ELSEVIER

Biochemical Engineering Journal 8 (2001) 31–37

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Chemometric optimisation of parameters for biocatalytic reduction of copper ion by a crude enzyme lysate of *Saccharomyces cerevisiae* grown under catabolic repression conditions

C. Bennett Chandran [a,*], T.V. Subramanian [a], P. Arthur Felse [b,1]

[a] *Department of Chemical Engineering, Anna University, Chennai, India*

[b] *Biotechnology Research Center, Department of Chemical Engineering, Indian Institute of Technology, Chennai, India*

Received 10 February 2000; accepted 15 November 2000

Abstract

The capacity of the microbes to reduce the metal has been demonstrated. The immobilised induced microbes with toxic chemical $CuCl_2$ was used to reduce the Cu ions as elemental metal and by using the response surface methodology the parameters such as the inducer concentration, the time of inducer addition which are concerned with the growth and formation of specific enzymes and the initial substrate concentration, initial pH of the substrate solution and the time of reaction which are concerned with the biocatalytic reduction of the metal ions were optimised for maximum reduction. The elemental copper reduced and removed experimentally from its ionic state at the optimum conditions was 54.82 ppm.

Keywords: Biocatalytic reduction; Removal of copper ions; Cytochrome P_{450}; Response surface methodology

1. Introduction

Heavy metals find their way into the water cycle via the natural process of erosion, weathering and volcanic activity [1]. In addition, the human activities like electroplating, tanning, mining, smelting, burning fossil fuels and trash, surface treatment and agrochemical processes contribute significantly to the metals found in natural water [2]. These metals tend to persist indefinitely, circulating and eventually accumulating in the food chain, thus posing a serious threat to the environment, animals and humans. The metal ions are highly water soluble and highly toxic, thus necessitating the treatment of wastewater, soils and sediments containing them. The toxicity of copper to microorganisms is well documented [3]. In addition, even the presence of relatively low concentrations of Cu^{++} in sewage could significantly reduce the efficiency of biological sewage treatment, such as the activated sludge process [4]. Haemolytic anaemia and hypertension in humans and reduced productivity of plants are some of the adverse effects of copper chloride. The present study involves biocatalytic reduction and removal of $CuCl_2$ in aqueous–organic phase by induction of copper degrading reductase in catabolic repressed yeast by addition of inducer ($CuCl_2$ itself) during the growth phase of yeast (*Saccharomyces cerevisiae*).

2. Materials and methods

2.1. Production of cytochrome P_{450} by S. cerevisiae

Shake flask studies of yeast grown with excess of glucose under catabolic repression conditions was used to induce the Cyt P_{450} in the yeast cells [5]. The conditions of cell growth were pH 4.9, agitation 180 rpm, and temperature 28°C [6] with a headspace of more than 150 ml in a 250 ml shaker flask. Cell culture was continued for 43 h to get the maximum Cyt P_{450} yield. Similar growth of cells was done in an external loop airlift reactor for higher production. The reactor had the following dimensions: diameter of the riser was 10×10^{-2} m, diameter of the downcomer was 10×10^{-2} m, height of the riser was 122×10^{-2} m, height of the downcomer was 90×10^{-2} m, working volume was 12×10^{-3} m^3, total volume was 20×10^{-3} m^3, and air flow rate was 2.5 m^3/(m^3 min).

* Corresponding author. Present address: School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK. Tel.: +44-2870-324053.

E-mail address: bc.chandrasekhar@ulst.ac.uk (C. Bennett Chandran).

[1] Present address: Biocatalysis and Bioprocessing Laboratory, Department of Chemical Engineering and Chemistry, Polytechnic University, 6 Metrotech Centre, Brooklyn, NY 11201.

1369-703X/01/$ – see front matter
PII: S1369-703X(00)00131-5

Nomenclature

r^2	regression coefficient
x	variables
$\hat{Y}$	predicted response

Greek symbol

β_i	linear effect
β_{ii}	squared effect
β_{ij}	interaction effect
β_0	offset term

Downstream processing of the cells were done by harvesting the cells in a centrifuge at 4°C, 5000*g*, for 15 min. The harvested biomass was lysed using cell disrupter and the microsomes were obtained [7].

