

# Application of statistical method to evaluate immobilization variables of trypsin entrapped with sol-gel method

Shi-Yong Lu, Jun-Qing Qian\*, Zhang-Gui Wu, Wei-Dong Ye, Guo-Feng Wu, Yi-Bin Pan, Kai-Yuan Zhang

Received: 06 February 2009 / Received in revised form: 08 March 2009, Accepted: 11 May 2009, Published online: 16 May 2009  
© Sevas Educational Society 2008

## Abstract

Bioimmobilization with sol-gel method is a complicate system, involving many parameters that can affect sol-gel structure and immobilized biomolecules activity. In this study, trypsin, a model enzyme, was entrapped into sol-gel glasses produced from tetraethoxysilane (TEOS) and water to study the relationships between the immobilization parameters, namely ratio of water to TEOS, volume of poly (ethylene glycol) (PEG), concentration of buffer solution, pH of buffer solution by using statistical methods. Two regression equations and analysis of variance (ANOVA) were obtained with the entrapped enzyme activity and enzyme loading yield as the response values, respectively. Statistical calculations showed that the effects of the four variables on the entrapped enzyme activity and enzyme loading yield were different. Entrapped trypsin activity (35.6%) and trypsin loading yield (68.3%) were obtained by using optimized immobilization conditions. The results of this study may help to manipulate the properties of sol-gel to get high immobilization efficiency.

**Keywords:** Sol-Gel, Trypsin, Enzyme, Entrapment, Response surface methodology

## Introduction

The application of sol-gel glass to entrap active biomolecules is now well documented and widely demonstrated (Gill 2001; Jin and Brennan 2002; Li et al. 2004). Sol-gel glass offers a better way to immobilize biomolecules within its porous matrix compared to other typical immobilization techniques, such as covalent attachment,

physical adsorption, cross-linking and encapsulation in polymer and inorganic matrixes (Bhatia 2000). This is due to mild sol-gel processing conditions, possibility to design the material structure and property and possibility of tailoring for specific requirements (Li et al. 2004; Cichna-Markl 2006; Gupta and Chaudhury 2007). However, biomolecules immobilized by sol-gel method is a complicate system involving many parameters. Sol-gel process begins with hydrolysis of a precursor to form an aqueous sol. After hydrolysis, the aqueous sol is mixed with a buffered aqueous solution containing the biomolecules and additives, resulting in gelation of the sol and entrapment of the biomolecules. After gelation, the wet silica network needs to age a period of days to promote further condensation and strengthen the network (Livage 1997; Collinson 2002; Siouffi 2003). There are many parameters involved in the sol-gel process that can affect the sol-gel matrix and the native activity of biomolecules (Gupta and Chaudhury 2007). Therefore, immobilization parameters should be studied and optimized to preserve the native activity of biomolecules and to achieve high immobilization efficiency.

Different strategies can be used for the optimization of immobilization. The classical method of experimental optimization involves changing one variable at a time keeping the others constant. The method, however, requires a large number of experiments to illustrate the effect of individual factors. In addition, this does not consider the effect of interactions of various parameters (Myers and Montgomery 1995). Use of statistical methods helps to select important parameters from a large number of factors and the interactions between important variables can be understood easily. Response surface methodology (RSM) is a statistical and mathematical method that involves main and interactions effects to improve optimal process settings. RSM has been widely used to evaluate the interactions among various process parameters. RSM was applied to optimize process parameters for various processes in biotechnology (Gonzalez-Saiz and Pizarro 2001; Melo et al. 2005; Mundra et al. 2007; Potumarthi et al. 2008; Prakash et al. 2008; Senthilkumar et al. 2008; Kumar and Satyanarayana 2007).

