JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 231-238

Optimization of the Medium Composition using Response Surface Methodology for Production of Trehalose Synthase from *Corynebacterium glutamicum*

Maohua Qu, Juan Du and Weiping Chen*

Key Lab of Agricultural Products processing and quality control of Nanchang City, Jiangxi Agricultural University, Nanchang - 330045, China.

(Received: 12 June 2014; accepted: 14 August 2014)

The medium was optimized for trehalose synthase production by *Corynebacterium Glutamicum*. Effects of factors on trehalose synthase production were examined to improve trehalose synthase yield. Experimental designs were applied to optimize medium. Results of factional factorial design (FFD) showed that maltose, NaCl, K_2HPO_4 and beef extract were the most significant factors affecting trehalose synthase production. Further central composite design (CCD) experiments showed that optimal maltose and NaCl concentrations were 22g/L and 13.36g/L, respectively. Trehalose synthase activity increased to 580 U/mL, an approximate 1.76-fold increase over the previous activity (330 U/mL) using an optimized fermentation medium with the main composition maltose (22g/L), beef extract (7.49g/L), NaCl (13.36g/L), K_2HPO_4(11.33g/L) and peptone (15.00g/L).

Key words: Trehalose synthase, Optimization, Response surface methodology.

The compound of trehalose is a disaccharide with non-reducing consisted of two glucose molecules linked by a-1,1 bond [1]. Trehalose is capable of stabilizing and protects biological membranes and proteins [2,3], allowing organisms to survive extreme conditions such as high temperature, freezing, salinity and dehydration [4]. Trehalose is used as a compatible solute under osmotic stress and can be used as carbon energy source [5,6].

There are five biosynthetic pathways for trehalose. They are OtsAB, TreYZ, TreS, TreP, TreT pathways [7,8]. TreS pathway is important among them which converting maltose to trehalose with reversible activity. This enzyme was first found in Pimelobacter sp. and then this protein has been found in other organisms. In this work, the medium was optimized for producing trehalose synthase by *Corynebacterium Glutamicum*.

The enzymes production has a close relationship with the medium and fermentation conditions using microorganisms. "One factor at a time" was usually used in optimizing conditions in the past time. The shortcoming of this method is that it doesn't consider the interactions between factors and make analysis with mathematic model. In twentieth century, orthogonal design and response surface methodology appeared. Orthogonal method has advantage with simple operation and can consider the interactions among factors. The disadvantage of orthogonal is that it can't get a mathematic model to explain the effects extent in visual. Response Surface Methodology, RSM in simple, consider the interaction with a processing and conclusion showing in visual by making mathematic model. The software in common using such as SARS and Design-Expert can do analysis using RSM. Central Composite Design,

* To whom all correspondence should be addressed.
E-mail : iaochen@163.com

CCD in simple, is a common method of RSM.

The single dimensional approach such as "one factor at a time" is time consuming and laborious for large number of variables and without guarantee for the preciseness of optimal conditions [9], so we use response surface methodology (RSM) for statistical experimental design to optimize the parameters affecting production of trehalose synthase. RSM can be used in evaluating the parameters even in the existing of interactions [10,11]. The application of RSM in fermentation can result in increased trehalose synthase yields. Central composite design(CCD) is the most widely used design in optimization [12].

Although many experiments used *C. Glutamicum* for gene engineering to obtain mutants with high yield of trehalose or trehalose synthase [13,14], there was no report about using response surface methodology for optimizing medium components for trehalose synthase production by *C. Glutamicum*. The media components concentrations were optimized for the maximum production of trehalose synthase in shake flask culture.

MATERIAL AND METHODS

Microorganism and cultivation

In this study, we used *C. Glutamicum* ATCC13032, an aerobic Gram-positive bacterium, obtained from China General Microbiological Culture Collection Center (CGMCC). The strain was cultured on LB slants at 30! and then incubated into DMCG I medium (mltose was used as carbon source)for trehalose synthase production in shake flask culture. The culture medium used for the production of trehalose synthase contained: KH_2PO_4(1g), sodium citrate(1.1g), $(NH_4)_2SO_4$ (5.0g), $Na_2B_4O_7$(0.002g), $MgSO_4$(0.2g), $FeSO_4$(0.025g), $CuCl_2$(0.0002g), K_2HPO_4(8g), $(NH_4)_6Mo_7O_{24}$ (0.0001g), $CaCl_2$ (0.05g), $MnSO_2$(0.002 g), $FeCl_3$(0.002g), NaCl(1g) and maltose (20g) per liter of distilled water. All fermentations were performed in 250mL baffled flasks containing 50mL medium. The flasks were incubated at 30! in a shaker, agitated at 200rpm for 30h.

