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Full Length Research Paper

Optimization of medium composition for *cis*-epoxysuccinate hydrolase production in *Escherichia coli* by response surface methodology

Hai-feng Pan[1], Wen-na Bao[2], Zhi-peng Xie[1]* and Jian-guo Zhang[1,2]

[1]Institute of Biochemistry, Zhejiang University, Hangzhou 310058, PR China.
[2]Hangzhou Bioking Biochemical Engineering Co., Ltd., Hangzhou 311106, PR China.

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Response surface methodology was applied to identify and optimize the medium composition for the *cis*-epoxysuccinate hydrolase production in recombinant *Escherichia coli*. Plackett-Burman design was used in the first step to evaluate the effects of 8 variables on the enzyme activity. $CaCl_2$, corn steep liquor and lactose were screened as significant factors and their concentrations were further optimized using response surface methodology based on 2^3 full factorial rotatable central composite design. The optimum predicted medium for maximum expression of recombinant *cis*-epoxysuccinate hydrolase was found to comprise: 17.1 g/l $Na_2HPO_4 \cdot 12H_2O$, 2.0 g/l KH_2PO_4, 0.5 g/l NaCl, 1.0 g/l NH_4Cl, 0.0111 g/l $CaCl_2$ and 0.5 g/l $MgSO_4 \cdot 7H_2O$, 17.18 ml/l corn steep liquor and 9.74 g/l lactose, with a predicted enzyme activity of 35490 U/g biomass, which was very close to the experimental activity of 36318 U/g biomass resulting in 1.7-fold increment after optimization.

Key words: Medium, optimization, response surface methodology, *cis*-epoxysuccinate hydrolase, *Escherichia coli*.

INTRODUCTION

Epoxide hydrolases (EHs, EC 3.3.2.3) catalyze the hydrolysis of epoxides to their corresponding diols. They are ubiquitous in nature and have gained considerable attention in recent years due to their role in cellular detoxification processes and the metabolism of a number of biologically important compounds (Kotik et al., 2007). From a biotechnological perspective, EHs of high enantioselectivity are useful biocatalysts for the production of optically active epoxides and diols, which can serve as chiral building blocks in the synthesis of biologically active drugs (Kotik and Kyslík, 2006). Mammalian EHs have been investigated for a long time, but their use as biocatalysts in biotransformation reactions on a larger scale is often hampered by their limited availability. On the other hand, microbial EHs offer the advantage of access to large amounts of enzyme (Morisseau et al., 1999). The industrial synthesis of L- and meso-tartaric acids was the first application of an epoxide hydrolase catalyzed epoxide hydrolysis (Bučko et al., 2005).

D(-)-Tartaric acid, hardly existing as a natural resource, is mainly used as chiral auxiliary, resolving agents and building blocks in the pharmaceutical industry (Pabba and Vasella, 2005). Traditionally, D(-)-tartaric acid is prepared through chemical or biological separation of DL-tartaric acid (Huang, 1990). Recently, a novel approach for D(-)-tartaric acid production by some microorganisms, such as genus *Pseudomonas*, *Alcaligenes* and *Bordetella* (Asai et al., 2000; Yamagishi et al., 1996; Pan et al., 2008), has become overwhelming. In these microorganisms, the enzyme *cis*-epoxysuccinate hydrolase (CESH), an epoxide hydrolase, catalyzes the cleavage of the epoxy group of easily obtained *cis*-epoxysuccinic acid or salts thereof resulting in the formation of D(-)-tartaric acid (Liu et al., 2007).

Because of the relatively low CESH activity in wild-type bacteria (Pan et al., 2008), an engineered bacteria was constructed using *Bordetella* sp. BK-52 gene in our laboratory. Production of recombinant CESH is mainly dependent on culture conditions such as medium

*Corresponding author. E-mail: xier1975@163.com.

Table 1. Plackett-Burman design for screening important factors affecting CESH activity[a].

