Media Optimization of *Bacillus thuringiensis* PBT-372 Using Response Surface Methodology

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Original scientific paper
Received: May 3, 2003
Accepted: January 17, 2004

Statistical design of experiments, to determine the optimal levels of the sugar concentration from molasses, initial pH of the medium and the inoculum for the production of *Bacillus thuringiensis* in shake flask fermentation, was used. Initial screening of locally available raw materials viz. fish meal, molasses, sago, paddy straw and whey for maximizing biomass and pesticidal crystal protein (PCP) production from *B. thuringiensis* was performed. Optimized values of 4.19 % of inoculum, initial pH of 5.62 and 11.9 g L^{-1} of molasses resulted in higher biomass and PCP production. Using response surface methodology, good interactions between these factors were found to be present in optimizing the biomass yield. Among the interactions between these factors, the one between pH and sugar concentration was the most significant.

Key words:
Bacillus thuringiensis, pesticidal crystal protein, response surface methodology, microbial insecticide

Introduction

Bacillus thuringiensis is a rod shaped Gram-positive bacterium, which produces parasporal crystal inclusions during sporulation.[1] The parasporal crystals comprise approximately two polypeptide chains each with an estimated molecular mass of 130 kDa.[2] Upon ingestion by the insect larvae, the high molecular weight 130 kDa toxic protein is cleaved to low molecular mass 68 kDa protein by the combined effects of proteases and alkaline pH in the insect gut.[3] Consequently, the brush border membrane vesicles (BBMVs) develop lesions or pores leading to ion leakage and ATP hydrolysis, ultimately leading to death of the larvae.[4]

For over 40 years, mixtures of *B. thuringiensis* spores and δ-endotoxins produced by industrial fermentation is being marketed for traditional spray application. In this form, *B. thuringiensis* accounts for 90 % of world sales of non-chemical insecticides. These original products contain δ-endotoxins active against lepidopteran and dipteran pests.

Although *B. thuringiensis* based microbial insecticides have been available for use in agricultural pest management, its high cost makes large-scale application impracticable, especially in developing countries. Therefore, production of this insecticide should be based on locally available materials to derive the desired economic advantage.[5] Formulations of *B. thuringiensis* using dried bovine blood and extracts of leguminous seeds[6,7] and natural sources[8] have been developed for field application either to combat the agricultural pests or annul the menace of vectors of human diseases. Using either fed batch or continuous culture, *Rossa* et al.[9] have achieved sizeable increase in biomass and spore production of *B. thuringiensis*.

Knowledge of the growth parameters and their interrelationships is necessary for achieving optimum results in either laboratory or plant scale production of *B. thuringiensis.* Statistical procedures like Response Surface Methodology (RSM) offer a convenient and less expensive methodology for optimization. This statistical technique, based on the fundamental principles of randomization and replication also provides an understanding of the effects of individual variables and their interactions on final result.[10]

The objective of the present study was to select a locally available cheap source of raw material for the production of *B. thuringiensis* and then to optimize the process conditions for its production. The raw materials studied were generally waste products from agro industries. In order to reduce the

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number of experiments and get more information about possible interaction effects, the optimization was carried out using a 2^3 full factorial central composite design (CCD).

Materials and Methods

Microorganism

Bacillus thuringiensis strain PBT-372 was obtained from the culture collection of Department of Biotechnology, Pondicherry University, Pondicherry, India.

Raw materials

Five locally available raw materials viz. fishmeal, molasses, sago, soya and paddy straw (after pre-treatment, wherever necessary), were screened initially for maximum biomass production of *B. thuringiensis* PBT-372. Following pre-treatment, the final total sugar mass fraction of each of the raw material was adjusted to 0.8 %. Paddy straw was soaked in tap water for 3 h and then filtered. Sago was boiled in tap water for 15 min and filtered through cheesecloth. The filtrates thus obtained were used in media preparation. 100 ml. of the media and basal salts as recommended by *Faloci* et al.[11] were taken in 250 ml Erlenmeyer flasks and sterilized in an autoclave at 121 °C for 15 min. The flasks were inoculated with overnight grown *B. thuringiensis* culture (2 %) and incubated on rotary shaker (180 rpm) at 30 °C for 72 h.

Optimization studies

A 2^3 full factorial central composite design (CCD) for 3 independent variables, each at two levels with 4 star points ($\alpha = \pm 1.682$) and six replicates at the center point as given in Table.1, was used to optimize the biomass production. Initial pH of the medium, inoculum (%) and sugar concentration (g L^{-1}) were chosen as independent variables.