Analysis of enzyme activity

After 30h incubation, cells were collected by centrifuging at 16000×g, 4°C, for 30min. Activity of trehalose synthase was determined according to methods described by Nishimoto [15]. One unit (U) of enzyme was defined as the amount of the enzyme to produce 1ug substrate per minute under the assay conditions and the specific activity was defined as the number of units per mg of protein.

Experimental design

Concentrations of NaCl, beef extract, maltose and K_2HPO_4 were varied with significant impact on trehalose synthase production, while concentrations of other components in the medium were kept constant. Two-level fractional factorial design (2FFD) was adopted to screen the most significant variables. The design and analysis was described by Mukerjee [16]. The 2FFD identified the significant parameters affecting trehalose synthase production and then central composite design(CCD) could be performed to optimize medium components. The ranges of variables for 2FFD are listed in Table 1. Each variable was tested at a high level(+1), a low level(1) and center points(0). Fractional design with 1/2 fraction which contains 18 runs is shown in Table 2. The confidence level is above 95% ($P \leq 0.05$).

The most significant variables of NaCl, maltose, K_2HPO_4 and beef extract obtained from 2FFD were further optimized using CCD. All experiments were made in triplicate and results are average values. The ranges of variables for CCD are listed in Table 3. Each variable was tested at five different levels (<2,1, 0, +1, +2). CCD matrix containing 30 runs is shown as Table 4. Data were analyzed by using Expert-Design ver. 8.0.5 software (Stat-Ease Inc., Minneapolis, MN, USA) to estimate t values, P values and confidence levels.

The statistical determination of variable levels was according to Eq. 1:

$$X_i = (x_i - x_{0i})/\Delta x_i \quad i = 0, 1, 2, \ldots N \qquad ...(1)$$

In Eq. 1, X_i is coded value of real variable; x_i, x_{0i} is the real value of X_i at the center point level and the denominator is step change value.

The model involving several factors is expressed as follows:

$$R2 = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 \qquad ...(2)$$

In Eq. 2, R1 is the predicted response of trehalose synthase activity; b_0 is the intercept coefficient; b_1, b_2, b_3 and b_4 are linear coefficients; b_{11}, b_{22}, b_{33} and b_{44} are the quadratic coefficients;

b_{12}, b_{13}, b_{14}, b_{23}, b_{24} and b_{34} are interaction coefficients. X1, X2, X3 and X4 are the four variables(maltose, beef extract, NaCl and K_2HPO_4).

RESULTS AND DISCUSSION

Effect of factors using 2FFD

Five medium components were evaluated by using 18 runs including values at two center points. Results of Trehalose synthase activity were shown as Table 2. Those results differed significantly at confidence level under 0.05($P< 0.05$). The maximum and minimum activity obtained were 441 and 317, respectively. The run No. 16 had the maximum activity which consisted of (g/L): NaCl 15, maltose 14, K_2HPO_4 4, beef extract 9 and peptone 15. The lowest activity was No.12,which consisted of(g/L) : NaCl 5, maltose 14, K_2HPO_4 4, beef extract 3 and peptone 15.

Analysis of variance (ANOVA) for 2FFD was shown in Table 5. According to the statistical evaluation, variables with above 95%confidence levels had a significant effect on trehalose synthase activity with probability > F ($P<0.05$). Maltose, beef extract, NaCl and K_2HPO_4 were most significant variables affecting trehalose synthase activity. Peptone was found to be insignificant one for trehalose synthase production by *C. glutamicum*. A model of 2FFD describing the relationship between the five variables and trehalose synthase activity was constructed as follows:

R1 trehalose synthse activity (U/mL) = +384.28 + 14.06 A + 14.69 B + 9.56 C + 9.69 D + 0.69 E - 5.69 AB - 1.31 AC + 4.31 AD + 10.56 AE + 7.06 BC - 2.56 BD - 6.81 BE - 18.19 CD - 0.19 CE - 0.062 DE ...(3)

R1 represents trehalose synthase activity and A, B, C D and E is coded values of NaCl, maltose, K_2HPO_4, beef extract and peptone respectively.