In the present study, trypsin, a model enzyme, was immobilized by sol-gel method to illustrate the relationships between the variables of the sol-gel process. Although several studies have been published

---

Shi-Yong Lu, Jun-Qing Qian\*

Department of Biochemical Engineering, Zhejiang University of Technology, Zhejiang, China

Zhang-Gui Wu, Wei-Dong Ye, Guo-Feng Wu, Yi-Bin Pan, Kai-Yuan Zhang

ZheJiang Medicine CO., LTD., Zhejiang, China

\*Tel.: 086 0571 88320317; Fax: 086 0571 88320913;  
E-mail: qjq@zjut.edu.cn

with trypsin immobilized in sol-gel glass matrix as catalyst or biosensor (Shtelzer et al. 1992; Park and Clark 2002; Sakai-Kato et al. 2002; Sakai-Kato et al.2003; Kato et al.2005) , the studies of relationships between the variables of the sol-gel process have never been reported. The purpose of this paper is to better understand relationships between the variables of sol-gel process and the response and to obtain the optimum conditions by using RSM.

## Materials and methods

### Materials

Trypsin was a commercial enzyme, obtained from Amresco, tetraethoxysilane (TEOS), N-alpha-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA, 99%) and Poly (ethylene glycol) 6000 (PEG 6000) were purchased from Acros Organics. All other chemicals were purchased from standard sources with reagent grade.

### Preparation of sol-gel entrapped trypsin

Preparation of sol-gel entrapped trypsin was performed according to the following procedures. Briefly, TEOS (0.1ml) was mixed with appropriate volume of PEG 6000 (200 mg/l), appropriate amount distilled water, and H<sub>3</sub>PO<sub>4</sub> (2μl, 1M) and then sonicated for 20 min to produce a homogeneous solution. The homogeneous solution was then mixed with appropriate buffered trypsin solution and allowed to gel at room temperature for 5 min. Then PBS (0.2ml) was added to the gel. The gel was then stored at 4°C until tested.

### Determination of the amount of entrapped trypsin

Entrapped trypsin sample was washed with PBS (1.5ml), and the washer was collected and mixed with Coomassie Blue dye solution (1.5ml), and determined in absorbance at 595 nm (Dulay et al. 2005). The amount of the entrapped trypsin is got as follows:

$$W_e = W_t - W_u$$

Where  $W_e$  is the amount of entrapped trypsin;  $W_t$  is the total trypsin involved in the process;  $W_u$  is the amount of unentrapped trypsin, which is got from the absorption at 595nm.

### Measurement of trypsin activity

Entrapped trypsin sample was washed with PBS (1.5ml) to remove the unentrapped free trypsin. The enzymatic activity of free and entrapped trypsin were determined in Tris-Hcl buffer solution (0.8ml, 50mM , pH 7.8) containing distilled water (2 ml), and using BAPNA (0.3 ml, 10 mM) as the substrate. The mixtures were incubated at 37°C for 5 min and 20 min, respectively. After incubation, acetic acid (0.5ml, 33%) was added to stop the reaction. The mixtures were filtered and then determined in absorbance at 410nm.

### Entrapment efficiency

The efficiency of entrapped trypsin was evaluated in terms of relative activity (%) and trypsin loading yield (%) as follows (Chang et al. 2008):

$$\text{Relative activity (\%)} = \frac{\text{Activity of entrapped trypsin}}{\text{Activity of equal amount of free trypsin}} \times 100\%$$

$$\text{Trypsin loading yield (\%)} = \frac{\text{Amount of entrapped trypsin}}{\text{Amount of trypsin introduced}} \times 100\%$$

### Experimental design and optimization by RSM

To find out the optimum levels of the most effective variables and to study their relationships, RSM using central composite design (CCD) was applied. Based on the one-factor experimental results, four critical variables selected were ratio of water to TEOS, volume of the PEG 6000 (200mg/ml), concentration and pH of buffer solution. Every variable at five coded levels (-α, -1, 0, 1, α) and non-coded values was shown in Table 1. Because in this study four variables were considered, so  $k = 4$ ,  $\alpha = [2^k]^{1/4} = 2$  [15].

