column represents a different variable. The statistical analyses were performed by using of multiple regressions. Such that the experimental design is based on the simple matrix of Hadamard, very economic on the number of runs, times and instruments. It will permit a rapid screening of important parameters in each cell state. In 12 experiments, 11 parameters were changed simultaneously at two levels. The values of changing levels of each parameter were taken from literatures. One milliliter of spore suspension was added to each flask and incubated at 30 °C on an orbital shaker. The situation of cultures at 3 h, 8 h and after 24 h of fermentation was analyzed microscopically –as explained in the next section- to evaluate the influences of media compositions on the germination, growth and sporulation respectively.

Table 1: Plackett-Burman design for medium optimization and measured response with eleven variables representing nutritional components of the medium with high (+) and low (-) concentrations

Run	Glucose	Glycerol	Sodium Acetate	$(NH_4)_2SO_4$	Corn Steep Liquor	Yeast Extract	Hydrolyzed Casein	$CaCl_2$	$MgSO_4.7H_2O$	$MnSO_4.1H_2O$	$FeSO_4.7H_2O$	Germination	Growth	Sporulation
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Cell/ml X 10^{-7}	Cell/ml X 10^{-7}	%
1	+	+	-	+	+	+	-	-	-	+	-	1,7	5,0	0,0
2	-	+	+	-	+	+	+	-	-	-	+	2,4	4,0	2,5
3	+	-	+	+	-	+	+	+	-	-	-	1,0	3,5	14,0
4	-	+	-	+	+	-	+	+	+	-	-	4,3	3,3	0,0
5	-	-	+	-	+	+	-	+	+	+	-	4,4	4,3	0,0
6	-	-	-	+	-	+	+	-	+	+	+	2,5	35,0	75,0
7	+	-	-	-	+	-	+	+	-	+	+	5,1	4,7	0,0
8	+	+	-	-	-	+	-	+	+	-	+	3,3	31,0	12,0
9	+	+	+	-	-	-	+	-	+	+	-	1,9	12,0	65,0
10	-	+	+	+	-	-	-	+	-	+	+	1,4	3,7	95,0
11	+	-	+	+	+	-	-	-	+	-	+	3,0	4,8	0,0
12	-	-	-	-	-	-	-	-	-	-	-	2,5	6,4	99,0
Concentration	g/L	g/L	g/L	g/L	ml/L	g/L	g/L	mg/L	mg/L	mg/L	mg/L			
(-)	5	1	0	1	2	1	1	20	50	5	0			
(+)	50	10	5	10	20	10	10	400	700	100	20			

Microscopic analysis

Germination, growth, sporulation and formation of inclusion bodies were monitored using a phase contrast microscope (Olympus Bx60). Cell counts (vegetative and sporulated cells) were carried out using this microscope and a Thoma chamber. Unlysed sporulated cells were counted as spores. Sporulation was determined as the ratio of lysed and unlysed sporulated cells, which were distinguished due to their refractile nature, over the total cell count. The percentage of sporulated lysed cells vs. the total cell count has also been calculated as a mature spore count.

RESULTS AND DISCUSSION

Pareto graphs of the effects of all variables on germination, growth and spore production have been presented in the Figs. 1-3. These graphs allow visually identifying the important effects and comparing the relative magnitude of the various effects. They display the absolute value of the effects and draw a reference line on the graph. Any effect that extends past this reference line is potentially important.

Fig. 1 reveals that the effect of all terms on germination is insignificant. Growth was affected by corn steep liquor, $MnSO_4$, sodium acetate (Fig. 2). Spore production was just

affected by corn steep liquor (Fig. 3). Eleven compositions were studied to evaluate the approximate polynomial (first-order model) for all dependent variables, explaining their effects on the composition of the composite. Table 1 presents the experimental design and results of 12 Plackett-Burman (PB) experiments. The data of germination, growth and spore production was analyzed based on Eq. (1) and the models (coded units) without omitting insignificant effects are given as follows:

$$Y_1=2.78-0.13X_1-0.30X_2-0.44X_3-0.47X_4+0.69X_5-0.25X_6+0.08X_7+0.46X_8+0.44X_9+0.04X_{10}+0.16X_{11} \quad (2)$$

$$Y_2=9.80+0.35X_1+0.03X_2-4.43X_3-0.60X_4-5.46X_5+0.4.00X_6+0.61X_7-1.39X_8+5.25X_9+0.98X_{10}+4.05X_{11} \quad (3)$$

$$Y_3=30.21-15.04X_1-1.12X_2-0.79X_3+0.46X_4-29.79X_5-12.96X_6-4.13X_7-10.04X_8-88X_9+8.96X_{10}+0.54X_{11} \quad (4)$$

Where Y_1, Y_2 and Y_3 refer to germination, growth process and spore production, respectively. If we omit insignificant effect, the models are given as follows:

$$Y'_2=9.80-4.43X_3-5.46X_5+.98X_{10} \quad (5)$$

$$Y'_3=30.21-29.79X_5 \quad (6)$$

These statistical models (5, 6) were able to predict satisfactory growth and spore production at different levels of medium compositions, respectively. Model 2 demonstrates that sodium acetate and corn steep liquor have positive effects on growth but $MnSO_4.H_2O$ has negative effect on it. Model 3 reveals that only corn steep liquor has negative significant effect on spore production.