2.2. Bioencapsulation

The crude cell lysate was mixed with sodium alginate in the ratio 1:1 at 4°C till a homogeneous solution was obtained. It was then dropped into a solution containing $CaCl_2$, $BaCl_2$ and $SrCl_2$ in the ratio 5:3:2 in a drop-wise fashion with a syringe from a convenient height to obtain beads of constant size with a diameter of 0.496 cm [8].

2.3. Analysis

Analysis of Cyt P_{450} was done by the method of Omura and Sato [9]. Cytochrome in its reduced state with CO to strive a complex, which has a λ_{max} at 450 nm. It was reduced by the addition of sodium dithionite while CO was added for complexing with Cyt P_{450}. CO was passed through the sample tube with Cyt P_{450} reduced with sodium dithionite at a controlled rate of 80 bubbles per minute for 10 min. Thus, the sample was analysed in the double beam spectrophotometer and the peak was noted.

Glucose was estimated by anthrone method. Anthrone reagent was prepared by adding 100 mg of anthrone powder (AR) in 50 ml of concentrated sulphuric acid (AR). The standards were prepared in the glucose concentration range 10–100 μg/ml. The samples were also collected in 1 ml volume. To 1 ml of standard and the sample, 4 ml of anthrone reagent was added and mixed well. The tubes were covered with marble top and boiled for 10 min; it was then cooled and the absorbance was measured at 620 nm in a double beam spectrophotometer. The protein was estimated by lowery method.

Samples were analysed in atomic absorption spectrophotometer of their copper content. At the end of the reaction the beads were separated and crushed to powder washed with double distilled water to remove the unreduced metal ions. The filtrate was then digested with concentrated HNO_3 to dissolve the reduced metal inside the bead and was then taken for analysis after suitable dilution with double distilled water.

The effect of initial pH, time of reaction and initial substrate concentration was optimised using response surface methodology for maximum metal reduction capacity of immobilised cell lysate of *S. cerevisiae*. The time of inducer addition and the amount of inducer were also optimised using response surface methodology to produce maximum substrate specific enzymes.

3. Experimental design

The classical method of studying one variable at a time while holding all others constant is extremely insufficient in many cases [10]. Statistically designed experiments are effective because they supply the needed information about the shape of the response surface. They are also efficient because they expend minimum resources. Response surface experiments attempt to identify the output or response of a system as a function of the explanatory variables. It is a very powerful statistical tool to design experiments for optimisation. It consists of a group of techniques, which are used to study the relationships between one or more measured responses and a number of input (independent) variable [11]. The results of response surface experiments are used to identify a mathematical relationship between independent variable levels and the response, and to optimise the system response. The interactions among variables are taken into consideration and the block effects are nullified with response surface methodology experimental design. Mathematics models have been widely used to help explain and predict the biochemical reactions. Henika [12] developed and used response surface techniques for many industrial applications.

A central composite experimental design is used to acquire data that will fit an empirical, full second-order polynomial model. A central composite experimental design coupled with a full second-order polynomial model is a very powerful combination that provides an adequate representation of most continuous response surfaces over a relatively broad factor domain.

3.1. Predicted response surface

The response surface is the one that relates the response with the variable considered in the study. If $X_1, X_2, \ldots, X_k$ are the variables considered, then the response predicted is given as

$$\eta = \phi(X_1, X_2, \ldots, X_k) \quad (1)$$

The function ϕ is called the true response surface and is assumed to be a continuous function of X_i.

The structural form of ϕ is usually unknown and therefore an approximating form is sought using a polynomial or some other type of empirical equation. Observations are made with

different combinations of the variables $(X_1, X_2, \ldots, X_k)$ and the parameters of the model is estimated. Tests are then performed on the magnitudes of the coefficient estimates as well as on the model from itself and if the fitted model is considered to be satisfactory, it can be used as a prediction equation. The response may be a simple linear equation or may be a polynomial equation of higher order. However, the number of observations made should be always greater than the number of parameters to be estimated.