**Table 1.** Coded and non-coded values of the experimental variables

Variables	Symbols	-α	-1	0	1	α
Ratio of water to EOS	A	37.14	43.32	49.5	55.7	61.89
(mol: mol) Volume of PEG 6000 (200g/l) (ml)	B	0.06	0.08	0.10	0.12	0.14
Concentration of buffer solution (HEPES) (mM)	C	250	300	350	400	450
pH of buffer solution	D	6.90	7.00	7.10	7.20	7.30

According to this design, the total number of experimental runs was  $2^k + 2k + x_0$ , where  $k$  is the number of variables and  $x_0$  is the number of repetitions of the experiments at the center point. Thus, for this design, a total of 30 experiments were performed according to the central composite design given in Table 2.

**Table 2.** Response surface central composite design and experiments

Run order	A	B	C	D	Trypsin relative activity measured (%)	Trypsin loading yield (%)
1	1	-1	-1	-1	33.7	32.6
2	1	1	1	-1	34.2	53.9
3	0	0	0	0	33.6	59.9
4	-1	-1	1	-1	34.1	56.9
5	1	-1	1	1	29.9	56.5
6	-1	1	1	1	33.2	67.8
7	0	0	0	0	37.6	54.8
8	1	1	-1	1	32.5	67.7
9	-1	-1	-1	1	43.1	55.0
10	-1	1	-1	-1	37.7	63.0
11	1	1	1	1	32.1	63.2
12	-1	1	-1	1	40.6	51.3
13	-1	-1	1	1	40.2	47.4
14	1	1	-1	-1	38.0	41.4
15	-1	-1	-1	-1	34.1	43.8
16	1	-1	-1	1	40.6	36.5
17	-1	1	1	-1	41.6	51.7
18	1	-1	1	-1	18.7	35.4
19	0	0	0	0	36.1	54.9
20	0	0	0	0	37.6	53.5
21	0	0	0	-2	21.4	50.5
22	0	2	0	0	30.0	67.3
23	0	0	0	0	29.6	56.1
24	0	-2	0	0	25.8	42.1
25	0	0	0	2	31.6	64.0
26	-2	0	0	0	32.2	63.0
27	2	0	0	0	19.7	42.1
28	0	0	0	0	33.2	60.4
29	0	0	-2	0	30.2	61.0
30	0	0	2	0	34.2	60.1

The relationship between the variables and the response was calculated by the second order polynomial equation.

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

Where  $Y$  is the predicted response,  $X_i, X_i^2, X_j$  are variables in coded values;  $\beta_0$  is the constant;  $\beta_i$  is linear effect;  $\beta_{ii}$  is squared effect and  $\beta_{ij}$  is interaction effect.

The analysis of results was performed with statistical and graphical analysis software (Design Expert, Version 7.1.3). The software was used for regression analysis of the data obtained and to estimate the coefficient of regression equation.

## Results and discussion

### Interpretation of regression analysis

By applying multiple regression analysis to the experimental data, the experimental results of the central composite design were fitted with a second-order full polynomial equation. The empirical relationship between entrapped trypsin relative activity ( $Y$ ) and the four variables in coded units is given below:

$$Y = 34.78 - 2.91A + 1.00B - 1.18C + 1.69D + 0.77AB - 1.47AC + 0.056AD + 1.31BC - 2.89BD - 0.41CD - 1.02A^2 - 0.53B^2 + 0.54C^2 - 0.88D^2$$

Where  $Y$  is entrapped trypsin relative activity;  $A$  is ratio of water to TEOS;  $B$  is volume of PEG 6000 (200mg/ml);  $C$  is concentration of buffer solution (HEPES);  $D$  is pH of buffer solution.

The analysis of variance (ANOVA) summary for the model of entrapped trypsin relative activity is shown in Table 3.

**Table 3.** ANOVA analysis for the model of entrapped trypsin relative activity

Source	DF	F-value	Prob>F	
Model	14	3.61	0.0133	significant
significant				
A	1	17.07	0.0012	significant
B	1	2.00	0.1812	
C	1	2.80	0.1182	
D	1	5.73	0.0324	significant
AB	1	0.79	0.3894	
AC	1	2.89	0.1126	
AD	1	0.004246	0.9490	
BC	1	2.29	0.1542	
BD	1	11.24	0.0052	significant
CD	1	0.22	0.6457	
A <sup>2</sup>	1	2.39	0.1459	
B <sup>2</sup>	1	0.65	0.4340	
C <sup>2</sup>	1	0.68	0.4253	
D <sup>2</sup>	1	1.79	0.2038	
Residual	13			
Lack of Fit	10	3.42	0.1701	Not significant
Pure Error	3			
Cor Total	29			

