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Application of statistical design to the optimization of culture medium for prodigiosin production by *Serratia marcescens* SWML08

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ABSTRACT

Combination of Plackett – Burman design (PBD) and Box – Behnken design (BBD) were applied for optimization of different factors for prodigiosin production by *Serratia marcescens* SWML08. Among 11 factors, incubation temperature, and supplement of $(NH_4)_2PO_4$ and trace salts into the culture medium were selected due to significant positive effect on prodigiosin yield. Box - Behnken design, a response surface methodology, was used for further optimization of these selected factors for better prodigiosin output. Data were analyzed step wise and a second order polynomial model was established to identify the relationship between the prodigiosin output and the selected factors. The media formulations were optimized having the factors such as incubation temperature 30 °C, $(NH_4)_2PO_4$ 6 g/L and trace salts 0.6 g/L. The maximum experimental response for prodigiosin production was 1397.96 mg/L whereas the predicted value was 1394.26 mg/L. The high correlation between the predicted and observed values indicated the validity of the statistical design.

Keywords: *Serratia marcescens*, prodigiosin, Plackett - Burman design, Box - Behnken design

INTRODUCTION

For several decades, prodigiosin has been known to be a natural compound showing a broad range of cytotoxic activity (Furstner, 2003) and is also produced by *Vibrio psychroerythrus* (D'Aoust and Gerber, 1974), *Serratia marcescens*, *Pseudomonas magnesiorubra* and other eubacteria (Lewis and Corpe, 1964). Recently, prodigiosin has been considered effective as an immunosuppressive, antifungal and antiproliferative properties (Azuma *et al.*, 2000; Han *et al.*, 2001; Montaner and Perez-Thomas, 2001; Soto-Cerrato *et al.*, 2004). Owing to these characteristics, prodigiosin may have potential for medical application, for instance, it may be used to develop antitumor drugs (Perez-Thomas *et al.,* 2003; Furstner, 2003). In regard to its potential commercial values, there is a demand to develop high throughput and cost effective bioprocesses for prodigiosin production. However, medium components have not yet been assigned a definite role in prodigiosin production and no detailed information was reported about their optimum concentration to ensure high prodigiosin production by *S. marcescens*. Thus it is essential to carry out the research on the effect of different media components on prodigiosin accumulation in *S. marcescens.*

The growth and pigment production of the organism are strongly influenced by medium composition. Thus optimization of medium components and culture condition are the primary task in a biological process (Djekrif-Dakhmouche *et al.,* 2006). The main strategy used is medium engineering for which the optimal operating condition of a parameter is optimized by changing one parameter at a time and keeping the others at a constant level (Liu and Tzeng, 1998). The optimization studies do not consider the interaction effects among the variables as any process is influenced by several variables (Silva and Roberto, 2001). Limitations of the single factor optimization can be eliminated by employing response surface methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process (Elibol, 2004). Single variable optimization methods are not only tedious, but can also lead to misinterpretation of results, especially when interaction effects between different factors are overlooked (Wenster-Botz, 2000). But response surface methodology (RSM), consisting of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables is an ideal methodology to infer facts scientifically.

Statistical methodologies such as Plackett - Burman design (PBD) (1946) and Box - Behnken design (BBD) (1960) have shown to be efficient and effective approach to systematic investigation on the target factors. PBD is an effective screening design which considerably diminishes the number of experiment and gives information for the evaluation of the target factors as much as possible. Only the most effective factors with positive

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significance are selected for further optimization. The less significance or high negative effect on response value would be omitted for further experiments (Plackett and Burman, 1946). PBD has been widely applied in many fields such as medium optimization, formulation of multi component and so on (Loukas, 2001; Naveena *et al.,* 2005). BBD can be used to optimize target parameters within the designed scopes. The number of trials is equal to the maximum number of the designed levels of the target factor and therefore presents the advantage.

In the present work, combination of PBD and BBD was applied to select the medium components that significantly influenced the accumulation of prodigiosin and also to ascertain the optimum concentrations of those components in fermentation medium for prodigiosin production by *S. marcescens* SWML08.

MATERIALS AND METHODS

Microorganism and preparation of inoculum

S. marcescens SWML08 strain was grown on the nutrient agar (Hi – medium, Mumbai, India) slants at 37 °C for 24 h and subcultured every two weeks. Nutrient broth was inoculated with a 24 h old culture and grown for 18 h and was used as inoculum.

