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# Optimized preparation of insulin-lauryl sulfate complex loaded poly (lactide-co-glycolide) nanoparticles using response surface methodology

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Insulin-lauryl sulfate (INS-SDS) complex loaded poly (lactic acid-co-glycolic acid) (PLGA) nanoparticles were prepared by spontaneous emulsion solvent diffusion method. To improve the insulin entrapment efficiency (E.E), a five-level-two-factor central composite design and surface response methodology (RSM) was used to determine the optimum levels of PLGA/INS complex weight ratio and PVA/acetone volume ratio, two important variables during nanoparticles fabrication. A quadratic model to express the E.E as a function of the two studied factors was developed. Only 10 experimental runs were necessary and the obtained model was adequate ($P < 0.05$). By partial derivative resolution of regression model, the optimum weight ratio of PLGA/INS complex and volume ratio of PVA/acetone was determined as 25/1 and 10/1, respectively. This preparing condition resulted E.E of insulin as high as 91% during nanoparticles production. Validation of the model was accomplished by experiments carried out on optimized formulation conditions. The experimental results were in good agreement with those predicted by the model. The results indicated that RSM represents an effective and potential technique for formulation optimization.

## 1. Introduction

Efficient delivery of therapeutic protein and peptide drugs to the systemic circulation and targeting cell or organ locations has received considerable attention in pharmaceutical fields due to recent advances in biotechnology. Among the possible strategies for successful delivery, nanoparticulate carriers made from biodegradable polymers represent an exciting approach to increase the uptake and transport of these bioactive agents. Compared with other colloidal carriers, like sub-micron emulsions and liposomes, biodegradable polymeric nanoparticles offer a higher stability when they are in contact with biological fluids (Hans and Lowman 2003). The polymeric nature of nanoparticles protects the drug from adverse external conditions and controls its release (Sakuma et al. 1997; Calvo et al. 1996; Takeuchi et al. 2001).

Among the methods available for preparing nanoparticles, spontaneous emulsion solvent diffusion (SESD) method presents distinctive advantages (Niwa et al. 2001; Murakami et al. 1999), in that it enables the production of nanosacle particulates (100–300 nm) without extend energy input such as shearing, sonication or high temperatures. So this method is very suitable for loading bioactive protein and peptide drugs due to their instability. The spontaneous formation of nanoparticles is governed by the so-called Marangoni effect, under which interfacial turbulences were generated at the interface of the water miscible solvent and the aqueous phase and result from diffusion and surface tension variations (Quintanar-Guerrero et al. 1998). Nevertheless, the method available for preparing biodegradable nanoparticles is basically applicable to lipophilic drugs, which are soluble in an organic phase such as ethanol or acetone, to encapsulate water-soluble drugs still remains a challenge. Because of the weak affinity between hydrophilic drug substances and liposoluble polymers, the drug encapsulated always has a tendency to move from the organic phase to the outer aqueous phase, which induces drugs leaking from the precipitating matrix and led very low drug entrapment efficiency.

On account of expensive for most biotechnology substances, a high drug encapsulation in nanoparticles is indispensable for reducing manufacturing costs. In our previous studies, a hydrophobic ion pairing (HIP) strategy was employed to achieve this task (Cui et al. 2006). After complexed with amphiphilic surfactant, the model drug insulin displayed much more hydrophobic and an increase in partition coefficient. The enhanced hydrophobicity of the complex improves the affinity of insulin and hydrophobic PLGA and also permits insulin co-dissolving with the polymer in nonaqueous solvents during nanoparticle preparation. Thus satisfactory drug entrapment efficiency was expected to be achieved.

In the present study, insulin-sodium lauryl sulfate complex (INS-SDS) loaded PLGA nanoparticles were prepared by the spontaneous emulsion solvent diffusion (SESD) method. The weight ratio of PLGA/INS and volume ratio of PVA/acetone, two important factors affect-

ing nanoparticle preparation were selected to find the optimum levels to maximize drug entrapment efficiency. The optimization process was employed through the central composite design and response surface methodology, which allows estimating the main and interaction effects of the investigated factors simultaneously and predicting the best performance conditions with a minimum number of experiments.