$R^2$ , 0.7953; adjusted  $R^2$ , 0.5749; CV, 10.38%; Adeq Precision, 8.7390

The model F-value of 3.61 implied the model was significant. Values of “Prob>F” less than 0.0500 indicated model terms were significant,  $A, D, BD$  were significant model terms. The “Lack of Fit F-value” of 3.42 implied the Lack of Fit was not significant relative to the pure error. Non-significant lack of fit is good. The coefficient of determination ( $R^2$ ) calculated was 0.7953, indicating that the

model could explain 79.53% of the variability. A relatively lower value of the coefficient of variation (CV = 10.38%) indicated a better precision and reliability of the experiments. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 8.7390 obtained in this model indicated an adequate signal. In addition, Tukey Test was applied to test the ANOVA, and the results showed that different levels of each variable had no significant difference.

The empirical relationship between trypsin loading yield ( $Y$ ) and the four variables in coded units is given below:

$$Y = 56.6 - 3.81A + 6.1B + 1.65C + 3.90D - 2.16AB + 1.26AC + 3.41AD - 0.94BC + 0.83BD + 0.46CD - 1.81A^2 - 1.27B^2 + 0.19C^2 - 0.63D^2$$

Where  $Y$  is trypsin loading yield;  $A$  is ratio of water to TEOS;  $B$  is volume of PEG 6000 (200mg/ml);  $C$  is concentration of buffer solution (HEPES);  $D$  is pH of buffer solution.

The ANOVA summary for the model of trypsin loading yield is shown in Table 4.

**Table 4.** ANOVA analysis for the model of trypsin loading yield

Source	DF	F-value	Prob>F	
Model	14	11.35	<0.0001	significant
significant				
A	1	26.2	0.0002	significant
B	1	66.98	<0.0001	significant
C	1	4.93	0.0447	significant
D	1	27.48	0.0002	significant
AB	1	5.59	0.0343	significant
AC	1	1.90	0.1917	
AD	1	13.94	0.0025	significant
BC	1	1.07	0.3197	
BD	1	0.83	0.3788	
CD	1	0.25	0.6253	
A <sup>2</sup>	1	6.71	0.0224	significant
B <sup>2</sup>	1	3.31	0.0919	
C <sup>2</sup>	1	0.078	0.7842	
D <sup>2</sup>	1	0.82	0.3822	
Residual	13			
Lack of Fit	10	1.94	0.3199	Not significant
Pure Error	3			
Cor Total	29			

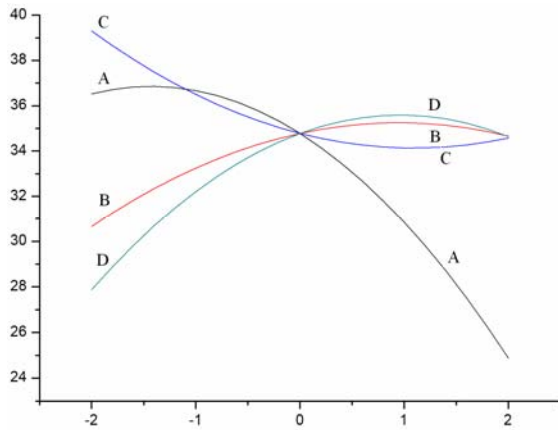
$R^2$ , 0.9244; adjusted  $R^2$ , 0.8430; CV, 6.78%; Adeq Precision, 12.5690

The model F-value of 11.35 implied the model was significant. Values of “Prob>F” less than 0.0500 indicated model terms were significant,  $A, B, C, D, AB, AD, A^2$  were significant model terms. The “Lack of Fit F-value” of 1.94 implied the Lack of Fit was not significant relative to the pure error. Non-significant lack of fit is good. The coefficient of determination ( $R^2 = 0.9244$ ) indicated that the model could explain 92.44% of the total variations. A relatively lower value of the coefficient of variation (CV = 6.78%) indicated a better precision and reliability of the experiments. Adeq Precision (12.5690) indicated an adequate signal. In addition, Tukey Test was applied to test the ANOVA, and the results showed that different levels of each variable had no significant difference.