The ANOVA (Table 5) showed that the variables of A, B, C and D were highly significant ($p<0.05$), and the interactive effect of AB, AE, BC, BE and CD were highly significant ($p<0.05$). It means that the NaCl, maltose, K_2HPO_4 and beef

Table 1. Factors and levels (g/L) for 2FFD

Code	Factor	-1	+1
A	NaCl	5	15
B	maltose	14	26
C	K_2HPO_4	4	12
D	beef extract	3	9
E	peptone	5	15

Table 2. Results of 2FFD experiments

Run	A NaCl	B maltose	C K_2HPO_4	D beef extract	E peptone	R1 trehalose synthse activity (U/mL)	
						Experimental	Predicted
1	1	1	1	1	1	420	422
2	-1	-1	1	1	1	340	341
3	-1	1	1	-1	1	407	406
4	-1	1	1	1	-1	410	409
5	-1	1	-1	-1	-1	368	371
6	1	1	-1	-1	1	367	366
7	1	-1	-1	-1	-1	335	337
8	0	0	0	0	0	386	383
9	1	-1	1	1	-1	371	371
10	-1	-1	1	-1	-1	367	370
11	0	0	0	0	0	384	383
12	-1	-1	-1	-1	1	317	316
13	1	-1	1	-1	1	410	410
14	1	1	1	-1	-1	425	426
15	1	1	-1	1	-1	417	416
16	1	-1	-1	1	1	441	440
17	-1	1	-1	1	1	377	374
18	-1	-1	-1	1	-1	375	372

extract have important effects on the trehalose synthase activity. The model neglecting the insignificant variables was presented as follows: R1 trehalose synthse activity (U/mL) = +384.28 + 14.06 A + 14.69 B + 9.56 C + 0.69 E - 5.69 AB + 10.56 AE + 7.06 BC - 6.81 BE - 18.19 CD ...(4)

Optimization of trehalose synthase production by CCD

The most significant variables screening from 2FFD were evaluated by using CCD. The main predicted and observed results were given in Table 4. The maximum and minimum activities obtained

Table 3. Factors and levels (g/L) for CCD

Code	Factor	-2	-1	0	+1	+2
A	maltose	11	16	21	26	31
B	beef extract	3	5	7	9	11
C	NaCl	3	7	11	15	19
D	K_2HPO_4	3	6	9	12	15

Table 4. CCD matrix of independent variables with experimental and predicted value for trehalose synthase activity

Run	A	B	C	DR2	Trehalose synthase activity (U/mL)	
	maltose	beef extract	NaCl	K_2HPO_4	Experimental	Predicted
1	-1	-1	-1	1	308	300
2	-1	-1	1	-1	364	396
3	-1	1	-1	-1	359	354
4	-1	1	1	1	427	436
5	1	-1	-1	-1	398	399
6	1	-1	1	1	453	468
7	1	1	-1	1	430	408
8	1	1	1	-1	389	421
9	0	0	0	0	556	566
10	0	0	0	0	559	566
11	-1	-1	-1	-1	354	356
12	-1	-1	1	1	390	398
13	-1	1	-1	1	323	357
14	-1	1	1	-1	335	359
15	1	-1	-1	1	337	335
16	1	-1	1	-1	469	457
17	1	1	-1	-1	383	397
18	1	1	1	1	484	506
19	0	0	0	0	554	566
20	0	0	0	0	558	566
21	-2	0	0	0	213	187
22	2	0	0	0	308	300
23	0	-2	0	0	395	400
24	0	2	0	0	474	436
25	0	0	-2	0	322	337
26	0	0	2	0	523	474
27	0	0	0	-2	538	508
28	0	0	0	2	529	523
29	0	0	0	0	609	566
30	0	0	0	0	560	566

Table 5. Analysis of variance (ANOVA) for the model of fractional factorial design

Variables	SS	df	MS	F-value	Probability > F	
Model	19154.44	15	1276.96	638.48	0.0310	Significant
A NaCl	3164.06	1	3164.06	1582.03	0.0160	
B Maltose	3451.56	1	3451.56	1725.78	0.0153	
C K2HPO4	1463.06	1	1463.06	731.53	0.0235	
D Beef extract	1501.56	1	1501.56	750.78	0.0232	
E Peptone	7.56	1	7.56	3.78	0.3024	
AB	517.56	1	517.56	258.78	0.0395	
AC	27.56	1	27.56	13.78	0.1675	
AD	297.56	1	297.56	148.78	0.0521	
AE	1785.06	1	1785.06	892.53	0.0213	
BC	798.06	1	798.06	399.03	0.0318	
BD	105.06	1	105.06	52.53	0.0873	
BE	742.56	1	742.56	371.28	0.0330	
CD	5292.56	1	5292.56	2646.28	0.0124	
CE	0.56	1	0.56	0.28	0.6896	
DE	0.06	1	0.06	0.03	0.8886	
Lack of Fit	1.17	1	1.17	0.59	0.5839	Not significant
Pure error	2	1	2			
Total	19157.61	17				
SD	1.26		R^2	0.9998		
Mean	384.28		Adj R^2	0.9986		
C.V.%	0.33		Pred R^2	0.8587		
PRESS	2707.41		Adeq precision	104.4086		