3.2. The response surface

The relationship $\eta = \phi(X_1, X_2, \ldots, X_k)$ between η and the levels of k factors may be represented by a "hypersurface". With k factors, the response surface is a subset of a $(k + 1)$-dimensional Euclidean space that can be represented in a two-dimensional space, whereas the solid surface is visualised in a three-dimensional space, the third dimension representing the height of the surface above the two-dimensional plane. In a two-dimensional response surface, the lines indicate the locus of points that gives a particular response. The location of the point of maximum response is called the "point of maximum response" and may be obtained by measuring the response function.

4. Results and discussion

4.1. Experimental design and response surface modelling

The variables that affect the biocatalytic reduction of the metal ions are [8]:

1. Initial concentration of the substrate (metal ion) to be reduced;
2. Initial pH of the reaction mixture;
3. Time of reaction;
4. Concentration of the inducer;
5. Time of addition of inducer.

While the first three parameters are optimised for the enzymatic reduction reaction, the next two parameters are concerned with the optimisation of *S. cerevisiae* growth under induction conditions for enhanced production of metal ion reducing enzyme. Hence, these three sets of variables need to be optimised separately. A 2^3 central composite design with six axial points ($\alpha = 1.682$) and eight replicates at the centre point leading to a total of 20 experimental points was used. The detailed experimental plan is given in Table 1. The independent variables were coded according to the equation below:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{2}$$

where x_i is the coded value of the variable, X_i the uncoded value of the variable, X_0 the value of X_i at the central point, and ΔX_i the step charge.

The coded levels of independent variables and their corresponding uncoded values are given in Table 2. For optimisation of parameters for *S. cerevisiae* growth under induction conditions for enhanced production of metal ion reducing enzyme, viz., concentration of inducer and time of addition, a 2^2 central composite experimental design with four axial points ($\alpha = 1.414$) and five replicates at the centre point leading to a total of 13 experiments was used. The detailed

Table 1
Experimental plan as required by central composite design along with observed and predicted (from model) values of responses for each combination — biocatalytic reduction of $CuCl_2$

S. No.	Initial substrate concentration (mg per 100 ml)	Time of reaction (h)	Initial pH of substrate solution	Amount of Cu formed (ppm)	
				Experimental	Predicted
1	18.00	20.00	6.5	24.53	23.86
2	30.00	24.00	8.5	29.53	29.36
3	18.00	28.00	6.5	21.03	18.87
4	42.00	28.00	6.5	28.28	29.55
5	30.00	24.00	8.5	28.09	28.36
6	42.00	20.00	6.5	29.73	29.30
7	18.00	28.00	10.5	27.69	26.16
8	42.00	20.00	10.5	28.73	28.94
9	30.00	24.00	8.5	29.33	27.36
10	18.00	20.00	10.5	28.70	27.47
11	30.00	24.00	8.5	29.66	28.36
12	42.00	28.00	10.5	30.18	29.88
13	30.00	24.00	8.5	28.55	26.18
14	30.00	30.73	8.5	22.49	24.95
15	50.18	24.00	8.5	24.33	24.72
16	9.82	24.00	8.5	10.44	12.82
17	30.00	24.00	11.86	15.22	16.57
18	30.00	24.00	8.5	30.12	26.18
19	30.00	24.00	5.14	10.16	11.58
20	30.00	17.27	8.5	28.05	28.36

Table 2
Coded levels of independent variables and their corresponding uncoded levels for $CuCl_2$ reduction

Coded levels	Uncoded levels		
	Initial substrate concentration (mg per 100 ml)	Time of reaction (h)	Initial pH of the substrate solution
−1.682	9.82	17.27	5.14
−1	18.00	20.00	6.50
0	30.00	24.00	8.50
1	42.00	28.00	10.50
1.682	50.18	30.73	11.86