### Interpretation of perturbation graph and contour plots

The regression model can be represented in the form of contour plots and perturbation graph. Perturbation graph shows the response

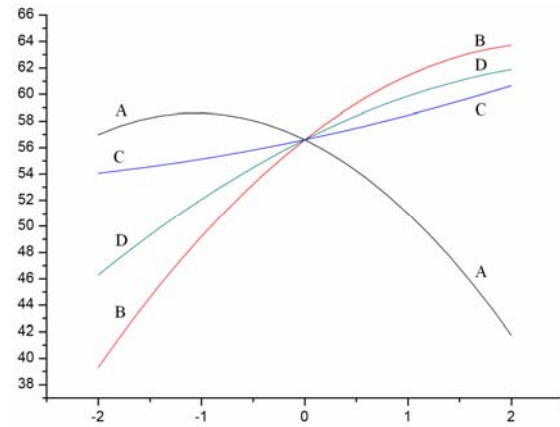
of each independent variable against other variables kept at their respective '0' levels.



**Fig. 1.** Perturbation graph showing the effect of each of the independent variables on entrapped trypsin activity while keeping other variables at their respective midpoint

The perturbation graphs (Fig. 1 and Fig. 2) show several trends. Firstly, when ratio of water to TEOS (A) was about -1, the maximums of entrapped trypsin activity and trypsin loading yield were achieved. Secondly, entrapped trypsin activity and trypsin loading yield increased when volume of PEG 6000 (B) and pH of buffer solution (D) increased. Thirdly, the effect of concentration of buffer solution (C) on entrapped trypsin relative activity was opposite to trypsin loading yield.

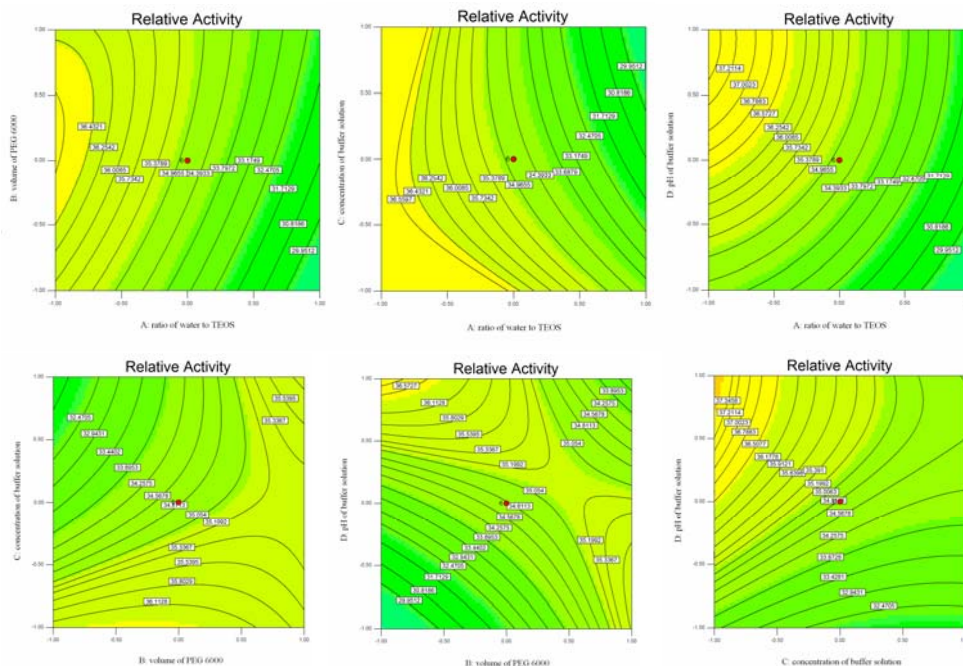
The perturbation graphs also show that when volume of PEG 6000 (B) and pH of buffer solution (D) were in relatively high level



**Fig. 2.** Perturbation graph showing the effect of each of the independent variables on trypsin loading yield while keeping other variables at their respective midpoint

while ratio of water to TEOS (A) was in relatively low level, the higher level of entrapped trypsin activity and trypsin loading yield were achieved. These findings are consistent with the previous report by Besanger et al., 2006.