Optimization of process parameters

Identification of suitable variables using Plackett - Burman design

The Plackett – Burman experimental design identifies the critical physico-chemical parameters required for elevated prodigiosin production by screening *n* variables in *n* + 1 experiments (Plackett and Burman, 1946). The variables chosen for the present study were medium type, pH, incubation temperature (°C), agitation speed (rpm), inoculum size (%), incubation time (h), the content of lactose (g/L), $(NH_4)_2PO_4$ (g/L), $CaCl_2$ (g/L), NaCl (g/L) and trace salts (g/L) in the culture medium. The experimental design for the screening of the variables was presented in Table 1. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). The effect of individual parameters on prodigiosin production was calculated by the following equation:

$$E = (\sum M_{+} - \sum M_{-}) / N$$

Where E is the effect of parameter under study and M_+ and M_- are responses (prodigiosin activities) of trials at which the parameter was at its higher and lower levels respectively and N was the total number of trials.

Response surface methodology

The levels of the significant parameters and the interaction effects between various variables that influenced the prodigiosin production were analyzed and optimized by Box – Behnken methodology (Box and Behnken, 1960). In this study, the experiment consisted of 17 trials and the independent variables were studied at three different levels, low (-1), medium (0) and high (+1). The experimental design used for the study was shown in Table 3. All the experiments were done in triplicate and the average of prodigiosin production obtained was taken as the dependent variable or response (Y). The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software (Version 7.1.5, Stat-Ease Inc., Minneapolis, USA) statistical package. The general form of the second degree polynomial equation is

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where Y_i is the predicted response, $x_i x_j$ are input variables which influence the response variable Y; β_o is the offset term; β_i is the i^{th} linear coefficient; β_{ii} is the i^{th} quadratic coefficient and β_{ij} is the ij^{th} interaction coefficient.

Extraction and determination of prodigiosin

For extraction of prodigiosin, one mL culture broth was centrifuged at 1200 x g for 10 minutes and the pellet was resuspended in 1 mL acidified methanol and mixed vigorously. The solution was then centrifuged at 1200 x g for 10 minutes. Optical density of the resulting solution was determined at 535 nm (OD_{535nm}). The total prodigiosin (mg/L) was calculated according to the following formula (Williams *et al.,* 1960; Chen *et al.,* 2006):

$$TP(mg/L) = \frac{ADV_1}{7.07 \times 10^4 V_2}$$

Where TP denotes the total pigment yield (mg/L), A the absorbance of methanol extract at 535 nm, D the dilution ratio, V_1 the volume of methanol added, 7.07 x 10^4 is extinction coefficient of prodigiosin and V_2 is the volume of fermentative liquid.

Statistical analysis

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included Fisher's F- test (overall model significance), it's associated probability p(F), correlation coefficient R, determination coefficient R^2 which measure the goodness of fit of regression model. For each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated using Design Expert software (Version 7.1.5, Stat-Ease Inc., Minneapolis, USA) statistical package.

Table 1: Screening of factors using Placket – Burman design for prodigiosin production by *S. marcescens* SWML08

Run	A: Media	B: pH	C: Temperature	D: Agitation	E: Inoculum conc	F: Incubation time	G: Lactose	H: $(NH_4)_2PO_4$	J: $CaCl_2$	K: NaCl	L: Trace Salts	Total Prodigiosin
			°C	rpm	%	h	g/L	g/L	g/L	g/L	g/L	mg/L
1	{ 1 }	1	-1	-1	-1	1	-1	1	1	-1	1	41.11
2	{ 1 }	-1	1	1	-1	1	1	1	-1	-1	-1	678.15
3	{ -1 }	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	388.26
4	{ -1 }	1	1	1	-1	-1	-1	1	-1	1	1	524.39
5	{ 1 }	1	1	-1	-1	-1	1	-1	1	1	-1	971.26
6	{ 1 }	-1	-1	-1	1	-1	1	1	-1	1	1	988.35
7	{ -1 }	1	1	-1	1	1	1	-1	-1	-1	1	998.24
8	{ -1 }	-1	-1	1	-1	1	1	-1	1	1	1	517.19
9	{ 1 }	-1	1	1	1	-1	-1	-1	1	-1	1	670.11
10	{ -1 }	1	-1	1	1	-1	1	1	1	-1	-1	660.25
11	{ -1 }	-1	1	-1	1	1	-1	1	1	1	-1	381.59
12	{ 1 }	1	-1	1	1	1	-1	-1	-1	1	-1	509.37

RESULTS AND DISCUSSION

Screening of suitable variables using Plackett – Burman design

The results (Table 1) indicated that there was a wide variation of total prodigiosin yield in the twelve trials (41.11 to 998.24 mg/L). These variations reflected the importance of medium optimization to obtain higher prodigiosin yield. The following first order polynomial model describes the variations of the results:

$$Y = \beta_0 + \sum \beta x_i$$

Where Y is the responsive value, β_0 the model intercept, β_i the linear coefficient and x_i is the level of the independent variable.