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Y_1	Y_2
1	17.1	2	1.0	0.5	0.01	0.5	20	10	1	-1	1	25029	24239.1
2	17.1	3	0.5	1.0	0.01	0.5	10	10	1	1	-1	21202	21677.4
3	15.0	3	1.0	0.5	0.02	0.5	10	5	1	1	1	14750	14496.3
4	17.1	2	1.0	1.0	0.01	1.0	10	5	-1	1	1	19282	19342.8
5	17.1	3	0.5	1.0	0.02	0.5	20	5	-1	-1	1	20905	20429.6
6	17.1	3	1.0	0.5	0.02	1.0	10	10	-1	-1	-1	16769	17022.8
7	15.0	3	1.0	1.0	0.01	1.0	20	5	1	-1	-1	19813	19752.3
8	15.0	2	1.0	1.0	0.02	0.5	20	10	-1	1	-1	20914	21703.9
9	15.0	2	0.5	1.0	0.02	1.0	10	10	1	-1	1	18433	17643.1
10	17.1	2	0.5	0.5	0.02	1.0	20	5	1	1	-1	18455	18930.4
11	15.0	3	0.5	0.5	0.01	1.0	20	10	-1	1	1	20527	20587.8
12	15.0	2	0.5	0.5	0.01	0.5	10	5	-1	-1	-1	17398	17651.8

[a] X_1: $Na_2HPO_4 \cdot 12H_2O$ (g/l); X_2: KH_2PO_4 (g/l); X_3: NaCl (g/l); X_4: NH_4Cl (g/l); X_5: $CaCl_2$ (g/l); X_6: $MgSO_4 \cdot 7H_2O$ (g/l); X_7: Corn steep liquor (ml/l); X_8: Lactose (g/l); X_9, X_{10}, X_{11}: Dummy variables; Y_1: Experimental CESH activity (U/g biomass); Y_2: Predicted CESH activity (U/g biomass).

composition. Investigation of the fermentation characteristics of recombinant cells in different media suggested that the careful optimization of medium composition may possibly lead to higher productivity in terms of CESH activity. Conventionally, the fermentation process is optimized using a one-at-a-time strategy, which is relatively simple and does not require statistical analysis. However, this single variable optimization strategy cannot reflect the interaction effects among variables and cannot depict the net effect of various medium constituents on enzyme productivity (He and Tan, 2006). Response surface methodology (RSM) (Myers and Montgomery 1995), which is a collection of statistical and mathematical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions, has successfully been used in the optimization of culture conditions to enhance enzyme production in *Escherichia coli* (Sunitha et al., 2000; Luz Paz Maldonado et al., 2007; Loa et al., 2007). Plackett-Burman design (Plackett and Burman, 1946) is usually used as the first step in RSM to screen for the most important independent variables. In this study, Plackett-Burman design and RSM based on rotatable central composite design (CCD) were applied to identify and optimize the medium composition that will maximize the intracellular over-expression of recombinant CESH.

MATERIALS AND METHODS

Expression system and culture conditions

E. coli BL21 (DE3) was used as the expression host. The CESH gene was isolated from *Bordetella* sp. BK-52 and cloned into the expression vector pET22b(+) (Novagen Inc., Madison, USA). This prokaryotic expression system was named as *pET22b-CESH-E. coli BL21*. CESH was expressed as intracellular enzyme from plasmid pET22b-CESH under the control of the strong T7/lac promoter. Recombinant cells were cultivated in 250 ml Erlenmeyer flasks containing 50 ml of medium specified according to the experimental design, in an orbital shaker at 37°C, 250 rpm for 8 h. Ampicillin, when added was used at a final concentration of 100 µg/ml.

CESH enzyme assay

The content of tartaric acid was determined by the ammonium metavanadate method (Liu and Yan, 1983) and the CESH enzyme activity was calculated according to the definition of one unit of enzyme as the amount of enzyme in 1.0 g of wet cells capable of generating 1 µmol of tartaric acid per hour under the experimental conditions described by Pan et al. (2008).

Experimental design and optimization

Preliminary results showed that higher activity of CESH was obtained in M9-based medium (17.1 g/l $Na_2HPO_4 \cdot 12H_2O$, 3.0 g/l KH_2PO_4, 0.5 g/l NaCl, 1.0 g/l NH_4Cl, 0.01 g/l $CaCl_2$ and 0.5 g/l $MgSO_4 \cdot 7H_2O$) supplemented with 10 g/l lactose and 10 ml/l corn steep liquor (dry matter content 35%) than in Luria-Bertani (LB) medium. Lactose was chosen as carbon source and inducer, while corn steep liquor was screened as best nitrogen source for CESH activity from four kinds of organic nitrogen sources (corn steep liquor, beef extract, yeast extract and peptone).