The contour plots described by the regression models were drawn to show the effects of the four variables, and combined effects of each independent variable on entrapped trypsin relative activity and trypsin loading yield.

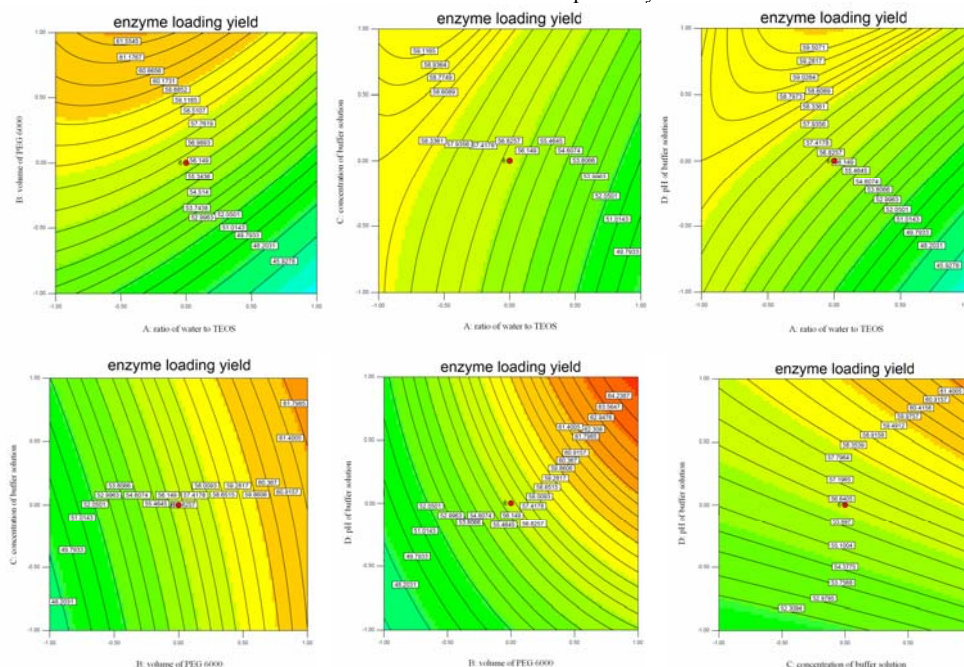


**Fig. 3.** (a-f) Contour plots of entrapped trypsin relative activity: the effect of two variables while the other two are held at 0 levels

Fig.3.a-c and Fig.4.a-c show ratio of water to TEOS (*A*) had significant effect on the entrapped trypsin activity and trypsin loading yield, respectively. When ratio of water to TEOS (*A*) decreased, the activity of entrapped trypsin and trypsin loading yield increased. This situation may be due to ratio of water to TEOS (*A*) which is the main factor affects the polymer structure, including the pore size and the network, etc. The enzyme activity responds to its microenvironment and the interactions to the solid matrix (Vidinha et al.2006). The protein-matrix interaction is of critical importance because it affects not only the protein conformation, but also the activity and stability ( Sotiropoulou and Chaniotakis 2005).

effect on the trypsin loading yield. When pH of buffer solution (*D*) increased, the trypsin loading yield increased. Ratio of water to TEOS (*A*) and pH of buffer solution (*D*) had an interaction effect on the trypsin loading yield. This could be attributed to the fact that enzyme entrapment and subsequent processing must be biocompatible in the biological pH range, pH has an impact on the polymer structure and enzyme activity ( Jin and Brennan 2002).

The contour plots also show that the effects of the four variables on the entrapped trypsin activity and trypsin loading yield were different, the order of effects were  $A > D > C > B$  and  $B > D > A > C$ , respectively.



