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Production of cytidine 5′-diphosphorylcholine with high utilization of ATP by whole cells of *Saccharomyces cerevisiae*

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ABSTRACT

Cytidine 5′-diphosphorylcholine (CDP-choline) was produced using a high efficiency ATP regeneration system and the Kennedy pathway in whole cells of *Saccharomyces cerevisiae* As 2.398. Out of eight variables, KH_2PO_4, glycerol and $(NH_4)_2SO_4$ were considered to be the most significant factors by response surface methodology including a Plackett–Burman design, path of steepest accent and central composite design. The optimum levels of the three variables were 20.13 g/L KH_2PO_4, 12.35 g/L glycerol and 0.49 g/L $(NH_4)_2SO_4$, respectively. Energy utilization efficiency increased from 10.59% to 16.72% and choline chloride conversion yields increased from 12.35% to 42.78%. A high efficiency ATP regeneration system improves CDP-choline production.

1. Introduction

Cytidine 5′-diphosphorylcholine (CDP-choline) is an essential intermediate in the biosynthetic pathway of structural phospholipids in cell membranes. CDP-choline supplementation can increase dopamine receptor densities (Giménez et al., 1991), elevate adrenocorticotropin independent of corticotropin-releasing hormone levels and amplify the release of other hypothalamic–pituitary–adrenal axis hormones such as luteinizing hormone, follicle stimulating hormone, serum growth hormone and thyroid stimulating hormone in response to hypothalamic releasing factors (Cavun and Savci, 2004). Therefore, it is a psychostimulant and nootropic. It may be indicated in the treatment of cerebral vascular disease, head trauma and cognitive disorders of different etiology (Secades and Lorenzo, 2006; Minnerup and Schabitz, 2009).

For CDP-choline production, the choline donors include phosphorylcholine and choline chloride. When choline chloride was used as a choline donor for CDP-choline production in *Saccharomyces cerevisiae*, CDP-choline concentration, cytidine 5′-monophosphate (CMP) conversion yield, choline chloride conversion yield and utilization efficiency of energy were 8.87 g/L, 87%, 8.7% and 6.53%, respectively (Watanabe et al., 1981). Using *Corynebacterium ammoniagenes* KY13505 and recombinant *Escherichia coli* MM294/pCKG55, CDP-choline was produced from orotic acid and choline chloride. CDP-choline concentration, orotic acid conversion yield and choline chloride conversion yield were 11 g/L, 45.74% and 35.85%, respectively (Fujio et al., 2004). When phosphorylcholine was added as a choline donor for CDP-choline production in *S. cerevisiae*, CDP-choline concentration, CMP and phosphorylcholine conversion yield and utilization efficiency of energy were 8.16 g/L, 80%, 32% and 6%, respectively (Watanabe et al., 1981). Baer (1947) reported the synthesis of phosphorylcholine utilizing diphenylphosphoryl chloride as phosphorylating agent. Although products were obtained with excellent yields and a high degree of purity, production suffered from the disadvantage that it requires considerable amounts of toxic organic solvents, such as pyridine. Therefore, it is necessary to replace phosphorylcholine by choline chloride for CDP-choline production. The choline chloride conversion yield was lower than the phosphorylcholine conversion yield (Watanabe et al., 1981), because the conversion of CMP to cytidine 5′-triphosphate (CTP) and choline chloride to phosphorylcholine demanded a high energy charge as a driving force. The phosphorylation of CMP and choline chloride competed with each other. Ample evidence supports a role for choline kinase as being rate-limiting and regulatory in some circumstances (Kent, 2005). The phosphorylation of choline chloride was more difficult than that of CMP. In our previous work, CTP was accumulated as 14.3 g/L after 3 h of reaction (Tang et al., 2009), but the synthesis rate of CDP-choline decreased slowly gradually. This was because the presence of CTP may allosterically inhibit choline phosphorylation (Kimura and Okuda, 1976). Furthermore, in this case, choline kinase was not phosphorylated or was dephosphorylated, and its activity was too low to catalyze the phosphorylation of choline, which led to the low choline conversion yield. Alkaline