At the first step, Plackett-Burman design was used to screen for significant factors of the medium (Table 1). Each independent variable was tested at high (+1) and low (-1) levels. The Plackett-Burman method allows evaluation of *N*-1 variables by *N* number of experiments (*N* must be a multiple of four). In addition to the variables of real interest, the Plackett-Burman design considered insignificant dummy variables, which were used to estimate the experimental error and check the adequacy of the first-order model. According to the above depiction, 12 experimental run with three dummy variables were carried out in this study. Minitab™ v 14.0 software (Minitab Inc., Pennsylvania, USA) was used for regression analysis of the obtained experimental data.

The factors screened by Plackett-Burman design were further optimized using RSM. The optimization of medium composition was

Table 2. Experimental design of $CaCl_2$ (X_5), corn steep liquor (X_7), lactose (X_8) and the result for CESH activity.

Run	$CaCl_2$ (g/l)	Corn steep liquor (ml/l)	Lactose (g/l)	CESH activity (U/g)	
				Experimental	Predicted
1	-1 (0.009)	-1 (15)	-1 (7.5)	29567	29571.7
2	1 (0.013)	-1 (15)	-1 (7.5)	29837	29913.5
3	-1 (0.009)	1 (25)	-1 (7.5)	22994	22515.8
4	1 (0.013)	1 (25)	-1 (7.5)	22496	22536.7
5	-1 (0.009)	-1 (15)	1 (12.5)	28043	27960.2
6	1 (0.013)	-1 (15)	1 (12.5)	28459	28895.0
7	-1 (0.009)	1 (25)	1 (12.5)	20469	20350.3
8	1 (0.013)	1 (25)	1 (12.5)	21011	20964.1
9	-1.682 (0.0076)	0 (20)	0 (10)	27056	27437.0
10	1.682 (0.0144)	0 (20)	0 (10)	28562	28240.6
11	0 (0.011)	-1.682 (11.59)	0 (10)	31651	31372.4
12	0 (0.011)	1.682 (28.41)	0 (10)	18432	18770.2
13	0 (0.011)	0 (20)	-1.682 (5.80)	25804	25995.5
14	0 (0.011)	0 (20)	1.682 (14.20)	23450	23318.1
15	0 (0.011)	0 (20)	0 (10)	32957	34382.8
16	0 (0.011)	0 (20)	0 (10)	35098	34382.8
17	0 (0.011)	0 (20)	0 (10)	33470	34382.8
18	0 (0.011)	0 (20)	0 (10)	34955	34382.8
19	0 (0.011)	0 (20)	0 (10)	34434	34382.8
20	0 (0.011)	0 (20)	0 (10)	35393	34382.8

designed based on 2^3 full factorial rotatable center composite design (CCD) with 6 axial points and 6 replicates at the central points, resulting in a total of 20 experiments as given in Table 2. The CESH enzyme activity was collected as the response. For statistical calculations, the relation between the coded values and real values were as described in the following equation:

$$X_i = (U_i - U_o) / \Delta U \qquad (1)$$

where X_i is the independent variable coded value, U_i the real value of the independent variable, U_o the real value of the independent variable on the center point, ΔU the step change and the central point was set with α of 1.682 for optimization of medium composition. Minitab™ v 14.0 software was used for analysis of the significance of each coefficient (linear or quadratic) and *P*-value (Probability > *F*) less than 0.05 indicated that the model terms are significant. Adequacy of the method developed was further analyzed. The optimal values were obtained solving the regression equation and analyzing the contour plot and response surface plot.