**Fig.4. (a-f)** Contour plots of trypsin loading yield: the effect of two variables while the other two are held at 0 levels

Fig.3.a, Fig.3.d and Fig.3.e show volume of the PEG 6000 (*B*) having no significant effect on the entrapped trypsin activity, and there is some interaction effect between volume of PEG 6000 (*B*) and pH of buffer solution (*D*). The entrapped trypsin activity increased when volume of PEG 6000 (*B*) and pH of buffer solution (*D*) increased. Fig.4.a, Fig.4.d and Fig.4.e show volume of PEG 6000 (*B*) having a significant effect on the trypsin loading yield. Increasing volume of PEG 6000 (*B*) resulted in the trypsin loading yield increasing. Ratio of water to TEOS (*A*) and volume of PEG 6000 (*B*) had an interaction effect on the trypsin loading yield which was due to PEG 6000 used as structure modifier (Gill 2001). So PEG 6000 affects the trypsin loading yield, and has little effect on the entrapped trypsin activity.

Fig.3.b, Fig.3.d and Fig.3.f show concentration of buffer solution (*C*) had no significant effect on the entrapped trypsin activity. Fig.4.b, Fig.4.d and Fig.4.f show that concentration of buffer solution (*C*) had less significant effect on the trypsin loading yield than the effects of other three variables. This is due to the concentration of buffer solution that is used to stabilize the pH of the system.

Fig.3.c, Fig.3.e and Fig.3.f show pH of buffer solution (*D*) had a significant effect on the entrapped trypsin activity. The activity of the entrapped trypsin increased with an increase in pH. Fig.4.c, Fig.4.e, and Fig.4.f show pH of buffer solution (*D*) had a significant

As shown in the perturbation graph and contour plots, when *A* was about -1 level, *B*, *D* was about 1-2 level, *C* was about 0 level, the optimized immobilization conditions were obtained. The experimental models were tested in the optimized immobilization conditions as above. The experiments were performed six times. The entrapped trypsin activity (35.6%, mean value of six experiments) and the trypsin loading yield (68.3%, mean value of six experiments) were close to the predicted response, which proved the validity of the models.

The study of entrapping trypsin in sol gel glass matrix will be useful to manipulate the properties of sol-gel glass when another enzyme besides trypsin was entrapped with the same method, which will provide a method having potential in both affinity chromatography and bioreactor applications.

## Conclusions

The present study was aimed to study the relationships and optimization of the immobilization parameters to achieve high entrapment efficiency. With the help of response surface methodology, the models of entrapped enzyme activity and enzyme loading were established with ratio of water to TEOS, volume of PEG 6000 (200mg/ml), concentration of buffer solution and pH of buffer solution as four variables. The results of this study may help

to manipulate the properties of sol-gel to get high immobilization efficiency when enzymes were entrapped in sol gel glass matrix.

### Acknowledgements

Authors are grateful to ZheJiang Medicine CO., LTD for providing financial assistance during the course of this investigation.

