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Optimization of growth medium for the production of spores from *Bacillus thuringiensis* using response surface methodology

Bing-Lan Liu, Yew-Min Tzeng

Abstract The effects of cultivation medium compositions including tapioca, fishmeal, $CaCO_3$ and $(NH_4)_2SO_4$ for the growth of *Bacillus thuringiensis* YMB 96-1988 were accessed by using response surface methodology (RSM). The two-level (2^{4-1}) fractional factorial designs (FFD) which involve two concentrations of each nutrient, and the paths of steepest ascent were effective in searching for the major factors of the bacteria growth. This allows the fitting of a first order linear model to the data. In this study, supplementary $CaCO_3$ showed a negative effect on the spore production based on the first order regression coefficients derived from SAS programme. Subsequently, a 2^3 central composite design (CCD) was used for allocation of treatment combinations. Preliminary studies showed that tapioca and fishmeal is believed to be the major factors for the growth of *B. thuringiensis*. Estimated optimum compositions for the production of spores by *B. thuringiensis* are as follows: tapioca, 5.01%; fishmeal 5.86%; $(NH_4)_2SO_4$ 0.06% and resulted in a maximum spore count of 8.56×10^8/ml was obtained. This value is close to the 8.35×10^8/ml spore density as counted from actual experimental observations.

1 Introduction

The potential of *Bacillus thuringiensis* for the production of toxin component and the use as a biological control agent has been well documented [1, 2]. During sporulation, *B. thuringiensis* produces a parasporal crystal protein called δ-endotoxin, the most important insecticidal component of this microorganism, that causes paralysis of the larval gut on ingestion [3]. This property has given rise not only to scientific inquiry but also commercial interest to *B. thuringiensis*. For widespread field use, large quantities of spore-crystal preparation of a high insecticidal potency are required. Apart from the genetic approach, another way to successful commercialization of δ-endotoxin production is the development of an optimal fermentation medium [4]. Accordingly, a number of studies have been carried out and have given improvement to culture conditions, in particular the carbon and nitrogen sources for increased spore and/or crystal protein production [5–8].

The conventional method for multifactor experimental designs are time-consuming and incapable of detecting the true optimum, due especially to the interactions among factors [9, 10]. One of the worthwhile technique to identify the explanatory variable in the system is response surface methodology (RSM) [11, 12]. RSM can be used to evaluate the relative significance of several factors. Especially, this method can be used in the presence of complex interactions [13]. In this study, RSM was used to optimize the culture medium for the growth of *B. thuringiensis* YMB 96-1988 [14]. The major variables affecting the performance of the culture in terms of spore production as a function of the levels of carbon (tapioca), nitrogen (fishmeal) sources and inorganic salts ($CaCO_3$, $(NH_4)_2SO_4$) were investigated.

2 Materials and methods

2.1 Organism and growth medium

Bacillus thuringiensis YMB 96-1988 [14], obtained from Institute of Biochemistry, National Yang Ming University, was used in this study. The strain was maintained on NB (0.8% nutrient broth and 0.3% yeast extract) agar at 4 °C and −70 °C for short term and long term storage, respectively. Seed culture grown at 30 °C for 10 to 12 hours was used for building up of inoculum in 100 ml Erlenmeyer flask containing 25 ml NB medium. All optimization study experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of medium with various concentrations of tapioca, fishmeal, $CaCO_3$ and $(NH_4)_2SO_4$. The inoculum level of 2% (v/v) seed culture broth was fixed throughout the study. The inoculated flasks were incubated on an orbital shaker at 210 rpm and 30 °C for 72 hours. After harvest, the number of viable spore was counted.

2.2 Experimental design

Four factors were studied for spore production namely: tapioca, fishmeal, $CaCO_3$ and $(NH_4)_2SO_4$ concentrations. This was studied at the centre of the design to find the accuracy of results of statistical experimentation. At the beginning of the studies, a 2^{4-1} fractional factorial design (FFD), involving two concentrations of each factor (Table 1), was effective in searching for the direction of the optimum domain. Total viable spore counts for each

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Bing-Lan Liu, Yew-Min Tzeng
Institute of Biotechnology,
National Dong Hwa University,
Shoufeng, Hualien, Taiwan, ROC

Correspondence to: Yew-Min Tzeng

Table 1. Assigned concentrations (in %, w/v) of variables of different levels of the 2^{4-1} factorial fractional design