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phosphatase treatment of choline kinase resulted in a 60% decrease in choline kinase activity (Kim and Carman, 1999). In contrast, phosphorylation of choline kinase with protein kinase A and protein kinase C resulted in a 1.9-fold (Kim and Carman, 1999) and 1.6-fold (Choi et al., 2005) stimulation in choline kinase activity, respectively. Furthermore, choline kinase was inhibited by ADP and activated by ATP (Kim et al., 1998). Therefore, an important factor to activate phosphorylcholine formation could be a high ratio of ATP/ADP or phosphorylated and activated choline kinase that may directly promote synthesis of phosphorylcholine.

In general, the glycolytic flux was shown to be inhibited by high energy charge (Atkinson, 1968). At the same time, ATP could flow into other metabolic pathways. In addition, the results of metabolic flux analysis revealed that NADH could not be oxidized via the electron transfer chain under anaerobic conditions, but could be oxidized via glycerol production, which expended a large number of ATP molecules (Kidd et al., 1999; Li et al., 2002). Therefore, one of requirements for the high CDP-choline production was the improvement of ATP supply. Based on these findings, the addition of ammonium ions ($(NH_4)_2SO_4$) and phosphate (KH_2PO_4) may reverse the inhibitory effect of ATP on phosphofructokinase (PFK) activity and stimulate glycolysis (Hofmann and Kopperschläger, 1982). Because the membrane could be destroyed by the extracellular osmolarity, intracellular acetaldehyde was depleted by diffusion (Stanley et al., 1997). Acetaldehyde addition was used to inhibit the High-Osmolarity-Glycerol pathway which needed NADH to reduce dihydroxyacetone phosphate by snatching NADH (Posas and Saito, 1997). The metabolic flux through the glycolytic pathway into the glycerol decreased as did the inefficient use of ATP in biosynthesis of glycerol. Through these methods, a higher-energy level in the system could facilitate the phosphorylation reaction of CMP and choline, which will improve the CDP-choline yield and energy utilization efficiency.

Since CDP-choline production involves multiple pathways, they may interact with each other (Fig. 1). Response surface methodology (RSM) can use quantitative data from appropriate experiments to design experiments, build models, evaluate the effects of factors, and analyze the optimum conditions for the factors for desirable responses. This methodology has been successfully utilized to optimize the composition of fermentation medium for various kinds of chemical production (Mundra et al., 2007; Zhang et al., 2009), yeast production (Li et al., 2009), enzymes production (Deepak et al., 2008; Singh et al., 2008), and drugs production (Ceylan et al., 2008; Chen et al., 2009; Gao et al., 2009). We decided to use a response surface approach including a Plackett–Burman design, path of steepest accent and central composite design for the statistical optimization of medium constituents.

2. Methods

2.1. Media and culture conditions

S. cerevisiae As 2.398 (2*n*, diploid strain), which is a spontaneous mutant from *S. cerevisiae* 1002 (China Center of Industrial Culture Collection), was used in this study. The growth medium contained (w/w): 5% glucose·H_2O, 0.5% peptone, 0.2% yeast extract, 0.2% $NH_4H_2PO_4$, 0.1% $MgSO_4{\cdot}7H_2O$, 0.2% KH_2PO_4 at an initial pH of 5.8. The cultivation was carried out at 24 °C for 72 h, the cells were harvested aseptically by vacuum filtration at 4 °C, and washed twice with demineralized water. The wet cells were frozen and stored at −20 °C before they were used as catalysts.

2.2. Reaction conditions

Reactions were carried out in a closed environment in a 7.5-L bioreactor (Bioflo 110, New Brunswick Scientific, USA) containing 5 L reaction mixture at 30 °C. After addition of the yeast wet cells, the reaction mixture was agitated at 200 rpm. The initial pH of the reaction mixture was adjusted to 7.0 with 5 mol/L NaOH. D-glucose·H_2O (20 g/L) was added after 4 h. After 24 h, the reaction mixture were sampled and analyzed. All experiments were carried out in triplicate. All values for metabolite concentrations are the mean values of at least three independent extraction procedures.