Table 3
Experimental plan as required by central composite design along with observed and predicted (from model) values of response for each combination — $CuCl_2$ induction studies

S. No.	Inducer concentration (mg per 100 ml)	Time of addition (h)	Amount of Cu formed (ppm)	
			Experimental	Predicted
1	3.75	14.344	46.92	45.18
2	3.75	20.000	51.94	52.03
3	0.57	20.000	38.54	38.54
4	6.00	16.000	42.25	44.08
5	6.00	24.000	34.14	34.35
6	1.50	24.000	37.48	36.84
7	6.9	20.000	39.65	38.45
8	1.50	16.000	40.74	41.72
9	3.75	20.000	52.99	52.03
10	3.75	20.000	52.19	52.03
11	3.75	20.000	51.93	52.03
12	3.75	20.000	50.09	52.03
13	3.75	25.656	34.30	34.85

experimental plan is given in Table 3. The independent variables were coded according to Eq. (2). The coded levels of independent variables and their corresponding uncoded values are given in Table 4.

4.2. Response surface methodology

After obtaining the responses for the experimental combinations in Tables 1 and 3, response surface methodology was used to describe the behaviour of the variables in the factorial space considered. Multiregression analysis was performed on the experimental data to obtain a suitable planar model that will describe the variation of the response with the change in independent variables. It was observed that linear models were insufficient to explain the behaviour of the response (results not shown) and hence, a second-order polynomial equation including any possible interaction terms was used. The polynomial used is of the form given below:

$$\hat{Y} = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_{ij} \quad (3)$$

The above equation was maximised by an iteration procedure to obtain the optimum combination of the variables. The software package MATLAB was used for this purpose. Another software package, design expert version 2.05 (Stat-Ease, Minneapolis, MN), was used to generate the experimental design as well to obtain the coefficients in Eq. (3). The same package was used for the generation of response surface contour plots for all the five variables considered.

Table 4
Coded levels of independent variables and their corresponding uncoded levels for self-induction studies of $CuCl_2$

Coded levels	Uncoded levels	
	Inducer concentration (mg per 100 ml)	Time of addition (h)
−1.414	0.57	14.34
−1	1.50	16.00
0	3.75	20.00
1	6.00	24.00
1.414	6.93	25.56

4.3. Optimisation of parameters for biocatalytic reduction of Cu ion

Factors affecting the biocatalytic reduction of Cu were optimised first. This was followed by the optimisation of the Cu ion reducing enzyme (isoenzymes) production to prove the necessity for self-induction for maximum reduction of the metal, Cu^{++}.

The parameters affecting the biocatalytic reduction are initial substrate concentration, time of reaction and initial pH of the reaction mixture. Experiments were conducted as per the central composite design plan given in Table 1. The experimental and predicted values of the response are given in Table 1. It is clear that all the variables being studied influenced the response significantly. The response varied from a value as low as 10.16 ppm to a value of 30.18 ppm. The experimental values are average of two independent runs within an error of ±5%. After the responses were obtained, multiregression analysis was performed on the data and the following quadratic response surface model was obtained.

$$\hat{Y} = 30.27 + 3.54X_1 - 1.01X_2 + 1.48X_3 - 2.62X_1^2 + 0.17X_2^2 - 4.28X_3^2 + 0.56X_1X_2 - 1.24X_1X_3 + 0.92X_2X_3 \quad (4)$$

Analysis of variance (ANOVA) for the selected model reveals that the model is highly significant as shown by F- and P-values. The value of $F_{\text{tabulated}}$ 99.5% confidence level is 5.97 and $F_{\text{statistic}}$ is 6.93 and hence the model accepted at 99.5% confidence level and the probability that the model will not explain the variations in the response is 0.004 (Table 5). The coefficient of determination r^2 is 0.92, which means that 8% of the total variations in response will not be accounted by the model.The coefficient of X_1 (initial substrate concentration) is higher than the other two variables signifying that the initial substrate concentration has