Factor	Symbol	Level		
		−1	0	+1
Tapioca	X_1	3.0	5.0	7.0
Fishmeal	X_2	4.0	5.0	6.0
$(NH_4)_2SO_4$	X_3	0.5	1.0	1.5
$CaCO_3$	X_4	0.5	1.0	1.5

shake-flask run was determined after a growth period of 72 hours, and the experimental data was subjected to segregate of each factor and their interactions. The actual concentration of each medium component was coded to facilitate multiple regression analysis. From the experimental results, an approximate polynomial's relationship for each dependent variable was obtained. The analysis of a simple polynomial model (first-order model), therefore, is represented by:

$$Y = \sum a_i x_i + c_0 + \varepsilon \ , \qquad (1)$$

where, Y is the predicted response (spore count); the parameters a_i are functions of x_i, c_0 is the intercept term and the remaining term, ε, represents random error in the yield values.

If the fitted first-order model is adequate, a series of single experiments has to be performed along with the path of steepest ascent toward to the optimum region. From here, the new region of the central point can be detected in which the desirable values of the response are suspected to be within the boundaries of the operability region. Finally, to describe the nature of the response surface in the optimum region, a 2^3 factorial central-composite design (CCD) with six star points ($\alpha = 1.673$) and three replications of the central points, leading to a total 17 sets, was used at five levels of inocula. The levels of each factor are given in Table 2. The model fitted to the centred data on the response of spore production were of second-order polynomial function:

$$Y = c_0 + \sum_{i=1}^{n} a_i x_i + \sum_{j \le i}^{n} b_{ij} x_i x_j \ , \qquad (2)$$

where, Y is the predicted response, subscripts i and j takes values from 1 to the number of variables (n); the c_0 is the intercept term; the a_i values are linear coefficient; the b_{ij} values are quadratic coefficient; x_i and x_j are the level of the independent variables. The analysis of data was carried out using SAS system. Response surface plots were generated by Statistica™ software (Tulsa, OK).

Table 2. Coding and assigned concentrations (in %, w/v) of variables of different levels of the central-composite design ($\alpha = 1.673$)

Factor	Symbol	Coded level				
		$-\alpha$	−1	0	+1	$+\alpha$
Tapioca	X_1	5.0	5.5	6.0	6.5	7.0
Fishmeal	X_2	6.0	8.0	10	12	14.0
$(NH_4)_2SO_4$	X_3	0.06	0.10	0.14	0.18	0.22

3 Results and discussions

Four 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 3 presents the experimental design and results of 2^{4-1} FFD experiments. The data was analysed based on Eq. (1), and the model is given as follows:

$$Y = 2.06 \times 10^8 + 3.81 \times 10^6 X_1 + 4.93 \times 10^7 X_2 + 1.15 \times 10^8 X_3 - 2.94 \times 10^8 X_4 \ . \qquad (3)$$

According to Eq. (3), all the factors have positive effects, except X_4 ($CaCO_3$). This equation also shows that X_3 ($(NH_4)_2SO_4$) is the most significant factor, with its coefficient effect being most pronounced. The second most conspicuous change is the response of X_2 (fishmeal). Partial response of tapioca starch (X_1) seems moderate. Calcium carbonate, however, at the experimental level gives opposite effect on the spore production. In summary, preliminary studies indicated that all the variables have to be rearranged to search for the direction of the optimum region. Based on the model equation obtained, Eq. (3), the path of steepest ascent experiment (data not shown) was conducted to find the more accuracy domain towards the optimum region. The central point for this experiment was reallocated as follow: tapioca 5.5% (w/v), fishmeal 4.0%, $(NH_4)_2SO_4$ 0.1% and $CaCO_3$ 0.1%.

Interestingly, the $CaCO_3$ concentration getting zero while the maximum spore count and protein concentration were observed (data not shown). This inferred that this component seems not necessary for the production of the crystal protein and spore in this case. It is tricky to say that $CaCO_3$ is an inhibitor for the spore production as the calcium is essential for cell growth and endotoxin production has been reported elsewhere [15]. Indeed, the calcium is required for the activation of amylase and exoprotease of *B. thuringiensis* during the vegetative growth and transition phases of metabolism [2]. The result obtained above can be interpreted as the calcium enriches in the fishmeal that provided the essential trace minerals for the metabolism demands. Therefore, no more extra

Table 3. Experimental design and results of 2^{4-1} fractional factorial design

Run	Tapioca (X_1)	Fishmeal (X_2)	$(NH_4)_2SO_4$ (X_3)	$CaCO_3$ (X_4)	Spore count (#/ml)
1	1	1	1	1	2.8×10^8
2	−1	−1	1	1	1.05×10^8
3	−1	1	−1	1	1.92×10^8
4	1	−1	−1	1	1.3×10^8
5	−1	1	1	−1	3.1×10^8
6	1	−1	1	−1	1.9×10^8
7	1	1	−1	−1	7.4×10^8
8	−1	−1	−1	−1	2×10^8

$X_1 = X_2 * X_3 * X_4$

$CaCO_3$ was needed in the medium, and it was eliminated from the medium in the following experiments.