RESULTS AND DISCUSSION

Since the early development of recombinant DNA technology, *E. coli*, especially the T7-based gene expression system employed (*pET22b-CESH-E. coli BL21*), has been widely used as a host for high-level expression of recombinant proteins (Nikerel et al., 2006). LB medium is the commonest type of medium for *E. coli*, but minimal medium, such as M9-based medium is preferred for large-scale production. Glucose is the commonest carbon source, but many studies (Hao et al., 2005) reported that it could inhibit the induction of T7/lac promoter and non-glucose carbon source shall be used at the stage of induction. On the other hand, lactose, a carbon source for bacteria, has been proven to be as effective as Isopropyl-b-D-thiogalactoside (IPTG) for inducing recombinant proteins in *E. coli* (Denice and Jill, 2001). Furthermore, it was reported that lactose is a carbon source for bacteria to promote growth and increase number of bacteria in culture, which results in the increase of solubility of the target recombinant protein (Kim et al., 2007). Therefore, in this study, lactose with low-cost and non-toxicity was employed as the inducer and carbon source due to advantages for its application in large-scale fermentations.

Screening of important factors using Plackett-Burman design

Plackett-Burman design is a powerful technique for screening important variables. Using Plackett-Burman method, a total of 12 experiments was performed to analyze the effect of 11 variables (including three dummy variables) on CESH activity. The responses (Y_1, U/g biomass) were analyzed (Table 1) and the *P*-value was used to identify the effect of every factor on CESH activity. As shown in Table 3, $CaCl_2$ (X_5), corn steep liquor (X_7) and lactose (X_8) were significant factors due to the *P*-value, which was less than 0.05. The coefficient of determination, R^2, was found to be 0.9634 which implied that the sample variation of 96.34% for CESH activity

Table 3. Estimated effects and coefficients for CESH activity using Plackett-Burman design (coded units).

Factor	Effect	Estimated coefficient	Standard error coefficient	*t* ratio	*P*-value
Constant		19456	276.6	70.34	0.000
$Na_2HPO_4 \cdot 12H_2O$	1634	817	276.6	2.95	0.060
KH_2PO_4	-924	-462	276.6	-1.67	0.193
NaCl	-61	-30	276.6	-0.11	0.920
NH_4Cl	1270	635	276.6	2.30	0.105
$CaCl_2$	-2171	-1085	276.6	-3.92	0.029
$MgSO_4 \cdot 7H_2O$	-1153	-577	276.6	-2.08	0.128
Corn steep liquor	2968	1484	276.6	5.37	0.013
Lactose	2045	1023	276.6	3.70	0.034

was attributed to the independent variables. Meanwhile, the coefficient of determination (adjusted R^2) was calculated to be 0.8659, indicating that a good agreement between the experimental and predicted values of CESH activity (Y_2, U/g biomass). Additionally, the *F* statistic was found to be 9.88 corresponding to *P*-value of 0.043 (the confidence interval is 0.05) indicating that the model was adequate. In other words, the model was fit with the responses data collected.

According to results shown in Table 3, the optimum medium composition (except three major factors) were found to be 17.1 g/l $Na_2HPO_4 \cdot 12H_2O$, 2.0 g/l KH_2PO_4, 0.5 g/l NaCl, 1.0 g/l NH_4Cl and 0.5 g/l $MgSO_4 \cdot 7H_2O$. Chen et al. (2007) optimized the M9-based medium complemented with glucose and yeast extract as carbon and nitrogen source for production of MBP (maltose-binding protein)-heparinase I (HepA) in *E. coli* with enzyme activity as response data. From their findings glucose, yeast extract and calcium ion were selected as three major factors using one-at-a-time strategy. Similar results of significant variables, namely, $CaCl_2$ (X_5), corn steep liquor (X_7) and lactose (X_8), were screened in our study and chosen for further study.

Optimization of medium composition using RSM

Based on the Plackett-Burman design, three variables including $CaCl_2$ (X_5), corn steep liquor (X_7) and lactose (X_8), which significantly influence CESH activity, were investigated for their optimum concentrations. Twenty experiments with different combinations of variables using CCD design method were performed and CESH activity (*Y*, U/g biomass) was collected as the responses (Table 2).