Table 5
ANOVA for the quadratic response surface model for Cu biocatalytic reduction studies (coefficient of determination, $r^2 = 0.92$)

Source	Sum of squares	Degrees of freedom	Mean square	F-value	Probability $> F$
Model	579.74	9	64.42	6.934	0.0041
Error	83.61	9	9.29		
Total	1006.35	19			

profound effect on the reduction of Cu. The coefficients of interaction terms X_1X_2 and X_2X_3 are low indicating that X_1 and X_2, and X_2 and X_3 can be varied independently without any effect on the reduction. The negative coefficient of X_2 and X_1X_3 explains that they are inversely proportional to the formation of Cu.

Eq. (4) was maximised using a MATLAB subroutine to obtain the optimum levels of variables. The optimum points as predicted by response surface methodology are initial substrate concentration of 40.48 mg per 100 ml, time of reaction was 28.4 h and the initial pH of the substrate mixture was 8.8. The model at the optimum point predicted maximum Cu ion reduction of 31.12 ppm and a reduction of 35.2 ppm was obtained experimentally under optimum conditions.

The isoresponse contour plots between variables are given in Figs. 1–3 . The figures show that higher amounts of Cu is formed at higher range of initial concentration of substrate, and the reaction time, while the initial pH of the substrate mixture at slightly basic range gave best results. The contour plots (from their centroid points) confirm the optimum levels of variables obtained.

4.4. Optimisation of parameters for self-induction of copper ion reducing enzymes

Similarly, the two factors that affect the production of Cu ion reducing enzymes are the amount of inducer ($CuCl_2$) and the time of addition of the inducer thereby making the enzyme specific towards Cu ion. Experiments were performed according to the experimental plan given in Table 3. The experimental values obtained at each combination and the predicted response is also given in Table 3. The experimental values are the average of two independent experiments within an error of ±5%. When the concentration of inducer was increased from 3.75 to 0.57 maintaining the time constant at 20 h, a change of about 27% was observed in Cu formation (run numbers 2 and 3). Similarly, when the time was varied from 20 to 25.6 h maintaining the inducer concentration constant at 3.75 mg per 100 ml a change in Cu formation by 34% was observed (run numbers 12 and 13).

Multiregression analysis was performed on the data to obtain a quadratic response surface model of the form given in Eq. (3). The second-order response surface model

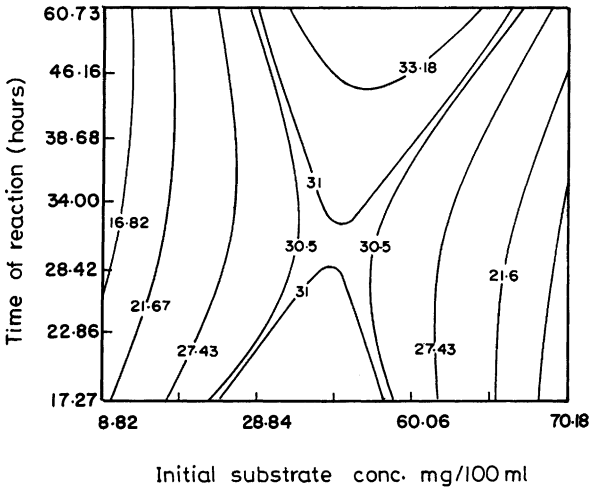

Fig. 2. Contour plot of formation of Cu at different levels of initial substrate concentration (mg per 100 ml) and time of reaction (h).

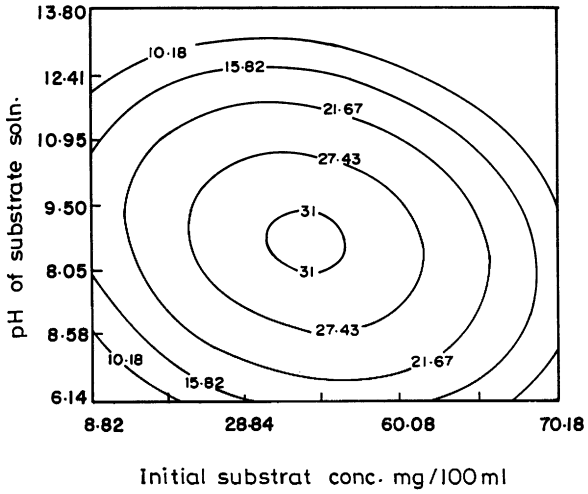

Fig. 1. Contour plot of formation of Cu at different levels of initial substrate concentration (mg per 100 ml) and pH of the substrate solution.