The medium components were screened and those with a p – value of < 0.1 using 90 % confident level were accepted as significant factors affecting the production of prodigiosin. According to ANOVA for the model, the model of regression was significant (p < 0.0002) (Table 2) which inferred that incubation temperature, $(NH_4)_2PO_4$ and trace salts as most significant variables influencing prodigiosin production. Kim *et al.* (2008) selected five medium components ($CaCl_2$, Na_2S0_4, Na_2SiO_3, $NaHCO_3$ and NH_4NO_3) through Plackett – Burman design for prodigiosin production by *Hahella chejuensis* KCTC 2396. In this study three medium components viz incubation temperature, $(NH_4)_2PO_4$ and trace salts were selected through Plackett – Burman design for production of prodigiosin by *S. marcescens* SWML08 and these medium components were selected for further optimization using Box – Behnken design. These results indicated that the Plackett – Burman design is a powerful tool for identification of the variables that could significantly affect prodigiosin production.

Response surface methodology

Statistical designs are effective tools that can be used to account for the main as well as the interactive influences of fermentation parameters on the process performance. Among them, response surface methodology (RSM) is a collection of certain statistical techniques for designing experiments, building models, evaluating the effect of the factors and searching for optimal conditions for desirable responses (Myers and Montgomery, 2002). Therefore, during the past decades, RSM has been extensively applied in the optimization of medium composition, fermentation conditions and food manufacturing processes (Vazquez and Martin, 1997; Ramirez *et al.,* 2001; Park *et al.,* 2005).

In this study, RSM (Box – Behnken design) employed to investigate the interactions among the selected factors (incubation temperature, $(NH_4)_2PO_4$ and trace salts) in the culture medium and also to determine their optimum levels for maximum prodigiosin production demonstrated markedly varied results, ranging from 411.29 to 1397.96 mg/L in prodigiosin production. The lowest prodigiosin

Table 2: Analysis of variance for prodigiosin production using Placket – Burman design

Source	Sum of square	Degree of freedom	Mean square	F - Value	p - Value
Model	35954.28	7	485.36	59.62	0.0002
Residual	1101.53	11	92.14		
Pure error	71.32	4	17.08		
Total	37055.81	18			

Table 3: Experimental design and results of Box Behnken design of response surface methodology

Run	Temperature (°C)	$(NH_4)_2PO_4$ (g/L)	Trace Salts (g/L)	Total Prodigiosin (mg/L)	
				Experimental	Predicted
1	0.000	0.000	0.000	834.16	833.64
2	0.000	0.000	0.000	831.12	833.64
3	0.000	1.000	-1.000	1397.96	1394.26
4	-1.000	-1.000	0.000	507.11	497.65
5	-1.000	0.000	1.000	518.59	520.54
6	0.000	1.000	1.000	724.59	727.27
7	0.000	0.000	0.000	827.15	833.64
8	1.000	0.000	1.000	411.29	408.15
9	1.000	0.000	-1.000	517.28	515.33
10	-1.000	1.000	0.000	712.35	707.72
11	0.000	0.000	0.000	829.65	833.64
12	0.000	-1.000	1.000	730.24	737.74
13	1.000	1.000	0.000	423.59	433.05
14	-1.000	0.000	-1.000	638.14	640.28
15	1.000	-1.000	0.000	521.35	525.98
16	0.000	-1.000	-1.000	789.35	786.67
17	0.000	0.000	0.000	847.11	833.64

Table 4: Analysis of Variance for prodigiosin production using Box – Behnken Design