The results indicate that main effects are significant at an α-level of 0.05. By Minitab software Estimated optimum compositions for the production of spores by *B. thuringiensis* are as follows: glucose, 5 g/L; glycerol, 1 g/L; sodium acetate, 0 g/L; $(NH_4)_2SO_4$, 1 g/L; Corn Steep Liquor, 2 mL/L; Yeast extract, 1 g/L; Hydrolyzed casein, 1 g/l; $CaCl_2$, 20 mg/L; $MgSO_4$, 50 mg/L; $MnSO_4$, 100 mg/L; $FeSO_4$, 0 mg/L and resulted in a maximum spore count of 100% was obtained.

The effect of each parameter could be interpreted based on these results as following:

Glucose: Despite it is the most utilized source of carbon during different phases of *B. thuringiensis* [26] but in this study it did not show any significant effect on all responses.

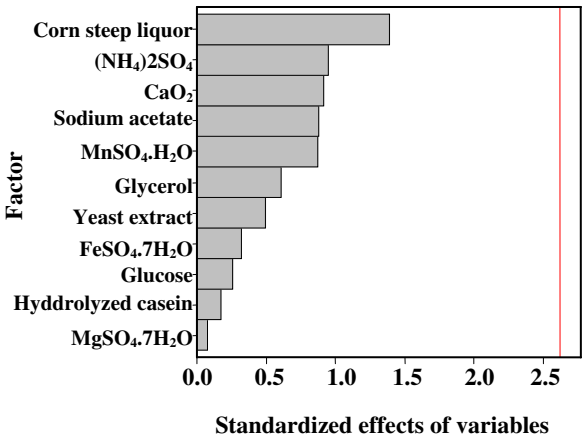

Fig. 1: Pareto graph showing the Standardized Effects of variables on germination.

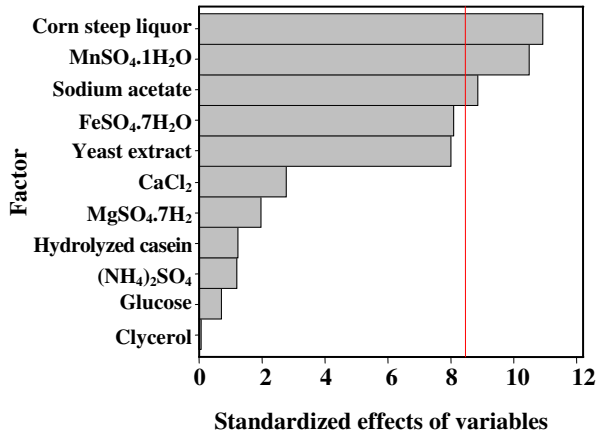

Fig. 2: Pareto graph showing the Standardized Effects of variables on growth.

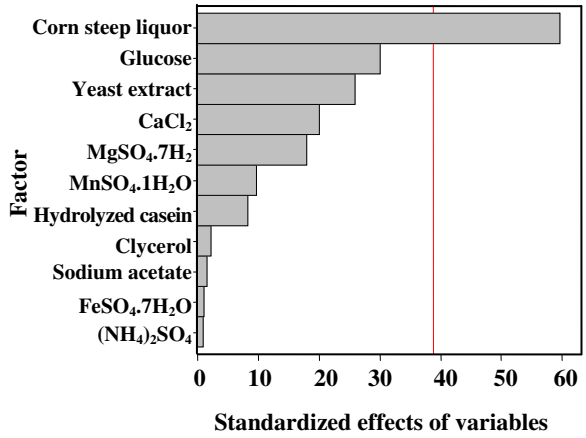

Fig. 3: Pareto graph showing the Standardized Effects of variables on spore production.

Glycerol: It affect none of responses significantly, however *Smith* [10] has reported that glycerol resulted in higher toxicity and spore production.

Sodium Acetate: It had no significant effect on germination and spore production but had negative effect on growth. Some authors showed that germination of *B. thuringiensis* spores are strongly inhibited by acetate [26].