Table 6. Analysis of variance (ANOVA) for CCD model

Variables	SS	df	MS	F-value	Probability > F	
Model	276896.05	14	19778.29	21.34	< 0.0001	Significant
A Maltose	18872.04	1	18872.04	20.36	0.0004	
B Beef extract	1926.04	1	1926.04	2.08	0.1700	
C NaCl	28085.04	1	28085.04	30.30	< 0.0001	
D K2HPO4	287.04	1	287.04	0.31	0.5861	
AB	0.0625	1	0.06	0.00	0.9936	
AC	351.563	1	351.56	0.38	0.5472	
AD	52.563	1	52.56	0.06	0.8150	
BC	1207.563	1	1207.56	1.30	0.2716	
BD	5439.063	1	5439.06	5.87	0.0285	
CD	5365.563	1	5365.56	5.79	0.0295	
A2	177974.074	1	177974.07	192.02	< 0.0001	
B2	37655.503	1	37655.50	40.63	< 0.0001	
C2	44000.074	1	44000.07	47.47	< 0.0001	
D2	4151.074	1	4151.07	4.48	0.0515	
Residual	13902.91667	15	926.86			
Lack of fit	11660.91667	10	1166.09	2.60	0.1516	Not significant
Pure error	2242	5	448.40			
Total	290798.9667	29				
SD	30.4		R2	0.9522		
Mean	430.03		Adj R2	0.9076		
C.V.%	7.08		Pred R2	0.7579		
PRESS	70395.36		Adeq precision	17.5725		

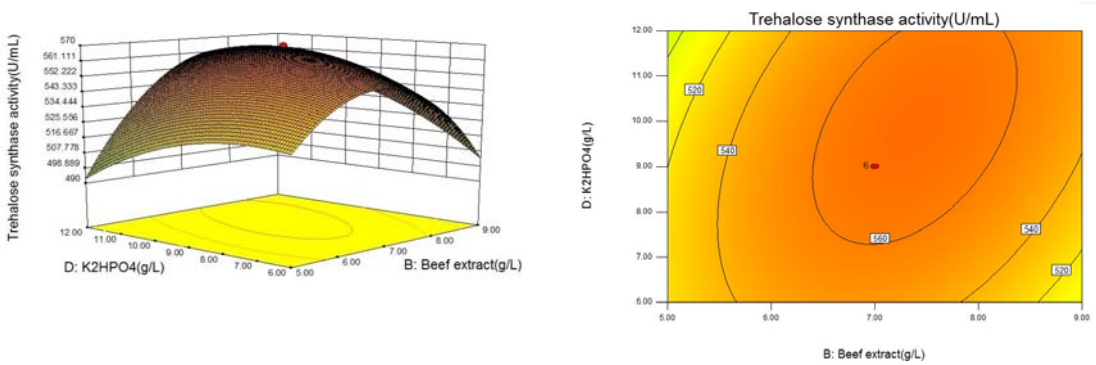

Fig. 1. Interactive effect between beef extract and K_2HPO_4 on trehalose synthase activity

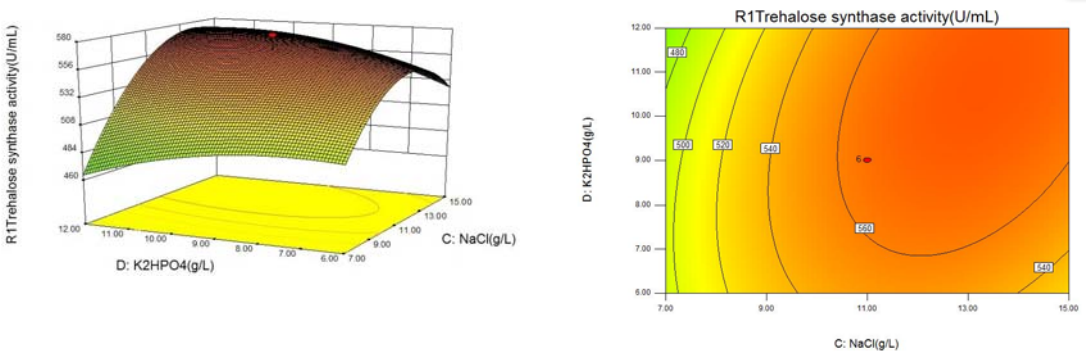

Fig. 2. Interactive effect between NaCl and K_2HPO_4on trehalose synthase activity