Source	Sum of squares	Degree of freedom	Mean square	F - Value	p - Value
Model	4.416E+005	9	49062.46	5.05	0.0221
A-Temperature	30341.70	1	30341.70	3.13	0.1204
B-DAP	6859.13	1	6859.13	0.71	0.4283
C-Trace Salts	27827.94	1	27827.94	2.87	0.1342
AB22952.25	22952.25	1	22952.25	2.36	0.1680
AC3.17	3.17	1	3.17	3.264E-004	0.9861
BC4765.83	4765.83	1	4765.83	0.49	0.5061
A23.363E+005	3.363E+005	1	3.363E+005	34.65	0.0006
B2415.83	415.83	1	415.83	0.043	0.8419
C24369.10	4369.10	1	4369.10	0.45	0.5238
Residual	67941.68	7	9705.95		
Lack of Fit	61019.14	3	20339.71	11.75	0.0188
Pure Error	6922.54	4	1730.64		
Cor Total	5.095E+005	16			

CV – 4.12; R^2 – 0.937

production of 411.29 mg/L was observed when incubation temperature was at 40 °C with $(NH_4)_2PO_4$ (0 g/L) and trace salts (0.1 g/L) (run 8). Prodigiosin production of 1397.96 mg/L was observed at incubation temperature 30 °C, $(NH_4)_2PO_4$ 6 g/L and trace salts 0.6 g/L (run 3). The results were presented in Table 3.

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis and the results were presented in Table 4. The model F value of 5.05 implied that the model was significant and also showed that there was 0.25% chance that the model F value could occur due to noise. The R^2 value (multiple correlation coefficients) closer to 1 denoted better correlation between the observed and predicted responses. The coefficient of variation (CV) indicated the degree of precision with which the experiments were compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (4.12) indicated that the experiments performed were highly reliable. The *p* values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables.

The results obtained from the BBD were fitted to a second order polynomial equation to explain the dependence of total prodigiosin production on the medium components.

$$Y = 833.64 - 61.58\ A + 29.28\ B - 58.98\ C - 75.75\ AB + 0.89\ AC - 34.52\ BC - 282.60\ A^2 - 9.94\ B^2 - 32.21\ C^2$$

Where Y is the predicted response (total prodigiosin production), A, B and C are the coded values of incubation temperature, $(NH_4)_2PO_4$ and trace salts respectively.

Kim *et al.* (2000) reported that, through Box - Behnken experimental design, the optimal concentrations of ($NaHCO_3$, Na_2SiO_3, NH_4NO_3, Na_2SO_4, and $CaCl_2$) were determined to be 0.45, 0.0045, 0.0045, 9.0, and 1.7115 g/L, respectively for prodigiosin production of 1.198 g/L by *Hahella chejuensis* KCTC 2396. In this study, the optimized values of the Box – Behnken design were found to be incubation temperature 30 °C, $(NH_4)_2PO_4$ 6 g/L and trace salts 0.6 g/L for production of 1.397 g/L prodigiosin by *S. marcescens* SWML08. Results obtained in this study are comparable to the study of Kim *et al.* (2000) by BBD for the production of prodigiosin and the statistical method employed was found to be a viable one for optimizing the medium factors. The optimized medium has shown the maximum prodigiosin yield by *S.marcescens* SWML08.

Chen and Johns (1993) and Juzlova *et al.* (1996) reported that ammonium chloride is a better inorganic nitrogen source for pigment production. The present study demonstrated that diammonium phosphate was a better nitrogen source for prodigiosin production in *S. marcescens* SWML08.

The fitted response for the above regression model was plotted in Figure 1 and 2. 3D graphs generated for the pair wise combination of the three selected factors for total prodigiosin production highlighted the roles played by these factors and also the physical constraints in the final yield of total prodigiosin.

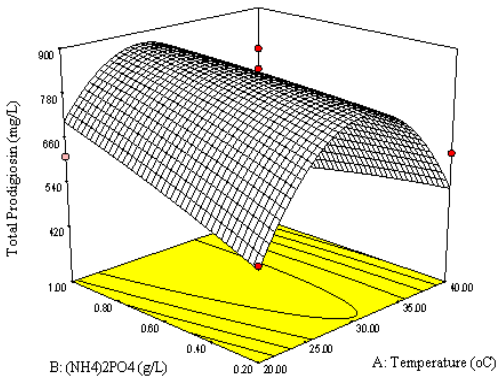


Figure 1: Response surface graph showing the effect of the interaction of incubation temperature and $(NH_4)_2PO_4$ on prodigiosin production