## 2. Investigations, results and discussion

### *2.1. Nanoparticles preparation*

It is well known that water-soluble peptides cannot be directly dissolved in nonaqueous organic solvents. However, it is possible to alter their liposolubility by using specific ampholytic surfactants, such as sodium lauryl sulfate (SDS). When the nonaqueous organic solvents were introduced into the insulin (INS)-SDS complex, the hydrophilic head-group of SDS was directed towards the hydrophilic areas of insulin and the hydrophobic tail was directed towards the organic phase to provide the correct orientation. A schematic representation of this process can be found in Fig. 1. As shown in Table 1, most of insulin entrapment efficiency reached up to 80–90% in the experimental plan, such as that of Run1, Run2, Run4, Run7 and Run9. The results were attributed to the improved affinity of insulin and PLGA matrix due to the formation of HIP complex. In addition, all of formulation conditions enabled the production of small nanoparticles (100–300 nm) within the designed experimental domain and uniform narrow unimodal distribution.

**Table 1: Experimental design matrix with observed values of the objectives variables**

| Run no. | $x_1$ | $x_2$ | Size (nm) | PDI | Drug recovery (%) |
|---|---|---|---|---|---|
| 1 | 1 | −1 | 261 | 0.069 | 88.95 |
| 2 | 0 | $-\sqrt{,2}$ | 240 | 0.055 | 85.46 |
| 3 | 0 | $\sqrt{,2}$ | 257 | 0.029 | 45.74 |
| 4 | $\sqrt{,2}$ | 0 | 259 | 0.041 | 80.75 |
| 5 | 1 | 1 | 282 | 0.096 | 63.50 |
| 6 | −1 | 1 | 214 | 0.087 | 38.85 |
| 7 | 0 | 0 | 257 | 0.063 | 82.11 |
| 8 | $-\sqrt{,2}$ | 0 | 173 | 0.092 | 42.62 |
| 9 | 0 | 0 | 252 | 0.057 | 82.50 |
| 10 | −1 | −1 | 211 | 0.037 | 62.46 |

### *2.2. Experimental design*

On the basis of initial studies, the weight ratio of PLGA to INS and volume ratio of aqueous PVA to acetone, both of which affecting drug E.E significantly, were selected as independent variables. E.E was considered as the response variable. Optimization was performed with central composite design, which is a progression from the factorial designs and was introduced by Box and Wilson (1951). In general, CCD needs a total of $(2^k + 2k + n)$ runs where k is the number of studied factors. For two controlled variables in this study, the design is performed with $2^2$ points from the original factorial design, $2 \times 2$ axial points at a distance 1.414 from the center point, and two center points for estimating the experimental error. Thus a set of 10 experimental points were needed and the schematic representation of this design can be found in Fig. 2.