To estimate the optimal CESH activity, the following full quadratic second order polynomial equation using coded units was found to explain the CESH activity. All terms regardless of their significance were included.

$$Y = 34382.8 + 238.9X_5 - 3746.7X_7 - 796.0X_8 - 2313.6X_5X_5 - 3292.1X_7X_7 - 3438.6X_8X_8 - 80.3X_5X_7 + 148.3X_5X_8 - 138.5X_7X_8 \quad (2)$$

As shown in Table 4, X_7, X_8, X_5X_5, X_7X_7, X_8X_8 were significant factors. The quality of the model could be checked using several criteria. The coefficient of determination, R^2, found to be 0.990 indicated that 99.0% of the total variability in the response could be explained by this model. Meanwhile, the coefficient of determination (adjusted R^2) was calculated to be 0.980, indicating a good agreement between the experimental and predicted values of CESH activity (Table 2). Additionally, ANOVA table (Table 5) was constructed for further check of the model adequacy. The *F* statistic was found to be 106.04 corresponding to *P*-value of 0.000 (the confidence interval is 0.05) indicating that the model was adequate. Furthermore, *P*-value for source 'lack of fit', which was greater than 0.05 indicated that the 'lack of fit' of model was insignificant. In other words, the model was fit with the responses data collected.

The contour plots and response surface plots described by the Eq. (2) are represented in Figures 1-2. Corn steep liquor (X_7) gave the most significant effect to the expression of recombinant CESH (P = 0.000), which indicated that nitrogen source had a marked effect on enzyme production. At the same time, lactose (X_8) was also an important factor (P = 0.003), which implied that carbon source and inducer significantly affected enzyme production. As is known, nitrogen source is mainly utilized to synthesize protein, nucleic acid and metabolites of nitrogen, while carbon source supplies energy to microorganism. Relatively lower content of nutriment is the limited factor for cell growth and protein expression, whereas higher content will lead separation of the plasmalemma from the wall, resulting in influence on their basic life activity. Therefore, 17.18 ml/l corn steep liquor and 9.74 g/l lactose were selected as the best for CESH

Table 4. Estimated regression coefficients of $CaCl_2$ (X_5), corn steep liquor (X_7) and lactose (X_8) for CESH activity (coded units).

Term	Estimated coefficient	Standard error coefficient	*t* ratio	*P*-value
Constant	34382.8	307.2	111.936	0.000
X_5	238.9	203.8	1.172	0.268
X_7	-3746.7	203.8	-18.384	0.000
X_8	-796.0	203.8	-3.906	0.003
X_5 X_5	-2313.6	198.4	-11.662	0.000
X_7 X_7	-3292.1	198.4	-16.594	0.000
X_8 X_8	-3438.6	198.4	-17.333	0.000
X_5 X_7	-80.3	266.3	-0.301	0.769
X_5 X_8	148.3	266.3	0.557	0.590
X_7 X_8	-138.5	266.3	-0.520	0.614

Table 5. ANOVA table for full quadratic model.

Source	Degree of freedom	Sequential sum of square	Adjusted sum of square	Adjusted mean square	*F* statistic	*P*-value
Regression	9	541337999	541337999	60148667	106.04	0.000
Linear	3	201140636	201140636	67046879	118.21	0.000
Square	3	339816560	339816560	113272187	199.70	0.000
Interaction	3	380803	380803	126934	0.22	0.878
Residual error	10	5672075	5672075	567207		
Lack of fit	5	943933	943933	188787	0.20	0.949
Pure error	5	4728142	4728142	945628		
Total	19	547010074	547010074			

expression by applying the regression analysis to the Eq. (2) using 'response optimizer' in Minitab software. In addition, $CaCl_2$ and the interactions among the medium composition gave insignificant effects towards the production of enzyme. This insignificant interaction effects simplified the scale-up process for the enzyme production and were desirable for most of the large-scale production. Based on Figures 1-2, optimum 0.0111 g/l $CaCl_2$ was selected as one of the optimized medium composition.

After analyzing the effect of $CaCl_2$ (X_5), corn steep liquor (X_7) and lactose (X_8), contour plots and response surface plots that were calculated according to the model Eq. (2) are shown in Figures 1-2. By using the plots, the effects towards the expression level of recombinant CESH were analyzed and the optimum value for each factor investigated was predicted. The optimum medium composition for maximum production of CESH was found to be 0.05 (0.0111 g/l) for $CaCl_2$, -0.564 (17.18 ml/l) for corn steep liquor and -0.104 (9.74 g/l) for lactose. The maximum value of enzyme activity calculated from the model according to the predicted optimum induction condition was 35490U/g biomass. It should be noted that this CESH activity was higher than the central point as well as any of those in the 20 experiments indicating that the optimization was quite successful. Verification experiments were carried out using the predicted optimum composition and the CESH activity was measured as 36318 ± 1028 U/g biomass which was 1.7-fold increment of CESH expression comparing to the CESH activity (21543 ± 638 U/g biomass) under original medium.