were 559U/mL and 233U/mL. The maximum activity was obtained in NO.10 and the minimum activity was in No. 21. The results obtained by CCD were analyzed by ANOVA as shown in Table 6. The following polynomial equation explaining the relationships between trehalose synthase activity and the four variables was constructed as follows:

R2 trehalose synthase activity (U/mL) = +566.00 + 28.04 A + 8.96 B + 34.21 C + 3.46 D + 0.062 AB + 4.69 AC + 1.81 AD - 8.69 BC + 18.44 BD + 18.31 CD - 80.55 A^2 - 37.05 B^2 - 40.05 C^2 - 12.30 D^2 ...(5)

R2 represents trehalose synthase production and A, B, C and D are coded values of maltose, beef extract, NaCl and K_2HPO_4, respectively.

The ANOVA (Table 6) for CCD showed that the variables of A, C, A^2, B^2 and C^2 were highly significant ($p<0.01$), and the interactive effect of BD and CD was highly significant ($p<0.05$). It means that the NaCl and maltose have important effects on the trehalose synthase activity. Beef extract and K_2HPO_4 have significant interactive effects on the trehalose activity. NaCl and K_2HPO_4 have significant interactive effects on the trehalose synthase activity, too. The model neglecting the insignificant variables was presented as follows:

R2 trehalose synthase activity (U/mL) = +566.00 + 28.04 A + 34.21 C + 18.44 BD + 18.31 CD - 80.55 A^2 - 37.05 B^2 - 40.05 C^2 ...(6)

The model was significant according to F test with a very low probability value. ANOVA for trehalose synthase production indicated F = 21.34, which implied that the model is highly significant and can approximate the CCD response surface properly. Determination coefficient R^2 examined the goodness of fit of the model. Coefficient of determination for trehalose synthase production was 0.9522, which can explain up to 95.22% variability of the response. Adequate precision is a measure of the signal to noise and a value greater than 4 is generally desirable. The adequate precision was 17.57 indicating an adequate signal and implying that the model can be adopted to navigate the design space. Coefficient of variance (C.V. %) was 7.08 indicating that the experiments were conducted with high precision. The lack of fit

compares the residual error to the pure error from replicated design points. The F value(2.6) for lack of fit implies insignificant and the chance is ÿ 0.05 that this value can occur due to noise. According to the model, trehalose synthase activity was significantly affected by NaCl and maltose. The interaction of BD and CD also affected significantly trehalose synthase activity.

According to the model of CCD, response surface plot was constructed to evaluate the effect of interactive factors. Fig. 1 represents the interaction of beef extract and K_2HPO_4 at the center point of other factors. The role of K_2HPO_4 in culture medium is to adjust pH value of medium environment to guarantee the efficiency of product synthesis in cells. The interactive effect of beef extract and K_2HPO_4 was assessed and other values of factors were kept constant at the center point value. The interaction is negligible between the variables if there's circular contour plots. The interaction between the variables is significant if there's elliptical or saddle nature for the plots. Fig.1 showed that there was highly significant interaction between beef extract and K_2HPO_4 because of the elliptical contour plots. Fig.2 shows the interaction of NaCl and K_2HPO_4 at the center point of other factors. NaCl made an osmotic environment to make force to the increasing of trehalose in medium. The maximum trehalose synthase activity (581 U/mL) was predicted by using CCD. The corresponding optimal medium composition for efficient trehalose synthase activity production was finalized as follows: maltose (22g/L), beef extract (7.49g/L), NaCl (13.36g/L), K_2HPO_4(11.33g/L) and peptone(15.00g/L).

Validation of the experimental model

The model constructed by CCD has a high R^2 value (0.9522) and a significant F-value (21.34). The high value of R^2 implied that the model was capable of representing the system under the experimental conditions. According to adjusted R^2 of the model, 90.7% of the variation of the model was due to the variation in the factors present in the model. The adequate precision value is 17.6 in the model which suggesting that it is suitable for navigating the space of design.