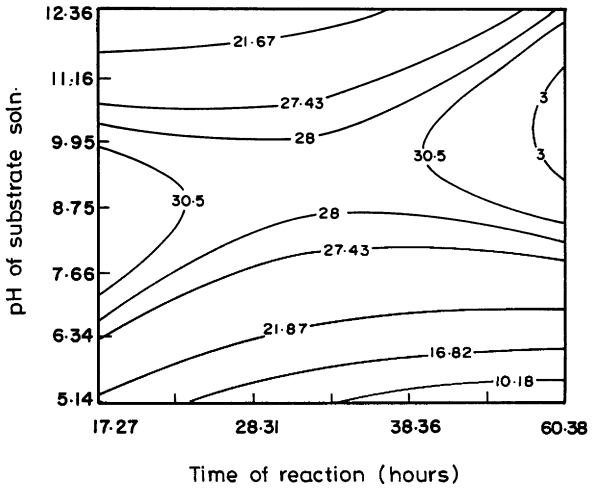

Fig. 3. Contour plot of formation of Cu at different levels of pH of the substrate solution and time of reaction (h).

Table 6
ANOVA for the quadratic response surface model for Cu induction studies (coefficient of determination, $r^2 = 0.97$)

Source	Sum of squares	Degrees of freedom	Mean square	F-value	Probability $> F$
Model	617.10	5	123.42	50.22	0.0001
Error	17.20	7	2.45		
Total	634.30	12			

obtained was

$$\hat{Y} = 52.03 - 0.03X_1 - 3.65X_2 - 6.77X_1^2 - 6.01X_2^2 - 1.21X_1X_2 \quad (5)$$

ANOVA (Table 6) of the regression model shows that the model is highly significant as evident from the low P-value (0.0001) and from the Fischer F-test. $F_{\text{statistic}} = 50.22$, while $F_{\text{tabulated}}$ for 99.5 confidence level is 9.52. Hence, the model is accepted at 99.5% confidence level. The coefficient of determination r^2 for this model is 0.97. The coefficient of determination is a measure of correlation between the experimental and predicted values and it gives the goodness of fit for the model. r^2-Value of 0.97 indicates that only 3% of the total variations are not explained in the model.Eq. (5) was maximised to obtain the optimum levels of variables. The optimum levels of variables were: concentration of inducer was 3.8 mg per 100 ml and time inducer addition was 18.8 h. Maximum Cu ion reduction of 53.79 ppm at optimum conditions was predicted by Eq. (5), while a maximum reduction of 54.82 ppm was obtained experimentally under optimum conditions.

The isoresponse contour plot between variables is given in Fig. 4. The contour plots (centre of the circle) confirm the optimum points obtained earlier.

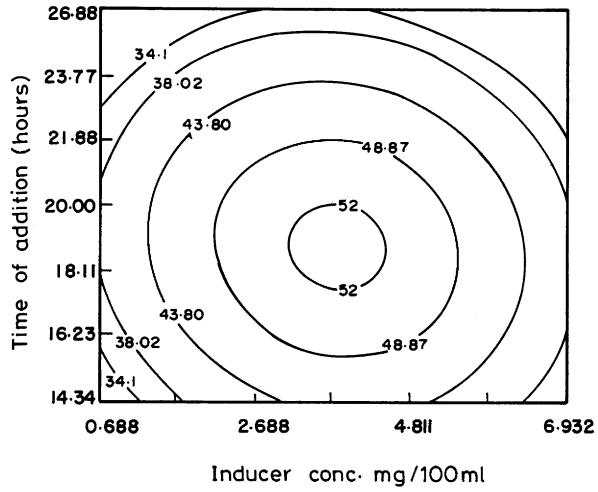

Fig. 4. Contour plot of formation of Cu at different levels of inducer concentration (mg per 100 ml) and the time of addition (h).