In conclusion, our study is the first to report on the optimization of medium composition for recombinant *E. coli* producing CESH by Plackett-Burman design and RSM with significant improvement of CESH enzyme activity. The final composition of optimized medium was as follows: 17.1 g/l $Na_2HPO_4{\cdot}12H_2O$, 2.0 g/l KH_2PO_4, 0.5 g/l NaCl, 1.0 g/l NH_4Cl, 0.0111 g/l $CaCl_2$ and 0.5 g/l $MgSO_4{\cdot}7H_2O$, 17.18 ml/l corn steep liquor and 9.74 g/l lactose. The enhanced production of CESH protein and relatively cheap medium (comparing to LB medium) will facilitate the production and application of this enzyme, especially for the industrial preparation of D(-)-tartaric

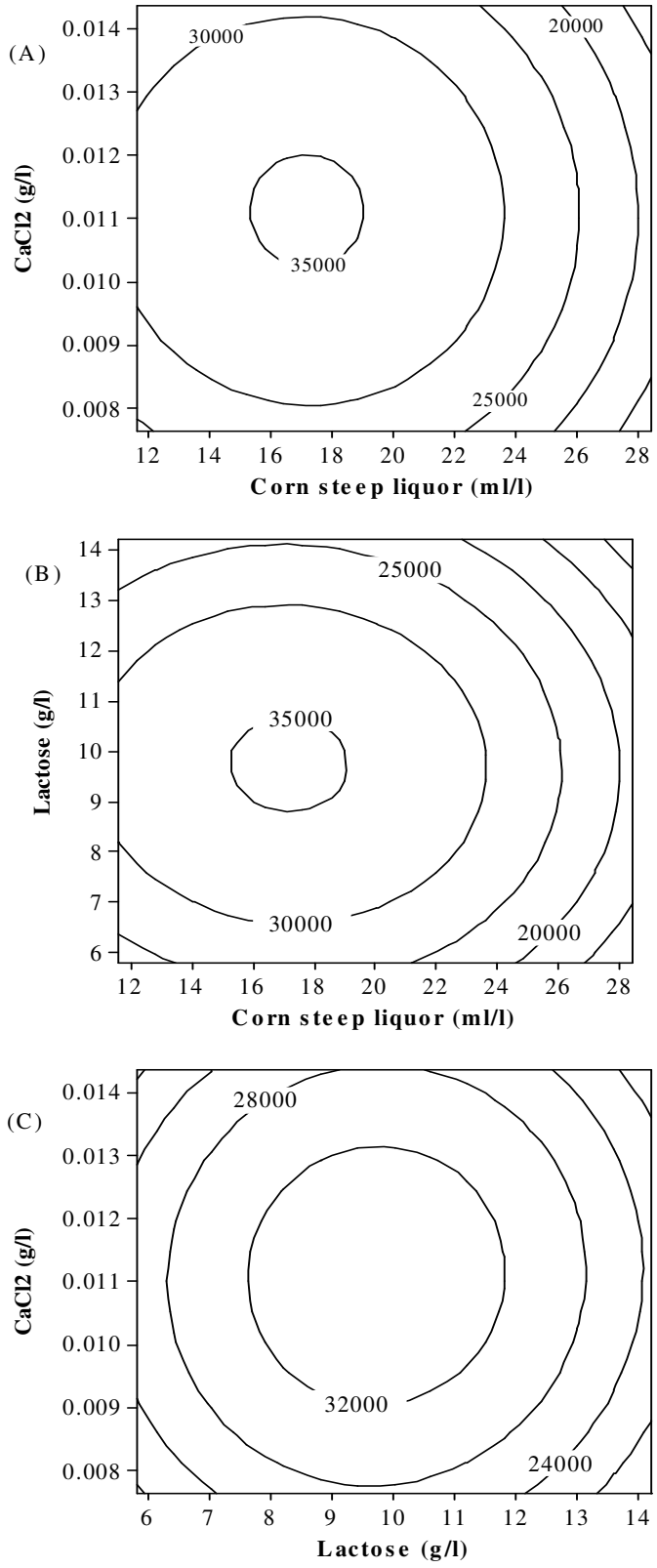

Figure 1. Contour plots of CESH activity. (A) Corn steep liquor vs. $CaCl_2$ with constant level for lactose (10 g/l); (B) Corn steep liquor vs. lactose with constant level for $CaCl_2$ (0.011 g/l); (C) Lactose vs. $CaCl_2$ with constant level for corn steep liquor (20 ml/l)