Ammonium sulfate: No significant effect on responses was observed, although it has been reported that it increase spore production [27].

Corn Steep Liquor: It affected growth and spore production strongly that was in agreement with other research works [15, 16].

Yeast Extract: No significant effect on the responses was observed which is in contradicted with other works that mention it as a growth initiator [28].

Hydrolyzed Casein: It is another source of protein utilized in the culture of *B. thuringiensis*, but *Pearson & Ward* [17] mentioned it as an improper element in medium composition and in this study does not have any significant effect on any of the responses either.

$CaCl_2$: It did not have any significant effect on any of the responses.

$MgSO_4$: It did not have any significant effect on any of the responses.

$MnSO_4$: positive effect on growth.

$FeSO_4$: The presence of the ion Fe^{2+} has also been mentioned as a determinant factor for the growth of this bacterium [19]. In this study it does not have any significant effect on any of the responses.

CONCLUSIONS

This work was another demonstration of use fullness of statistical analysis in biotechnology. Medium optimization could be effectively done in a limited number of essays that make the process economically more feasible. However if there is an interaction between parameters the number of essays should be increased. Plackett–Burman design allows considering a large number of variables and identifying the most important of them by a rapid screening of variables that affect growth, spore production, and germination. The results showed that the effects of corn steep liquor on all of three responses were obviously more important than other factors. For the future works, insignificant variables can be omitted to repeat the experiments with a lower number of parameters. Of course with limitation of concentration level of each parameters, it is necessary that high (+) and low (-) level of each parameters be considered again.

Acknowledgement

Author would like to acknowledge the financial support of University of Tehran for this research under grant number 8104956/1/01.

Received : Oct. 5, 2010 ; Accepted : May 15, 2012

REFERENCES

[1] Andrews G.E., Faust R.M., Wakbiko H., Raymond K.C., The Biotechnology of *Bacillus thuringiensis*, CRC Crit. Rev. *Biotechnol*, **6**, p. 163 (1987).

[2] Najafloo A., Sarrafzadeh M.H., Gerami, A., Statistical Analysis and Modeling of Sporulation Phase During Fermentation of *B. thuringiensis* to Study the Effect of Oxygen and Culture Time on its Bioinsecticide Activity., *J. Petrol. Chem. Eng.*, **42**(8), p. 1025 (2009).

[3] de Maagd R., Bravo A., Crickmore N., How *Bacillus thuringiensis* has Evolved Specific Toxins to Colonize the Insect World, *Trends in Genetics*, **17**, p. 193 (2001).

[4] Avignon Rossa C.A., Yantorno M.O., Arcas J.A., Ertola R.J., Organic and Inorganic Nitrogen Source Ratio Effects on *Bacillus thuringiensis* Var. *israelensis* Delta-Endotoxin Production, *World J. Microbiol. Rev*, **6**, p. 27 (1990).

[5] Faloci M.M., Yantorno O.M., Marino H.A., Arcas J.A., Ertola R.J., Effect of the Media Composition on the Growth Parameters and Biological Properties of *B. thuringiensis* Delta-Endotoxin, *World. J. Microbiol. Biotech*, **6**, p. 32 (1990).

[6] Goldberg I., Sneh B., Battat E., Klein D., Optimisation of a Medium for a High Yield Production of Spore-Crystal Preparation of *B. thuringiensis* Effective Against the Egyptian Cotton Leaf Warm, *Biotech. Lett*, **2**, p. 419 (1980).

[7] Salama H.S., Foda M.S., Dulmage H.T., El-Sharaby A., Novel Fermentation Media for Production of d-Endotoxins from *Bacillus thuringiensis*, *J. Invertebr. Pathol*, **41**, p. 8 (1983).

[8] Naveena B.J., Altaf M., Bhadriah K., Reddy G., Selection of Medium Components by Plackett-Burman Design for Production of Lactic Acid by *L. amylophilus* GV6 in SSF Using Wheat Bran, *Biores. Tech.*, **96**, p. 485 (2005).

[9] Chauhan K., Trivedi U.B., Patel K.C., Statistical Screening of Medium Components by Plackett-Burman Design for Lactic Acid Production by *Lactobacillus* sp. KCP01 Using Date Juice, *Bioresource Technology*, **98**, p. 98 (2007).

[10] Robert A. Smith., Effect of Strain and Medium Variation on Mosquito Toxin Production by *Bacillus thuringiensis* var. *israelensis*, *Can. J. Microbiol*, **28**(9), p. 1089 (1982).

[11] Sasaki K, Jiaviriyaboonya S, Rogers, P.L., Enhancement of Sporulation and Crystal Production in Culture of *Bacillus Sphaericus* 2362, *Biotechnol Lett*, **20**, p. 165 (1998).