5. Conclusion

Reduction of $CuCl_2$ varied from a very low value to a value as high as 52.99 ppm after optimisation. The interaction between all the parameters were not so significant indicating that the variables can be altered independently. The initial substrate concentration has got a predominant effect on the reduction of $CuCl_2$. The optimal condition for $CuCl_2$ induction is 3.8 mg per 100 ml as inducer concentration and the time of addition was 18.8 h. While the optimal conditions for biocatalytic reduction are, initial substrate concentration was 40.48 mg per 100 ml, time of reaction was 28.4 h and the initial pH of the substrate mixture was 8.8.

The regression models obtained were able to explain the experimental result with high levels of significance. ANOVA demonstrates that the polynomial model is adequate for describing the relationship between response and the variables in both the metal studies.

Thus, using the response surface technique it was possible to examine the biocatalytic reduction process with a relatively small set of experiments. It was also possible to assess the influence of individual variables on metal biocatalytic reduction and study interaction among them using the response surface model. The contour plots and the regression equations were able to predict the contribution of optimum levels of variables. The optimum points were verified experimentally and were found to be real optima as the response obtained under optimum conditions was higher than any other combination of variables.

Acknowledgements

One of the authors C. Bennett Chandran acknowledges the financial assistance given by the CSIR, Government of India during his tenure as Senior Research Fellow.

References

[1] M. Hutton, Cadmium in European community, MARC Report No. 26, Minority and Assessment Research Center, Chelse College, London, 1982, 99 pp.
[2] B.S. Wilhemi, J.R. Duncan, Metal recovery by *Saccharomyces cerevisiae* biosorption columns, Biotechnol. Lett. 17 (9) (1995) 1007–1012.
[3] Y.E. Collins, G. Stotzky, Factors affecting the toxicity of heavy metals to microbes, in: T.J. Beveridge, R.J. Doyle (Eds.), Metal Ions and Bacteria, Wiley, New York, 1989, pp. 31–90.
[4] G.N. McDermott, W.A. Moore, M.A. Post, M.B. Ettinger, Effect of copper on aerobic biological sewage treatment, J. Water Pollut. Control 35 (1963) 227–241.
[5] A. Wiseman, Rapid and economical production of microsomal cytochrome P_{450} in yeast resuspended in 20% glucose medium: relationship to the biosynthesis of mitochondrial cytochromes, Biochem. Soc. Trans. 5 (5) (1977) 1520–1522.
[6] J. Salihan, M.A. Winkler, A. Wiseman, Optimisation of culture conditions for yield of cytochrome P_{450} in *Saccharomyces cerevisiae*, Biochem. Soc. Trans. 2 (1983) 401.

[7] D.J. King, M.R. Azari, A. Wiseman, Immobilization of cytochrome P_{450} enzyme in *Saccharomyces cerevisiae* methods, Enzymology 137 (1988) 675–686.
[8] G. Balagopal, Studies on degradative detoxification using induced biocatalysts and biosensing of xenobiotics in liquid and vapour phases, Ph.D. Thesis, Anna University, Chennai, 1995.
[9] T. Omura, R. Sato, The carbon monoxide binding pigments of liver microsomes. II. Solubilisation, purification and properties, J. Biol. Chem. 239 (1964) 2370–2379.
[10] R.A. Stowe, R.P. Mayer, Efficient screening of process variables, Ind. Eng. Chem. 58 (1966) 36–40.
[11] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building, Wiley, New York, 1978.
[12] R.G. Henika, A Background Information Manual for Use with Foremost-McKesson's RSM Computer Program, Foremost-McKesson, Inc., Dublin, CA, 1984.