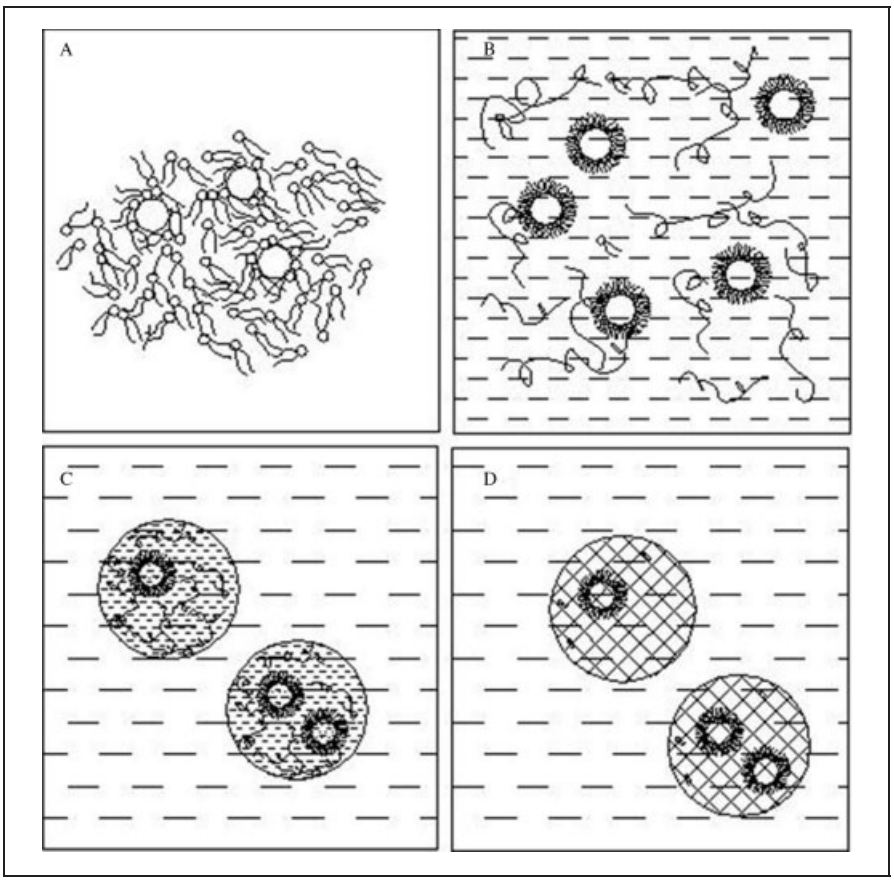


Fig. 1:
Schematic representation of the formation of nanoparticles loaded with INS-SDS complex. A: INS-SDS complex, B. Solubilization of insulin within nonaqueous solvent-containing polymers, C. Emulsion droplet-containing INS-SDS complex, D. Solidified nanoparticles in SESD

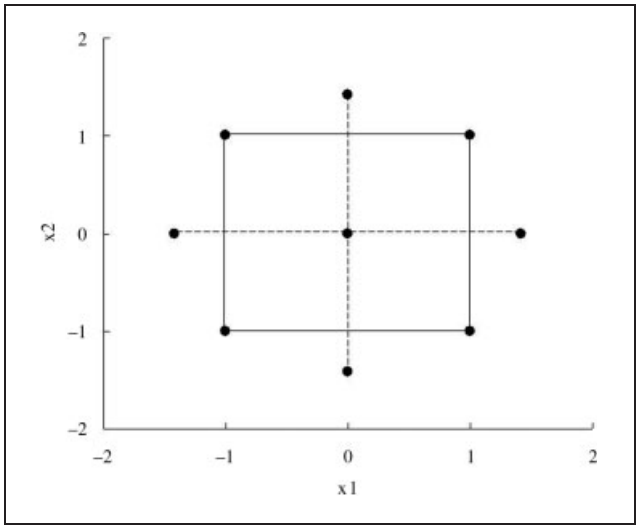


Fig. 2: Five-level-two-factor central composite design to investigate the effect of PLGA/INS weight ratio and PVA/acetone volume ratio on entrapment efficiency

### 2.3. Model fitting

The CCD experimental plan and results are presented in Table 1. Multiple regression analysis was employed to fit the second-order polynomial equation, expressing a mathematical relationship between the responses of E.E (Y) and independent variables.

$$Y = 82.30 + 13.13x_1 - 13.15x_2 - 10.36x_1^2 - 8.40x_2^2 - 0.46x_1x_2 \quad (1)$$

where $x_1$ and $x_2$ represent, respectively, coded values of PLGA/INS weight ratio and of PVA/acetone volume ratio.

The statistical significance of the model was verified by F-test, and the analysis of variance for the fitted second-order polynomial equation is summarized in Table 2. The calculated F value of the fitted model is much greater than $F_{0.05}(5,4)$, indicating that the model was significant at the probability level of $\alpha = 0.05$. On the other hand, the value of $P_{\text{lack of fit}} > F$ suggesting that the lack of fit was not significant and the developed model was adequate to represent the experimental data. In addition, the value of the determination coefficient ($R^2 = 0.9978$) indicates the goodness of fit of the model and only 0.22% of the variability in the responses was not explained by the model. An excellent value of the correlation coefficient (R), as high as 0.9911, suggested a good correlation the experimental and predicted values of E.E.

The significance of the investigated factors and their interactions, represented as coefficient of the model, was determined by Student's t test and p values. As listed in Table 3, the larger the magnitude of the t-value and smaller the P-value, the more significant is the corresponding coefficient. This meant that both the first-order effects of PLGA/INS weight ratio and of PVA/acetone volume ratio were highly significant ($P_{x1} < 0.005$, $P_{x2} < 0.005$). The quadratic main effects of the two factors ($P^2_{x1} = 0.007925$, $P^2_{x2} = 0.009771$) were less significant than their respective first-order effects. Whereas the interaction effect between them was not significant ($P_{x1x2} < 0.05$), indicating that both the two factors are the most important factors affecting the E.E during nanoparticles preparation.