2.3. Analyses

The concentrations of CDP-choline, CMP, and CTP in the reaction were measured by high-performance liquid chromatography (Agilent 1100, USA) analysis, using a Sepax HP-C18 column (Sepax Technologies, Inc., Changzhou, China) and a UV detector (Agilent

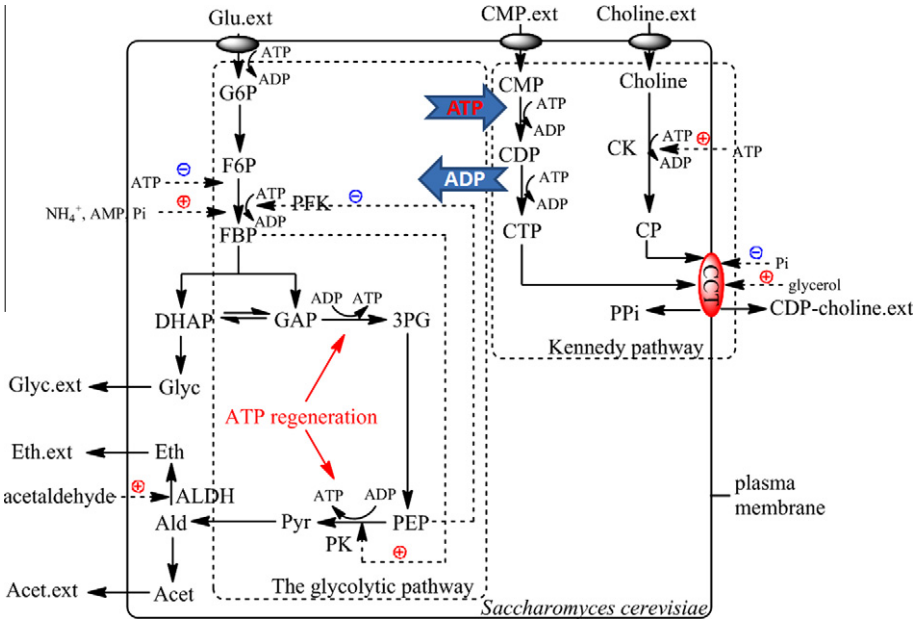

Fig. 1. Schematic representation of the metabolic pathway involved in CDP-choline production, the known regulation modes (dashed lines) and their locations within the cellular structure of *S. cerevisiae*.

G1314B VWD, USA, 280 nm). The mobile phase was 0.6% (v/v) phosphoric acid (the mixture was adjusted with triethylamine to pH 6.6) and the flow rate was 1.0 mL per min. The procedure was performed at 25 °C.

The glucose concentration was measured with a glucose oxidase electrode (Institute of Biology, Shandong Academy of Sciences SBA-40C). Ethanol and glycerol were analyzed by gas chromatography (SP-6890, LuNanRuiHong, Shangdong, China) in a column packed with diphenyl-polysiloxane copolymer (Agilent; HP-5, USA) and detected using a flame ionization detector. The injector temperature was 200 °C, and the temperature of the column was 190 °C. The detector temperature was 280 °C, and the carrier gas was nitrogen.

CTP: phosphorylcholine cytidyltransferase (CCT) activity was assayed by the method of Jiapeng et al. (Tang et al., 2009).

2.4. Utilization efficiency of energy (UEE)

Utilization efficiency of energy is defined as Eq. (1):

$$\mathrm{UEE} = \frac{n_{\mathrm{ATP/P}}}{n_{\mathrm{ATP/E}}} \tag{1}$$

where $n_{\mathrm{ATP/P}}$, the ATP demand quantity for the formation of products; $n_{\mathrm{ATP/E}}$, the ATP regeneration amount from energy source during the metabolic pathway. In this case, glucose was an energy source, and one glucose molecule can regenerate two ATP molecules via glycolysis under anaerobic conditions. The biosynthesis of one CDP-choline from CMP and choline chloride demands three ATP molecules. Therefore, Eq. (1) was considered as Eq. (2):