Table 1 – *Optimization of initial pH, inoculum and sugar mass concentration: independent variables in the 2^3 CCD*

Variable	Quantity	Level				
		–1.682	-1	0	1	+1.682
X_1	pH	3.636	5	7	9	10.4
X_2	Inoculum, %	0.977	2	3.5	5.0	6.023
X_3	Sugar concentration, g L^{-1}	1.59	5.0	10.0	15.0	18.0

Data analysis

The design-expert package (Design Expert software, version 2.05, 1990) and MINITAB (version 12.2, Minitab Inc., 1998) were used for regression and graphical analysis of the data obtained. Growth was monitored at the end of 72 h following inoculation by determining the OD_{600} of the culture and treated as the dependent variable. The biomass production was optimized within the levels of the variables using Monte Carlo optimization technique which was also confirmed from the response surface contour plots.[12]

Results and discussion

The complexity and the cost of ingredients that are used in the preparation of media for large-scale production of microbes, often decide their production cost and the demand for them in the agrarian sector. Screening studies with microorganisms[13] has proved *Bacillus thuringiensis* PBT-372 to be an effective microbial pesticide. However, the cost of the ingredients used in the production limits its large-scale application. With a view to find out alternative, cheap raw materials for production, naturally occurring raw materials viz. fishmeal, molasses, sago, paddy straw and whey were used as sole carbon source in batch fermentation studies. The biomass and protein yield from each of the material as sole carbon source was compared with those using LB broth as the sole carbon source and the results are given in Table 2. It is evident from this data that the growth of the organism was maximum in media containing LB broth followed by fishmeal as the carbon source. However, molasses was the

Table 2 – *Effect of various natural sources on biomass and cell protein productivity of B. thuringiensis strain PBT-372*

Nutrient Source*	OD_{600}	Cell Protein m/μg
Fishmeal	0.841	61.85
Molasses	0.787	65
Sago Paddy straw	0.372	57.5
Whey	0561	59
Luria Bertani Broth (LB)	0.928	62.5
Mean	0.713	57.06
S.D.	0.206	10.41
S.E	0.08	4.25

*All the media excepting Luria Bertani broth were supplemented with basal salts

substrate, which supported maximum cell protein production. *Ampofo*[5] suggested that the choice of substrate to be used in mass scale multiplication of *B. thuringiensis* PBT-372 should be based on their availability, cost and ease with which the bacterium utilizes the substrate. Hence, for further optimization study, molasses was chosen as the chief source of carbon in the preparation of media for *B. thuringiensis* production.

The input quantities, initial pH of the medium, inoculum and the substrate mass concentration were optimized for the enhanced biomass production of *B. thuringiensis* by applying a 2^3 full factorial central composite design. Conventional methods of optimization are time consuming and cannot indicate the effects of mutual interactions between quantities on the desired outcome.[10] When optimizing culture conditions with complex media components these problems compound and may affect the final outcome totally in an unexpected manner. Therefore, in the present study, Response Surface Methodology (RSM) was employed which ensures randomization and replication and simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. Several workers[14,15,16] have employed RSM for optimization in various fermentation experiments. Though there are many reports in the literature on the effects of quantities such as media composition, pH, aeration, amino acid/vitamin content[17–24] on the growth of *B. thuringiensis,* these provide limited information. Hence, a statistical design viz. full factorial central composite design (CCD) was used in this study to optimize three important quantities, pH, inoculum and sugar concentration of the substrate for maximum biomass growth.

Table 3 represents the design matrix of the variables in both coded and uncoded units along with the predicted and experimental responses viz. biomass growth as OD_{600}. It may be seen that the biomass growth was maximum (OD_{600} = 0.528) at pH 9, 5 % inoculum and 15 g L^{-1} sugar concentration. At high inoculum levels (>5 %), the influence of pH was significant. However, at high inoculum level of 3.5 % and sugar concentration (10 g L^{-1}), the biomass yield was not proportionally increased. Increase in sugar/molasses ratio did not greatly influence the biomass yield.