The predicted values were calculated using the obtained regression model (Eq. (1)), which were in contrast to observed values and illustrated in Fig. 3. Moreover, the residual analysis showed that the developed model for E.E was well with a normal distribution (as seen in Fig. 4).

**Table 2: Analysis of variance (ANOVA) for the model regression representing drug recovery**

| Source | Sum of squares | DF | Mean square | F-value | $F_{0.05}$ |
|---|---|---|---|---|---|
| Model | 3343.54 | 5 | 668.71 | 359.52 | 6.26 |
| Residue | 7.45 | 4 | 1.86 | | |
| Lack of fit | 7.37 | 3 | 2.46 | 30.75 | 251.7 |
| Pure error | 0.08 | 1 | 0.08 | | |
| Total | 3350.99 | 9 | | | |

**Table 3: Results of multiple regression analysis and parameter estimation**

| Term | Coefficients | Std error | t-value | p-value |
|---|---|---|---|---|
| Intercept | 82.3050 | 0.195000 | 422.077 | 0.001508 |
| $x_1$ | 13.1330 | 0.097500 | 134.697 | 0.004726 |
| $x_1^2$ | −10.3606 | 0.128980 | −80.327 | 0.007925 |
| $x_2$ | −13.1541 | 0.097500 | −134.914 | 0.004719 |
| $x_2^2$ | −8.4031 | 0.128980 | −65.150 | 0.009771 |
| $x_1x_2$ | −0.4600 | 0.137886 | −3.336 | 0.185402 |

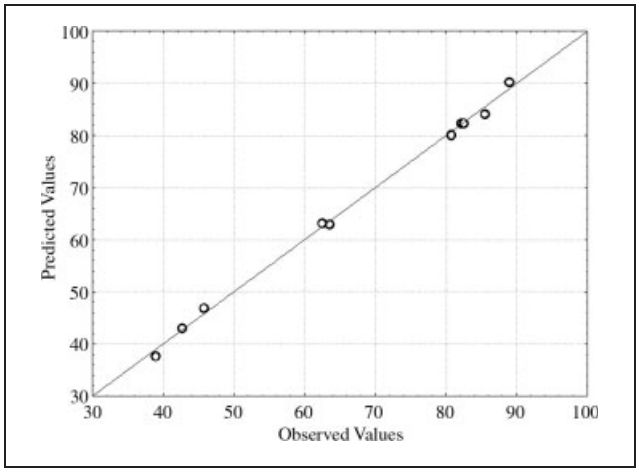


Fig. 3: Predicted versus observed values plot for entrapment efficiency

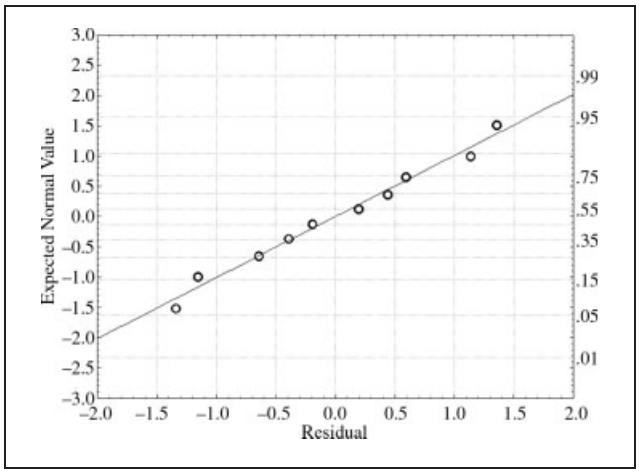


Fig. 4: Predicted versus observed values plot for entrapment efficiency

### 2.4. Analysis of the response surfaces

Response surfaces can be illustrated as three-dimensional contour curves by presenting the response in function of two variables and holding the others constant. Thus, it is possible to visualize and understand both the main and the interaction effects of these two factors within the experimental domain. Moreover, the response values for the variables can be predicted from these plots.