A verification test was carried out using the predicted optimum culture to confirm the efficiency of the model for maximum trehalose synthase activity. The maximum trehalose synthase activity obtained by the verification experiment was found to be 580U/mL. This is obviously fit for agreement with the model prediction. Trehalose synthase activity obtained was improved to 580U/mL, 1.76 fold as compared to that obtained using non-optimal medium (330U/mL).

CONCLUSION

Response surface methodology was used in optimizing medium composition for trehalose synthase production in this paper. The maximum trehalose synthase activity was obtained under the optimal medium conditions. The optimal medium consisted of maltose (22g/L), beef extract (7.49g/L), NaCl (13.36g/L), K_2HPO_4(11.33g/L) and peptone(15.00g/L) with the levels of other components being kept constant. The maximum trehalose synthase activity obtained using shake flask fermentation under optimal was 580U/mL, which is 1.76 fold as compared to yield in non-optimal medium.

REFERENCES

1. Elbein A D, Pan Y T, Pastuszak I, et al. New insights on trehalose: a multifunctional molecule[J]. *Glycobiology*, 2003; **13**: 17R-27R.
2. Crowe J H, Crowe L M, Chapman D. Preservation of membranes in anhydrobiotic organisms: the role of trehalose[J]. *Science*, 1984; **223**: 701-703.
3. Sasano Y, Haitani Y, Hashida K, et al. Simultaneous accumulation of proline and trehalose in industrial baker's yeast enhances fermentation ability in frozen dough[J]. *Journal of bioscience and bioengineering*, 2012; **113**: 592-595.
4. Garg A K, Kim J K, Owens T G, et al. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses[J]. Proceedings of the National Academy of Sciences, 2002; **99**: 15898-15903.
5. Foster S P, Johnson C P. Feeding and hemolymph trehalose concentration influence sex pheromone production in virgin Heliothis virescens moths[J]. *Journal of insect physiology*, 2010; **56**: 1617-1623.
6. Liu K, Dong Y, Huang Y, et al. Impact of trehalose transporter knockdown on Anopheles gambiae stress adaptation and susceptibility to Plasmodium falciparum infection[J]. *Proceedings of the National Academy of Sciences*,

2013; **110**: 17504-17509.
7. Pade N, Compaoré J, Klähn S, et al. The marine cyanobacterium Crocosphaera watsonii WH8501 synthesizes the compatible solute trehalose by a laterally acquired OtsAB fusion protein[J]. *Environmental microbiology*, 2012; **14**: 1261-1271.
8. Li H, Su H, Kim S B, et al. Enhanced production of trehalose in< i> Escherichia coli</i> by homologous expression of otsBA in the presence of the trehalase inhibitor, validamycin A, at high osmolarity[J]. *Journal of bioscience and bioengineering*, 2012; **113**: 224-232.
9. Singh S K, Singh S K, Tripathi V R, et al. Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic Pseudomonas putida SKG-1 isolate[J]. *Microb Cell Fact*, 2011; **10**: 114-127.
10. Khuri A I, Mukhopadhyay S. Response surface methodology[J]. Wiley Interdisciplinary Reviews: Computational Statistics, 2010; **2**: 128-149.
11. Ba_ D, Boyac1 0 H. Modeling and optimization I: Usability of response surface methodology[J]. *Journal of Food Engineering*, 2007, **78**: 836-845.
12. Khodadoust S, Ghaedi M. Optimization of dispersive liquid–liquid microextraction with central composite design for preconcentration of chlordiazepoxide drug and its determination by HPLC UV[J]. *Journal of separation science*, 2013; **36**: 1734-1742.
13. Becker J, Zelder O, Häfner S, et al. From zero to hero—Design-based systems metabolic engineering of Corynebacterium glutamicum for l-lysine production[J]. *Metabolic engineering*, 2011; **13**: 159-168.
14. Kim T K, Jang J H, Cho H Y, et al. Gene cloning and characterization of a trehalose synthase from Corynebacterium glutamicum ATCC13032[J]. *Food Science and Biotechnology*, 2010; **19**: 565-569.
15. Nishimoto T, Nakano M, Nakada T, et al. Purification and properties of a novel enzyme, trehalose synthase, from Pimelobacter sp. R48[J]. *Bioscience, biotechnology, and biochemistry*, 1996; **60**: 640-644.
16. Mukerjee R, Wu C F J. Two-Level Fractional Factorial Designs[J]. A Modern Theory of Factorial Designs, 2006,**23**: 49-84.