### References

- Besanger TR, Hodgson RJ, Guillon D et al (2006) Monolithic membrane-receptor columns: optimization of column performance for frontal affinity chromatography/mass spectrometry applications. *Anal Chim Acta* 561:107-118
- Bhatia RB, Jeffrey BC, Gupta AK et al (2000) Aqueous Sol-Gel Process for Protein Encapsulation. *Chem Mater* 12:2434-2441
- Chang SW, Shaw JF, Yang KH et al (2008) Studies of optimum conditions for covalent immobilization of *Candida rugosa* lipase on poly( $\gamma$ -glutamic acid) by RSM. *Bioresour Technol* 99:2800-2805
- Cichna-Markl M (2006) Selective sample preparation with bioaffinity columns prepared by the sol-gel method. *J Chromatogr A* 1124:167-180
- Collinson MM (2002) Recent trends in analytical applications of organically modified silicate materials. *Trends Anal Chem* 21:30-38
- Dulay MT, Baca QJ, Zare RN (2005) Enhanced proteolytic activity of covalently bound enzyme in photopolymerized sol gel. *Anal Chem* 77, 4604-4610
- Gill I (2001) Bio-doped nanocomposite polymers: sol-gel bioencapsulates. *Chem Mater* 13:3404-3421
- Gonzalez-Saiz JM, Pizarro C (2001) Polyacrylamide gels as support for enzyme immobilization by entrapment. Effect of polyelectrolyte carrier, pH and temperature on enzyme action and kinetics parameters. *Eur Polym J* 37:435-444
- Gupta R, Chaudhury, NK (2007) Entrapment of biomolecules in sol-gel matrix for applications in biosensors: problems and future prospects. *Biosens Bioelectron* 22:2387-2399
- Kato M, Inuzuka K, Sakai-Kato K et al (2005) Monolithic bioreactor immobilizing trypsin for high-throughput analysis. *Anal Chem* 77:1813-1818
- Kumar P, Satyanarayana T (2007) Optimization of culture variables for improving glucoamylase production by alginate-entrapped *Thermomucor indicata-seudaticae* using statistical methods. *Bioresour Technol* 98:1252-1259
- Jin W, Brennan JD (2002) Properties and application of proteins encapsulated within sol-gel derived materials. *Anal Chim Acta* 461:1-36
- Li W, Fries DP, Malik A (2004) Sol-gel stationary phases for capillary electrochromatography *J Chromatogr A* 1044:23-52
- Livage J (1997) Sol-gel processes. *Curr Opin Solid State Mater Sci* 2:132-138
- Melo LLMM, Pastore GM, Macedo GA (2005) Optimized synthesis of citronellyl flavour esters using free and immobilized lipase from *Rhizopus* sp. *Process Biochem* 40:3181-3185
- Mundra P, Desai K, Lele SS (2007) Application of response surface methodology to cell immobilization for the production of palatinose. *Bioresour Technol* 98:2892-2896
- Myers RH, Montgomery DC (1995) Response surface methodology: process and product optimization using designed experiments. Wiley-Interscience, New York
- Park CB, Clark DS (2002) Sol-gel encapsulated enzyme arrays for high-throughput screening of biocatalytic activity. *Biotechnol Bioeng* 78:229-235
- Potumarthi R, Subhaker C, Pavani A et al (2008) Evaluation of various parameters of calcium-alginate immobilization method for enhanced alkaline protease production by *Bacillus licheniformis* NCIM-2042 using statistical methods. *Bioresour Technol* 99:1776-1786
- Prakash O, Talat M, Hasan SH et al (2008) Factorial design for the optimization of enzymatic detection of cadmium in aqueous solution using immobilized urease from vegetable waste. *Bioresour Technol* 2008; doi:10.1016/j.biortech.2008.02.008
- Senthilkumar SR, Dempsey M, Krishnan C et al (2008) Optimization of biobleaching of paper pulp in an expanded bed bioreactor with immobilized alkali stable xylanase by using response surface methodology. *Bioresour Technol* 2008; doi:10.1016/j.biortech.2008.01.058
- Sakai-Kato K, Kato M, Toyo'oka T et al (2002) On-line trypsin-encapsulated enzyme reactor by the sol-gel method integrated into capillary electrophoresis. *Anal Chem* 74:2943-2949
- Sakai-Kato K, Kato M, Toyo'oka T et al (2003) Creation of an On-Chip Enzyme Reactor by Encapsulating Trypsin in Sol-Gel on a Plastic Microchip. *Anal Chem* 75:388-393
- Siouffi AM (2003) Silica gel-based monoliths prepared by the sol-gel method: facts and figures. *J Chromatogr A* 1000:801-818
- Shtelzer S, Rappoport S, Avnir D, et al (1992) Properties of trypsin and of acid phosphatase immobilized in sol-gel glass matrices. *Biotechnol Appl Biochem* 15:227-235
- Sotiropoulou S, Chaniotakis NA (2005) Tuning the sol-gel microenvironment for acetylcholinesterase encapsulation. *Biomaterials* 26:6771-6779
- Vidinha P, Augusto V, Almeida M et al (2006) Sol-gel encapsulation: An efficient and versatile immobilization technique for cutinase in non-aqueous media. *J Biotechnol* 121:23-33