The results of the experiment were fitted into a second order polynomial regression model as shown in equation 1.

$$Y = 0.323 - 0.01\,X_1 + 0.048\,X_2 + 0.067\,X_3 + \\ + 0.006\,X_1^2 + 0.009\,X_2^2 + 0.017\,X_3^2 + \\ + 0.009\,X_1 \cdot X_2 + 0.032\,X_1 \cdot X_3 + 0.006\,X_2 \cdot X_3 \quad (1)$$

Table 3 – *Full factorial central composite design for 3 variables (2^3 CCD), showing the predicted and observed biomass yields.*

Run No.	pH	Inoculum (%)	Sugar conc., γ/g L^{-1}	OD_{600}	
				observed	predicted
1	–1(5)	–1(2)	–1(5)	0.242	0.281
2	1(9)	–1(2)	–1(5)	0.228	0.213
3	–1(5)	1(5)	–1(5)	0.428	0.384
4	1(9)	1(5)	–1(5)	0.258	0.277
5	–1(5)	–1(2)	1(15)	0.354	0.337
6	1(9)	–1(2)	1(15)	0.353	0.400
7	–1(5)	1(5)	1(15)	0.45	0.408
8	1(9)	1(5)	1(15)	0.528	0.492
9	–1.682 (3.64)	0(3.5)	0(10)	0.356	0.359
10	1.682 (10.36)	0(3.5)	0(10)	0.331	0.322
11	0(7)	–1.682 (0.98)	0(10)	0.301	0.269
12	0(7)	1.682 (6.02)	0(10)	0.407	0.433
13	0(7)	0(3.5)	–1.682 (1.59)	0.258	0.259
14	0(7)	0(3.5)	1.682 (18.41)	0.494	0.487
15	0(7)	0(3.5)	0(10)	0.319	0.323
16	0(7)	0(3.5)	0(10)	0.329	0.323
17	0(7)	0(3.5)	0(10)	0.314	0.323
18	0(7)	0(3.5)	0(10)	0.339	0.323
19	0(7)	0(3.5)	0(10)	0.324	0.323
20	0(7)	0(3.5)	0(10)	0.316	0.323

*Numbers in parenthesis are the uncoded value of the variables.

Where Y is the predicted response and X_1, X_2 and X_3 are the coded forms of test variables. The coefficients of all the variables in equation 1 were tested for their significance using the student's 't' test, given in Table 4. The coefficients for 1st and 2nd order effects of sugar mass concentration and the interaction between the pH and sugar concentration were the most significant for this model. The summary of the analysis of variance (ANOVA) is shown in Table 5.

In the present study, the F-value of 11.92 is close to F (9, 10) indicating that the model is significant at 1 % confidence level. The probability P value is also relatively low (0.002) indicating the

Table 4 – *Coefficients of the regression equation and the least square fit of the quantity estimates*

Model term	Quantity estimate	Computed *t*-value	*P*-value
Intercept	0.323	24.558	–
X_1	–0.011	–1.248	0.240
X_2	0.048	5.571	0.000
X_3	0.067	7.754	0.000
$X_1 \cdot X_1$	0.006	0.712	0.493
$X_2 \cdot X_2$	0.009	1.148	0.278
$X_3 \cdot X_3$	0.017	2.062	0.066
$X_1 \cdot X_2$	–0.009	–0.842	0.419
$X_1 \cdot X_3$	0.032	2.856	0.017
$X_2 \cdot X_3$	0.006	0.602	0.560

Table 5 – *Regression analysis (ANOVA) for the biomass production of B. thuringiensis*

Sources of Variation	Sum of Squares	Degrees of freedom	Mean Squares	*F* value	Probability $P > F$
Model	0.112	9	0.12	11.92	0.002
Error	0.010	10	0.002		
Total	0.122	19			

$R = 0.915$, $R^2 = 0.838$, C.V. = 9.33

significance of the model. The correlation coefficient *R* and the coefficient of variation are generally used to provide correlation measures for the estimation of the regression model. The value of $R = 0.915$ indicates a high degree of correlation between the observed and predicted values. The lower value of the C.V. (9.33 %) ensures a greater reliability of the experiments in this study. The maximum biomass production predicted by the model ($OD_{600} = 0.582$) agreed with the experimental value ($OD_{600} = 0.515$) which was obtained after the experimental verification at the optimum values.

The response surface contours, which are the graphical results of the interactive effects, are shown in Figures 1a to 1c. The 3D response surface plots indicate that in general the biomass growth was favored at higher inoculum size and sugar concentration (Fig.1a and 1b). It appears that the effect of pH is influenced by the levels of other parameters.