New levels of nitrogen and carbon sources as well as inorganic salt were chosen as factors (Table 2) after path of steepest ascent experiment. For these three factors, a full 2^3 factorial design was investigated. Tables 4 and 5 show the experimental design and results of ANOVA for the 17 trials performed by the experimental design. Corresponding second-order response model for Eq. (2) that was found after SAS RSREG analysis for the regression is presented below:

$$\begin{aligned} Y &= 8.84 \times 10^8 - 1.35 \times 10^8 X_1 - 4.2 \times 10^7 X_2 \\ &\quad + 5.84 \times 10^6 X_3 - 1.38 \times 10^7 X_1 X_2 \\ &\quad - 2.38 \times 10^7 X_1 X_3 - 2.19 \times 10^8 X_2 X_3 \\ &\quad - 4.75 \times 10^8 X_1^2 - 4.43 \times 10^8 X_2^2 - 4.68 \times 10^8 X_3^2 \ . \end{aligned} \tag{4}$$

Statistical significance of the second-order model equation was checked by an *F*-test. The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.92, indicating that 92% of the variability in the response can be explained by the model. This revealed that Eq. (4) is a suitable model to describe the response of the experiment pertaining to spore production. The response taken from the Table 5 reveal that the linear term of tapioca starch (X_1) and quadratic coefficients of X_1^2, X_2^2, X_3^2 and $X_2 * X_3$ have remarkable effects on the spore yield. This suggests that the effect of second-order model is mainly from the interaction between these components ($p < 0.05$). Nevertheless, the significance for the linear terms, $X_1 - X_3$, was not significant at the 5% probability level. They were not omitted from the model equation, since its adequate fit could be confirmed. This also can be seen from the surface plots for these components as shown in Figs. 1 to 3 (see below). By moving along the two axes (Fig. 2), for example, it can be demonstrated that increasing the levels of X_1 and X_3 from the percentages has a conspicuous effect on overall linkage. As a result, the stationary ridge shape was observed in the surface

Table 4. The design and results of the central-composite experiments

Run	Coded level			Spore (#/ml)
	X_1	X_2	X_3	
1	1	1	1	1.15×10^8
2	−1	1	1	1.2×10^8
3	1	−1	1	5.4×10^8
4	−1	−1	1	1.1×10^8
5	1	1	−1	1.6×10^8
6	−1	1	−1	1.5×10^8
7	1	−1	−1	1.6×10^8
8	−1	−1	−1	6.1×10^8
9	1.673	0	0	1.49×10^8
10	−1.673	0	0	1.02×10^8
11	0	1.673	0	1.33×10^8
12	0	−1.673	0	1.14×10^8
13	0	0	1.673	2.1×10^8
14	0	0	−1.673	1.04×10^8
15	0	0	0	9×10^8
16	0	0	0	8.4×10^8
17	0	0	0	9.2×10^8

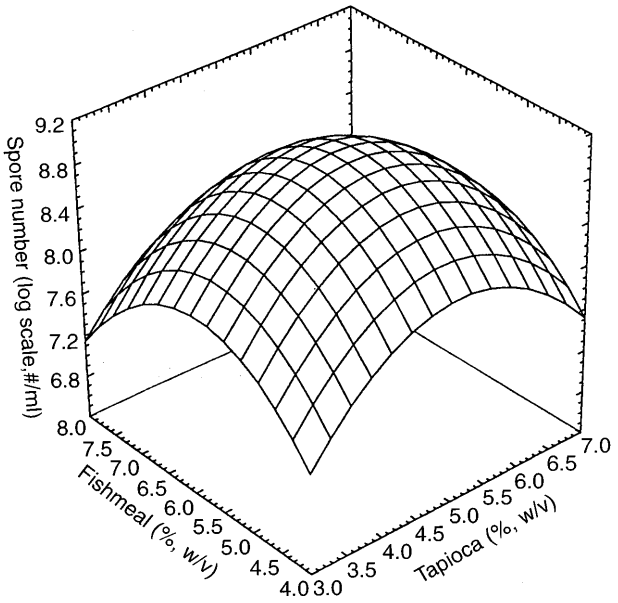

Fig. 1. Response surface graph of spore density affected by tapioca and fishmeal concentrations. The plot was obtained with the 2^3 factorial central-composite design