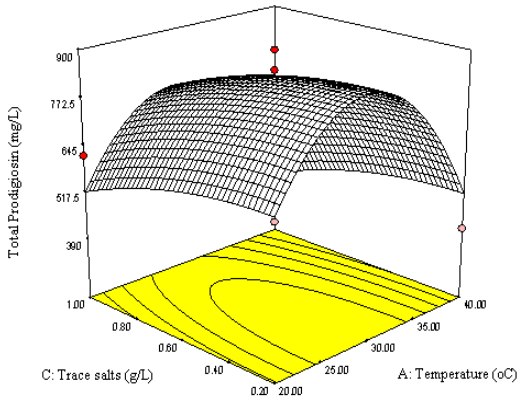
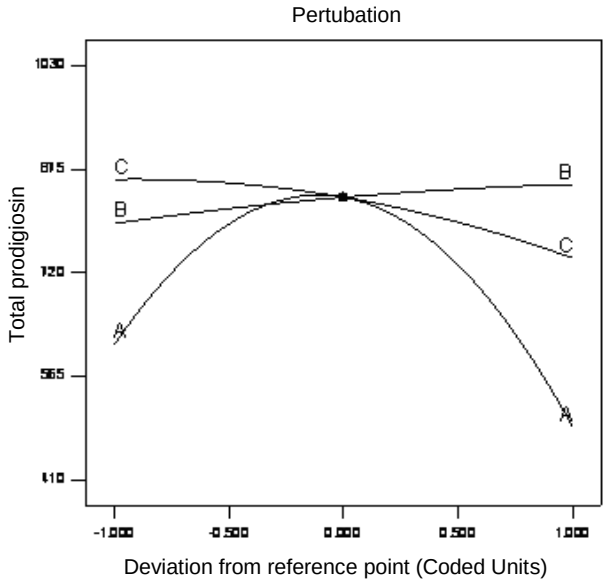


Figure 2: Response surface graph showing the effect of the interaction of temperature and trace salts on prodigiosin production



Design-Expert® Software
Total prodigiosin
● Total prodigiosin

Actual Factors
A: Temperature = 30 °C
B: DAP = $(NH_4)_2PO_4$ - 6 g/L
C: Trace Salts = 0.6 g/L

Figure 3: Perturbation graph showing the optimum values of the tested variables

Validation of the model

The maximum experimental response for prodigiosin production was 1397.96 mg/L whereas the predicted value was 1394.26 mg/L indicating a strong agreement between them. The optimum values of the tested variables are incubation temperature 30° C, $(NH_4)_2PO_4$ 6 g/L and trace salts 0.6 g/L as shown in perturbation graph (Figure 3). The model was also validated by repeating the experiments under the optimized conditions, which resulted in the prodigiosin production of 1390.91 mg/L (Predicted response 1394.26 mg/L), thus proving the validity of the model.

CONCLUSIONS

The combination of Plackett – Burman design with Box - Behnken design for optimizing the bioprocess variables for prodigiosin production by *S. marcescens* SWML08, is an effective and reliable tool to select the statistically significant factors and finding the optimal concentration of those factors in culture medium. The present work demonstrates the rewarding application of Box - Behnken design for quickly determining the conditions leading to the optimum yield of prodigiosin production. This study identified the effect of various factors in the production of prodigiosin by *S. marcescens* SWML08 in the culture medium and found that incubation temperature, $(NH_4)_2PO_4$ and trace salts are the significantly influenced factors for maximum prodigiosin production. This statistical methodology could be successfully applied to any bioprocess, where an analysis of the effects and interactions of many experimental factors are mandatory.

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REFERENCES

Azuma, T., Watanabe, N., Yagisawa, H., Hirata, H., Iwamura, M. and Kobayashi, Y. (2000). Induction of apoptosis of activated murine splenic T cells by cycloprodigiosin hydrochloride, a novel immunosuppressant. *Immunopharmacology and immunotoxicology* **46, 29-37.**

Box, G. E. P. and Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics* **2, 455-475.**

Chen, D., Han, Y. and Gu, Z. (2006). Application of statistical methodology to the optimization of fermentative medium for carotenoids production by *Rhodobacter sphaeroides. Process Biochemistry* **41, 1773-1778.**

Chen, M. H. and Johns, M. R. (1993). The effect of pH in nitrogen source on pigment production by *Monascus purpureus. Applied Microbiology and Biotechnology* **40, 132-138.**

D' Aoust, J. Y. and Gerber, N. N. (1974). Isolation and purification of prodigiosin from *Vibrio psychroerythrus. Journal of Bacteriology* **118, 756-757.**