Employing response surface design, *Muralidharan*[25] reported that pH 7.5 was ideal for maximal

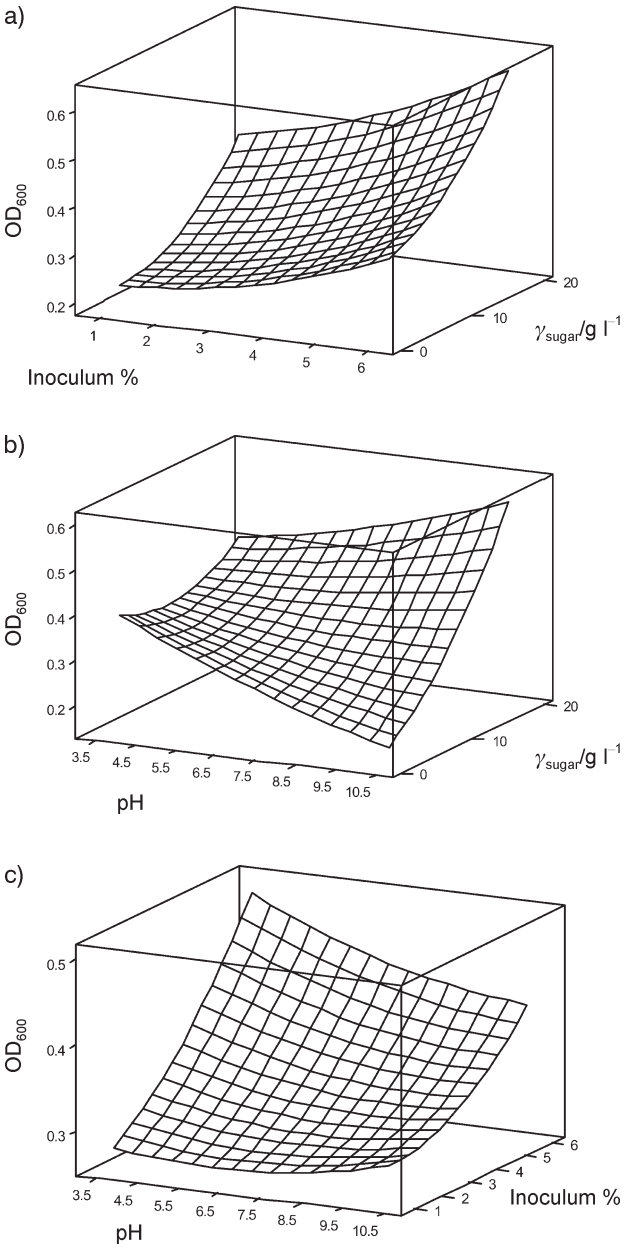

Fig. 1 – *Response Surface Contour plot showing the interaction on biomass yield of B. thuringiensis between a) inoculum and sugar concentration, b) pH and sugar mass concentration and c) inoculum and pH*

EPS production where the response surfaces were spheroids. Optimization of culture conditions with corn steep liquor, black strap molasses, fish meal and leguminous seeds, for maximum production of microbial biomass and cell protein, have been reported.[19] However, depending upon the nature of raw materials employed and the quantum of biomass required, fermentative production of *B. thuringiensis* was achieved using either batch[21] or continuous cultures.[24] *Murthy* et al.[10] have suggested that knowledge of stoichiometric co-efficients and growth quantities should help to achieve optimum results when industrial scale production is intended.

In this study, the results showed that molasses at pH 5.62 with inoculum size of 4.19 % and sugar concentration of 11.9 g L^{-1} supported maximum biomass production.

Conclusion

Molasses proved to be a good substrate among other locally available raw materials that could be used to support enhanced biomass yield of *Bacillus thuringiensis*. Statistical optimization studies were carried out for the production of *B. thuringiensis* using molasses as the sole carbon source and sugar concentration, initial pH of the medium and inoculum size as the design parameters. The sugar concentration and the interaction between pH and sugar concentration were found to be significant factors for the biomass growth. The optimum values of the variables as predicted by a second order regression model were 4.19 % of inoculum, initial pH of 5.62 and 11.9 g L^{-1} of sugar concentration. These conditions resulted in maximum biomass growth, which was confirmed by experimental observation. The regression model showed a high degree of correlation and confidence level.

ACKNOWLEDGEMENTS

Financial support by the Department of Biotechnology, Goverment of India is gratefully acknowledged. SRP was supported by Dr. K. S. Krishnan Research Fellowship by the Department of Atomic Energy (Goverment of India).

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