Table 5. Analysis of variance for the results of the central composite design

Effect	Sum of square	DF	*F*-Ratio	Regression coeffs.
X_1	6.18×10^{16}	1	3.38 (ns)	Constant = 8.84×10^8
X_2	5.99×10^{15}	1	0.33 (ns)	$X_1 = -1.348 \times 10^8$
X_3	1.16×10^{14}	1	0.01 (ns)	$X_2 = -4.201 \times 10^7$
$X_1 * X_2$	3.78×10^{14}	1	0.02 (ns)	$X_3 = 5.838 \times 10^6$
$X_1 * X_3$	1.13×10^{15}	1	0.06 (ns)	$X_1 * X_2 = -1.375 \times 10^7$
$X_2 * X_3$	9.57×10^{16}	1	5.24 (*)	$X_1 * X_3 = -2.375 \times 10^7$
$X_1 * X_1$	6.27×10^{17}	1	34.34 (**)	$X_2 * X_3 = -2.188 \times 10^8$
$X_2 * X_2$	5.46×10^{17}	1	29.90 (**)	$X_1 * X_1 = -4.749 \times 10^8$
$X_3 * X_3$	6.10×10^{17}	1	33.42 (**)	$X_2 * X_2 = -4.431 \times 10^8$
Block	1.22×10^{17}	1	6.68	$X_3 * X_3 = -4.684 \times 10^8$
Block	6.27×10^{16}	1	3.41	
Total error	9.13×10^{16}	5		

DF: Degree of freedom; Confidence levels: ns: not significant; *: $p < 0.05$, **: $p < 0.005$; $R^2 = 0.94$

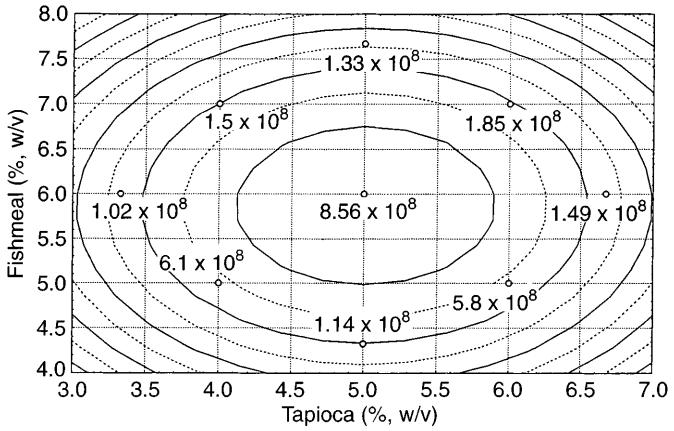

Fig. 2. Contour plot showing spore count in response to varying concentrations of tapioca and fishmeal. The strain depicted is YMB 96-1988

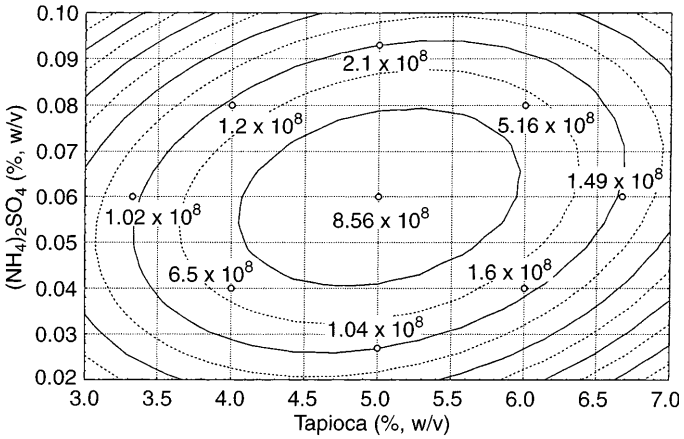

Fig. 4. Contour plot showing spore count in response to varying concentrations of tapioca and $(NH_4)_2SO_4$. The strain depicted is YMB 96-1988