Figure 5 depicts the response surface and contour plots showing the effects of PLGA/INS weight ratio and PVA/acetone volume ratio on drug recovery during nanoparticle preparation. It is evident that the E.E steadily increased with increasing the weight ratio of PLGA/INS up to 26.5/1 (w/w), but decreased slightly beyond this ratio at low PVA/acetone volume ratio. The results of the study were concordant with those of Lamprecht et al. (1999) who showed that an increase in weight ratio of polymer to drug, i.e. increase in polymer concentration, would lead to an increase in the drug payload because of the enhancement of emulsion droplets viscosity and consequently detention of complex distributing into the external aqueous phase. While at high PVA/acetone volume ratio, the increase in the drug E.E was insignificant as the PLGA/INS weight ratio was increased. In the case of higher volume ratio of PVA to acetone, the drug E.E of PLGA nanoparticles was mainly dependent on the diffusion speed of solvent phase into external aqueous phase, consequently affecting the polymer precipitation rate. Larger volumes of dispersion medium provide enough space for more oil/water interface formation, thus promoting particle precipitation as quickly as possible. On the other hand, when the polymer amount was not high enough, as low as 10/1 for the weight ratio of PLGA/INS, the E.E decreased as PVA/acetone volume ratio was increased in the range of 12–30/1 (v/v). The results indicated that under this ratio, E.E was mainly determined by polymer concentration and the effect of external volume was not significant.

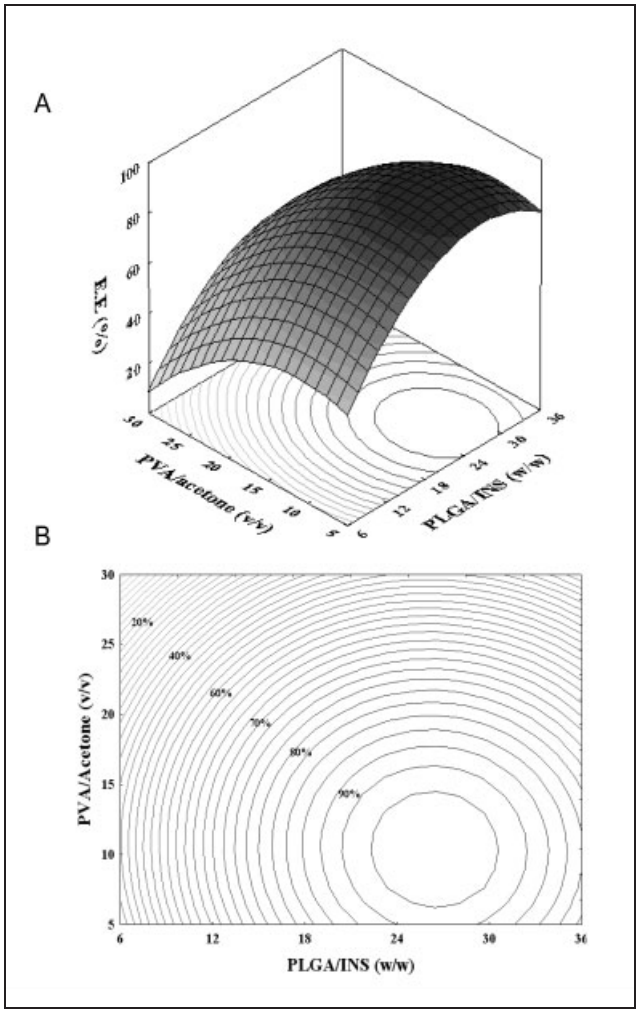


Fig. 5: Response surface (A) and contour plot (B) of PLGA/INS weight ratio and PVA/acetone volume ratio on entrapment efficiency

### 2.5. Attaining optimum conditions

The optimum point for nanoparticle preparation was obtained by graphic method and partial derivative resolution of Eq. (1). The most efficient condition for this preparation would use the lowest amount of polymer and external phase volume to achieve an acceptable E.E ($> 85\%$). Figure 5 (B) suggests what seems to be a reasonable zone of acceptable PLGA/INS weight ratio (20–30) and PVA/acetone volume ratio (10–15) should be employed for the practical near-optimum preparation. Under the recommended conditions, 85–90% of E.E was expected to be achieved.

The adequacy of the model predicted here was examined by additional independent experiments at the suggested optimal preparation conditions. Triple experiments were carried out under optimum experimental conditions with PLGA/INS weight ratio of 25/1 and PVA/acetone volume ratio of 10/1. Compared with predicted maximum E.E (92.2%) which was obtained from Eq. (1), the actual average value of E.E (91.0%) was obtained. The good correlation between these two results showed that the generated model adequately predicted the percent drug recovery. Thus, the optimised nanoparticle preparation for drug encapsulation improvement was successfully developed by RSM.