[12] Yerra K.R., Kuen-Juh T., Wen-Shi W.., Yew-Min T., Medium Optimization of Carbon and Nitrogen Sources for the Production of Spores from *B. amyloliquefaciens* B128 Using RSM, *Process Biochemistry*, **42**(4), p. 535 (2007).

[13] Vimala Devi P.S., Ravinder T., Jaidev C., Cost-Effective Production of *Bacillus thuringiensis* by Solid-State Fermentation, *Journal of Invertebrate Pathology*, **88**(2), p. 163 (2005).

[14] Abdel-Hameed A., Carlberg G., El-Tayeb O.M., Studies on *B. thuringiensis* H-14 Strains Isolated in Egypt-IV. Characterization of Fermentation Conditions for δ-Endotoxin, *World J. Microb. Biotech.*, **7**(2), p. 231 (1991).

[15] Liu Y-B., Tabashnik, B.E., Johnson M.W., Larval Age Affects Resistance to *Bacillus thuringiensis* in Diamondback Moth *(Lepidoptera: Plutellidae)*, *J. Econ. Entomol.*, **88**, p. 788 (1995).

[16] Prabakaran G., Balaraman K., Hoti S.L., Manonmani A.M., A Cost-Effective Medium for the Large-Scale Production of *B. sphaericus* H5a5b for Mosquito Control, *Biological Control*, **41**(3), p. 379 (2007).

[17] Pearson D., Ward OP., Effect of Culture Conditions on Growth and Sporulation of *B. thuringiensis* Subsp. *israelensis* and Development of Media for Production of the Protein Crystal Endotoxin, *Biotech Lett*, **10**, p. 451 (1988).

[18] Foda M.S., Salama H.S., Selim M., Factors Affecting Growth Physiology of *B. thuringiensis*, *Applied Microbiology and Biotechnology*, **22**, p. 50 (1985).

[19] Magda A. El-Bendary, Dr., *Bacillus thuringiensis* and *Bacillus sphaericus* Pesticides Production, *Journal of Basic Microbiology*, **46**(2), p. 158 (2006).

[20] Tasharrofi N., Adrangi S., Fazeli M., Rastegar H., Khoshayand M.R., Faramarzi M.A., Optimization of Chitinase Production by Bacillus Pumilus Using Plackett-Burman Design and Response Surface Methodology, *Iranian Journal of Pharmaceutical Research*, **10** (4), p. 759 (2011)

[21] Hoseyni S.M., Khosravi-Darani K., Mohammadifar M. A., Nikoopour, H., Production of Mycoprotein by Fusarium Venenatum Growth on Date Sugar, *Asian Journal of Chemistry*, **21**(5), p. 4017 (2009).

[22] Khosravi-Darani K., Zoghi A., Comparison of Pretreatment Strategies of Sugarcane Baggase: Experimental Design for Citric Acid Production, *Bioresource Technology*, **99**, p. 6986 (2008).

[23] Mokhtari-Hosseini Z.B., Vasheghani-Farahani E., Heidarzadeh-Vazifekhoran A., Shojaosadati S.A., Karimzadeh R., Khosravi-Daran K., Statistical Media Optimization for Growth and PHB Production from Methanol by a Methylotrophic Bacterium, *Bioresour. Technol.*, **100**, p. 2436 (2009).

[24] Ghaemi-Oskouie S.F., Tabandeh F., Yakhchali B., Eftekhar F., Response Surface Optimization of Medium Composition for Alkaline Protease Production by *Bacillus clausii, Biochemical Engineering Journal*, **39**, p. 37 (2008).

[25] Sarrafzadeh M.H., Belloy L., Esteban G., Navarro J.M., Ghommidh C., Dielectric Monitoring of the Growth and Sporulation of *Bacillus thuringiensis*. *Biotechnology Letters*, **27**, p. 511 (2005).

[26] Arcas J., Yantorno O., Ertola R., Effect of High Concentration of Nutrients on *Bacillus thuringiensis* Cultures, *Biotechnology Letters*, **9** (2), p. 105 (1987).

[27] Wan M., Wan Y., Mohd M., Chan-Choy M., Effect of Ammonium Sulphat on the Sporulation of *B. thuringiensis* (Local Isolate) During Batch Fermentation., *J. Tech.*, **39**, p. 53 (2003).

[28] Zeinat K.M., Nashwa A.H., Fetyan A., Mohamed A.I., Sherif E., Biodegradation and Detoxification of Malathion by *B. thuringiensis* MOS-5, *Australian Journal of Basic and Applied Sciences*, **2**(3), p. 724, ISSN 1991-8178 (2008).