Djekrif-Dakhmouche, S., Gheribi – Aoulmi, Z., Meraihi, Z. and Bennamoun, L. (2006). Application of a statistical design to the optimization of culture

medium for α amylase production by *Aspergillus niger* ATCC16404 grown on orange waste powder. *Journal of Food Engineering* **73, 190-197.**

Elibol, M. (2004). Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor* A3(2) with response surface methodology. *Process Biochemistry* **39, 1057-1062.**

Furstner, A. (2003). Chemistry and biology of roseophilin and the prodigiosin alkaloids: survey of the last 2500 years. *Angewandte Chemie International Edition* **42, 3582-3603.**

Han, S. B., Park, S. H., Jeon, Y. J., Kim, Y. K., Kim, H. M. and Yang, K. H. (2001). Prodigiosin blocks T cell activation by inhibiting interleukin – 2Rα expression and delays progression of autoimmune diabetes and collagen-induced arthritis. *Journal of Pharmacology Experimental Therapeutics* **299, 415-425.**

Juzlova, P., Martinkova, L. and Kren, V. (1996). Secondary metabolites of the fungus *Monascus*: a Review. *Journal of Industrial Microbiology* **16, 163-167.**

Kim, S. J., Hong, K. L. and Joung, H. Y. (2008). Statistical Optimization of Medium Components for the Production of Prodigiosin by *Hahella chejuensis* KCTC 2396. *Journal of Microbiology and Biotechnology* **18(12), 1903-1907.**

Lewis, S. M. and Corpe, W. A. (1964). Prodigiosin producing bacteria from marine sources. *Applied Microbiology* **12, 13-17.**

Liu, B. L. and Tzeng, Y. M. (1998). Optimization of growth medium for production of spores from *Bacillus thuringiensis* using response surface methodology. *Bioprocess and Biosystems Engineering* **18, 413-418.**

Loukas, Y. L. (2001). A Plackett – Burman screening design directs the efficient formulation of multicomponent DRV liposomes. *Journal of Pharmaceutical and Biomedical Analysis*. **26, 255-263.**

Montaner, B. and Perez-Thomas, R. (2001). Prodigiosin induced apoptosis in human colon cancer cells. *Life Science* **68, 2025-2036.**

Myers R. H. and Montgomery, D. C. (2002). Response Surface Methodology. Wiley, New York.

Naveena, B. J., Altaf, Md. and Bhadriah, K. (2005). Selection of medium components by Plackett – Burman design for production of L (+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran. *Bioresource Technology* **96, 485-490.**

Park, P. K., Cho, D. H., Kim, E. Y. and Chu, K. H. (2005). Optimization of carotenoid production by *Rhodotorula glutinis* using statistical experimental design. *World Journal of Microbiology and Biotechnology* **21, 429-434.**

Perez-Thomas, R., Montaner, B., Llagostera, E. and Soto-Cerrato, V. (2003). The prodigiosins, proapoptotic drugs with anticancer properties. *Biochemical Pharmacology* **66, 1447-1452.**

Plackett, R. L. and Burman, J. P. (1946). The design of optimum multifactorial experiments. *Biometrika* **33, 305-325.**

Ramirez, J., Gutierrez, H. and Gschaedler, A. (2001). Optimization of astaxanthin production by *Phaffia rhodozyma* through factorial design and response surface methodology. *Journal of Biotechnology* **88, 259-268.**

Silva, C. J. S. M. and Roberto, I. C. (2001). Optimization of xylitol production by *Candida guilliermondii* FTI 20037 using response surface methodology. *Process Biochemistry* **361, 119-124.**

Soto-Cerrato, V., Llagostera, E., Montaner, B., Scheffer, G.L. and Perez-Thomas, R. (2004). Mitochondria-mediated apoptosis operating irrespective of multidrug resistance in breast cancer cells by the anticancer agent prodigiosin. *Biochemical Pharmacology* **68, 1345-1352.**

Vazquez, M. and Martin, A. M. (1997). Optimization of *Phaffia rhodozyma* continuous culture through response surface methodology. *Biotechnology* **57, 314-320.**

Wenster-Botz, D. (2000). Experimental design for fermentation media development: Statistical design or global random search. *Journal of Bioscience and Bioengineering* **90, 473-483.**

Williams, R. P., Gott, C. L. and Green, J. A. (1960). Studies on pigmentation of *Serratia marcescens.* V. Accumulation of pigment fractions with respect to length of incubation time. *Journal of Bacteriology* **81, 376-379.**