## 3. Experimental

### 3.1. Materials

Bovine insulin (29 IU/mg) was purchased from SIGMA Chemical Co., Ltd., USA. Sodium lauryl sulfate (SDS) was supplied by Nacalai Tesque Inc, Japan. PLGA 7520 (75 : 25, Av.$M_W$ 20000) was obtained from the Wako Pure Chemical Ind. Ltd., Japan). Poly(vinyl alcohol) (PVA-403) was supplied by Kuraray Co., Ltd., Japan. All other reagents were of analytical grade.

### 3.2. Preparation of nanoparticles

PLGA nanoparticles were prepared according to a modified spontaneous emulsion solvent diffusion method (Kawashima et al. 1999). Briefly, PLGA, insulin and SDS were co-dissolved completely in the mixture of acetone (1 ml) and slight 0.01 M hydrochloric acid. The resultant polymer–drug solution was poured into 10 ml PVA aqueous solution (1.0%, w/v). The emulsified system was stirred at 400 rpm for 1 h using a propeller type agitator with three blades (Heidon 600G, Shinto Scientific Co., Ltd., Japan). The entire dispersed system was then subjected to centrifugation (40,000 g for 15 min; Kubota 7780, Kubota Co., Ltd., Japan). The nanoparticles were washed two times with distilled water to remove free drug and PVA before freeze-drying.

### 3.3. Determination of drug entrapment efficiency

The freeze-dried nanoparticles were dissolved in acetonitrile, to which 0.01 M hydrochloric acid was added to preferentially precipitate the polymer. The drug content in the supernatant after centrifugation (40,000 g for 15 min, Kubota 7780, Kubota Co., Ltd., Japan) was measured spectrophotometrically at 214 nm by means of an HPLC method (LC-20AD pump, SPD-20A detector, CTO-910AS column oven, Crestpak C18S column, Shimadzu Co., Ltd., Japan). Drug entrapment efficiency (E.E) was expressed according to Eq. (2).

$$\text{E.E}\,(\%) = \frac{\text{Mass of drug in NPs}}{\text{Mass of drug used in form}} \times 100 \qquad (2)$$

**Table 4: Independent variables and their levels investigated in the central composite design**

| Factor | Coded level | | | | |
|---|---|---|---|---|---|
| | $-\sqrt{,2}$ | −1 | 0 | 1 | $\sqrt{,2}$ |
| $X_1$ | 6 | 10 | 20 | 30 | 34 |
| $X_2$ | 5 | 8.7 | 17.5 | 26.3 | 30 |

* $X_1$ is the weight ratio of PLGA to insulin; $X_2$ is the volume ratio of aqueous PVA solution to acetone.

### *3.4. Particle size analysis*

The dried nanoparticle samples were suspended in distilled water and were sonicated before measurement. The obtained homogeneous suspensions were subjected to examination. The particle size distribution, expressed as mean diameter and polydispersity index, was determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano-ZS90 (Malvern Instruments, UK). Each measurement was performed in triplicate.

### *3.5. Experimental design*

A full factorial central composite design (CCD) was employed to identify the optimum level of the major variables previously selected by simple factor experiment. The experimental design with the coded ($x_i$) and actual ($X_i$) levels of independent variables are shown in Table 4. When developing the regression model, the test factors were coded in terms with the following Eq. (3)

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3, \ldots, k \tag{3}$$

where $x_i$ is the dimensionless value of independent variable, $X_i$ is the real value of independent variable, $X_0$ is the real value of the independent variable at the center point, and $X_i$ is the step change value.

### *3.6. Statistical analysiss*

The analysis of experimental data was carried out using a response surface methodology (RSM). A quadratic model as expressed as following equation was used to correlate the response variable (Y) to the independent variables (X).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \tag{4}$$

where, $\beta_0$, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are the regression coefficients, for intercept, linear, quadratic and interaction terms, respectively. The optimum levels of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour and surface plots. The statistical analyses were carried out using multiple regressions and ANOVA with the program STATISTICA (Version 6.0, StatSoft. Inc., USA).

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