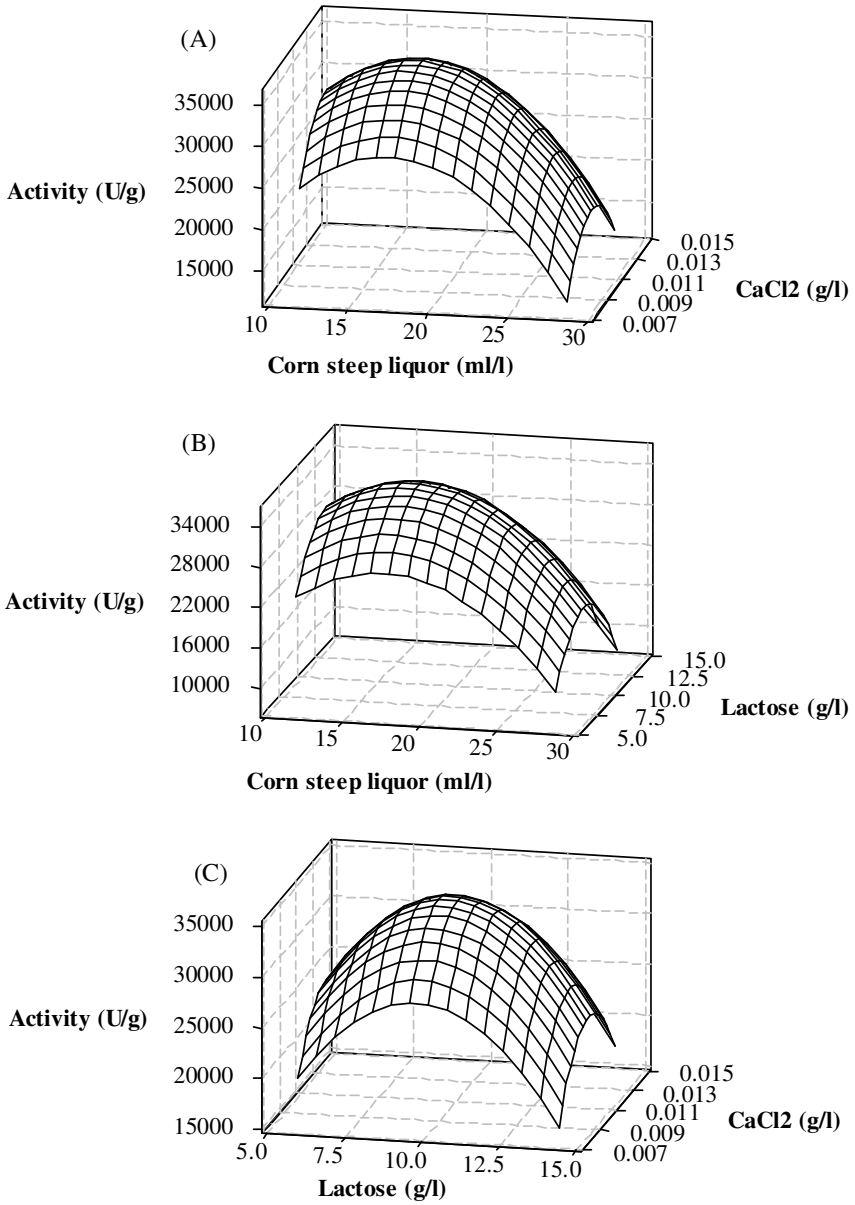

Figure 2. Response surface plots of CESH activity. (A) Corn steep liquor vs. $CaCl_2$ with constant level for lactose (10 g/l); (B) Corn steep liquor vs. lactose with constant level for $CaCl_2$ (0.011 g/l); (C) Lactose vs. $CaCl_2$ with constant level for corn steep liquor (20 ml/l).

acid.

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