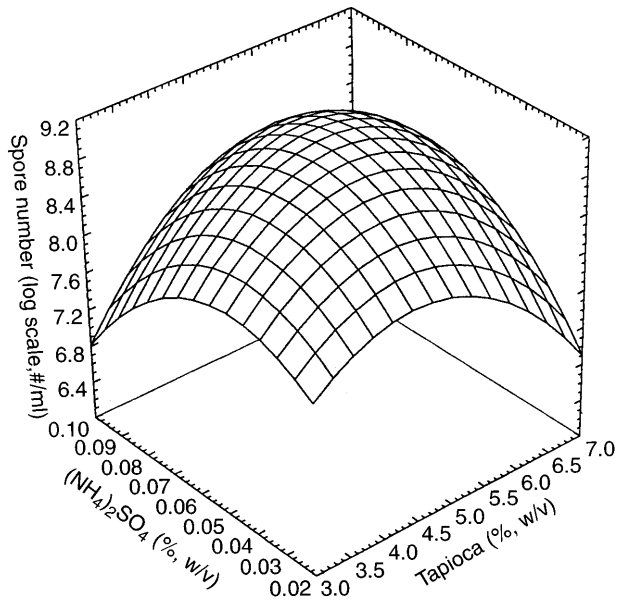

Fig. 3. Response surface graph of spore density affected by tapioca and $(NH_4)_2SO_4$ concentrations. The plot was obtained with the 2^3 factorial central-composite design

plot of these two components. According the Eq. (4), tapioca and fishmeal are believed to be the major factors for the growth of *B. thuringiensis* among the variables studied.

Yet, the influence of organic and inorganic source ratio on the spore production is probably more pronounced than the C:N ratio in the system. This observation has been drawn in which the different combinations of organic and inorganic source effect the metabolism of *Clostridium acetobutylicum* as reported [16]. Besides, no extra significant effect was found along with the fishmeal (Figs. 1 to 6). The reason for the less effect may attribute to overstock the demand of the fishmeal concentration under experimental condition. Probably, the inhibition of spore titre by the high level of main nitrogen source, fishmeal, can be interpreted as a some kind of competition occurred between the organic (fishmeal) and inorganic nitrogen (NH_4^+). It has been shown that the formation of exoprotease is repressed by the presence of ammonium ion during the exponential growth phase [2]. Thus, if the higher concentration of NH_4^+ exists in the beginning of fermentation medium, the lower spore count was observed (Table 4). This implied that the utilisation of inorganic nitrogen source is preferable during the vegetative phase of cell growth. Of course, the fishmeal itself may still play a key role in the spore production under different combination of these three components. This may reflect on the use of amino acids derived from the breakdown of bath cell and medium protein during stationary phase while the expression of parasporal crystal gene (C*ry* gene) is initiated as well.

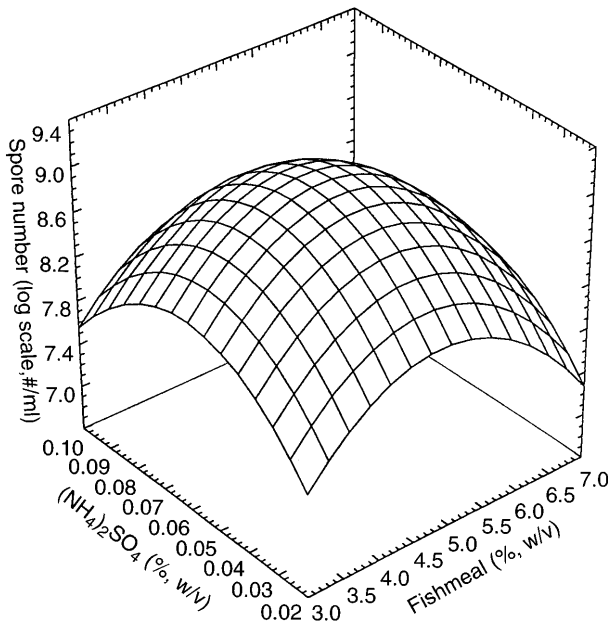

Fig. 5. Response surface graph of spore density affected by $(NH_4)_2SO_4$ and fishmeal concentrations. The plot was obtained with the 2^3 factorial central-composite design

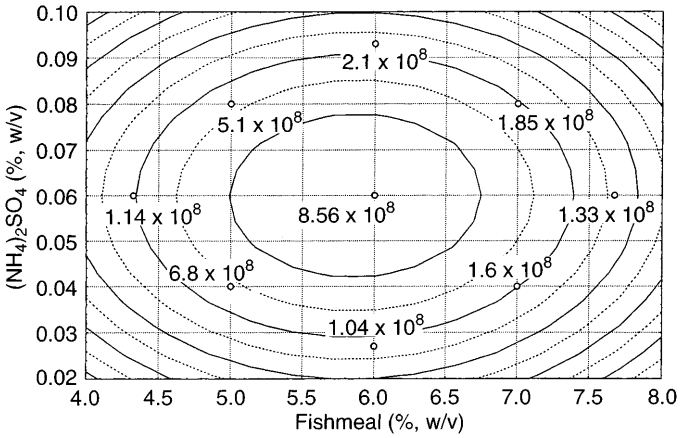

Fig. 6. Contour plot showing spore count in response to varying concentrations of $(NH_4)_2SO_4$ and fishmeal. The strain depicted is YMB 96-1988

Calculations for the carbon and nitrogen contents based on the compositions used in medium revealed that no good relationship can be detected from the experimental data. Although the higher carbonate content of medium might have prolonged the vegetative exponential phase of the cell growth, the delaying sporulation and lysis were observed. The C/N ratio, however, still could not explain the bioinsecticidal activity properly in the case of *B. thuringiensis* var. *thuringiensis* [17]. The above pattern of substrate utilisation is reflected in the spore count (Table 4) at this study. Whereas, the maximum spore counts still can be detected, under the condition of carbon and nitrogen sources content ratio of the medium is centred at the neighbourhood of 7. This indicates that the cultivation occurred under different stable physiological states while the C/N ratio varying from case to case. Simultaneously, the key factors controlling the cell growth and/or spore (or δ-endotoxin) production, as it occurs in nature, are not limited by single nutrient [18]. If this is the case, the further elucidation necessary requires a better knowledge on the role played by C:N ratio on *B. thuringiensis* physiology.

The values calculated for the Eq. (4) were the best estimates to be obtained from the experimental data, so that it would be inappropriate to conceive a hypothesis that these coefficients are equal to zero. For the analysis of the fitted surface, Equation (4) was transformed into its canonical equation:

$$Y = 8.56 \times 10^8 - 4.67 \times 10^8 Z_1^2 - 6.37 \times 10^8 Z_2^2 - 7.71 \times 10^8 Z_3^2 \quad . \qquad (5)$$

Since all coefficients of this equation are negative, the fitted surface has a true maximum. Based on this observation the concentration ranges of three components were toward to the maximum as confirmed from Figs. 1 to 6. Estimate optimum's concentrations of the factors are: tapioca starch, 5.01%; fishmeal, 5.86% and $(NH_4)_2SO_4$, 0.06% and the predicated spore yield were 8.56×10^8/ml. Applying this optimum point into the culture medium carried out on the same procedure as stated above and the 8.35×10^8/ml spore count was observed. The good correlation between predicted and measured result of this experiment verifies the validity of the response model and the existence of a high opportunity to reaching an optimum point in the system. The optimum concentration for these factors also can be easily found out from the contour plots of spore production (Figs. 4 to 6) obtained from the CCD experiments.

The optimum's composition, obtained from Eq. (5), shows that growth of *B. thuringiensis* YMB 96 was strongly affected by all variables. However, the Figs. 1 to 3 (or Figs. 4 to 6) are an evidence in which all the interactions of three variables exhibited a strong effect on the spore formation. This suggested that the whole system is extremely difficult to assigned to only one or few reason(s) due to the interaction between the factors. Although real situations may be more complicated than we presented here, the attempt for the medium optimisation has been made by RSM. In particular, the regression model is adequate to the response taken from the experimental data and to define a process of optimisation of the culture medium.

4 Conclusion

The present study shows that the RSM has proved satisfactory in this medium-screening exercise. Fitted model provides a suitable prediction in the response, indicating that the improvement of medium optimisation can be made, for practical purposes, through RSM procedure. However, the overall yield of spore count is somewhat less than predicted. Since not all the factors on their own, or simple interactions between them, could explain the variation in the data; there may be more complex interactions to be considered. Obviously, the responses using spore count itself show some kind of variation from case to case. Such a variation in spore count under experimental conditions suggests that the spore number cannot be quantitatively described by a single constant correlation coefficient over the entire range of concentrations. The results of this study also suggested that a cardinal analysis (e.g. total protein and/or bioassay), apart from spore counts, should be performed to find a unique extreme in the experimental region. This will show the direction to find the optimum condition in further experimentation. Clearly, spore morphology should be a factor to be considered when future work is performed with multiply limited microbial cultures involving complementary substrate. One potential solution is to directly measure a macromolecular component of the biomass, e.g. protein contents, rather than to measure the biomass concentration indirectly, e.g., spore count.

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