$$\mathrm{UEE} = \frac{3n_{\text{CDP-choline}}}{2n_{\mathrm{glucose}}} \tag{2}$$

2.5. Experimental design

2.5.1. Plackett–Burman design

Plackett–Burman experimental design (Plackett and Burman, 1946) was first carried out, which was based on the first-order model Eq. (3):

$$Y = \beta_0 + \sum \beta_i x_i \tag{3}$$

This was used to screen the important variables that influenced CDP-choline production by *S. cerevisiae*. Based on the results of an earlier study (Tang et al., 2009), eight variables (concentrations of CMP and choline chloride (the molar ratio of CMP to choline chloride, 1:2), glucose·H_2O, KH_2PO_4, $MgSO_4$, acetaldehyde, yeast, glycerol and $(NH_4)_2SO_4$) were denoted as numerical factors and investigated at two widely spaced intervals designated as −1 (low level) and +1 (high level) (Table 1). Eight variables were screened in 12 experimental runs. The experimental design is shown in Table 2. The *P*-value (significance level) of each concentration effect was determined using the Student's *t*-test:

$$t_{(\mathrm{xi})} = \frac{E_{(\mathrm{xi})}}{\mathrm{S.E.}} \tag{4}$$

where $E_{(\mathrm{xi})}$ was the effect of variable (A–H). The standard error (S.E.) of the concentration effect was the square root of the variance. The variables with significance levels greater than 95% were considered to significantly influence CDP-choline production.

2.5.2. Steepest ascent-tool-path

The direction of steepest ascent is the direction in which *Y* increases more rapidly. The most efficient direction in which to move the experiment is along the line perpendicular to the contours. One usually takes as the path of steepest ascent the line through the center of the region of interest and normal to the fitted surface (Box et al., 1978). Thus, the steps along the path are proportional to the regression coefficients β_i. The path of steepest ascent started from the optimal point of the first design. To move away from the first design center along the path of steepest ascent, we moved 0.12, −0.20, −1.0 in the direction of KH_2PO_4, glycerol and $(NH_4)_2SO_4$, respectively (Table 3). These new units were determined according to the concentration range of unity level from first design and the estimated coefficient ratio from the first-order model Eq. (3).

2.5.3. Central composite design

In order to maximize the response yield of CDP-choline, the independent variables X_1 (phosphate source concentration:

Table 1
Range of different reaction components in the Plackett–Burman design.

Variable	Levels	
	Low (−1)	High (+1)
(A) CMP (g/L)	8	12
(B) Glucose·H_2O (g/L)	26.7	33.3
(C) KH_2PO_4 (g/L)	16	24
(D) $MgSO_4$ (g/L)	10	16
(E) Acetaldehyde (g/L)	6.0	8.0
(F) Yeast (g/L)	200	300
(G) Glycerol (g/L)	10	15
(H) $(NH_4)_2SO_4$ (g/L)	1.0	2.0

Table 2
The experimental design using the Plackett–Burman method for screening of variables.

Run	Variables[a]								The CDP-choline yield[b] (%)
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	
1	+1	−1	+1	−1	−1	−1	+1	+1	46.54 ± 0.83
2	+1	+1	−1	+1	−1	−1	−1	+1	46.36 ± 1.00
3	−1	+1	+1	−1	+1	−1	−1	−1	84.43 ± 0.11
4	+1	−1	+1	+1	−1	+1	−1	−1	83.28 ± 1.64
5	+1	+1	−1	+1	+1	−1	+1	−1	46.39 ± 1.17
6	+1	+1	+1	−1	+1	+1	−1	+1	62.25 ± 1.27
7	−1	+1	+1	+1	−1	+1	+1	−1	61.91 ± 1.41
8	−1	−1	+1	+1	+1	−1	+1	+1	47.24 ± 0.87
9	−1	−1	−1	+1	+1	+1	−1	+1	45.36 ± 2.00
10	+1	−1	−1	−1	+1	+1	+1	−1	47.22 ± 0.93
11	−1	+1	−1	−1	−1	+1	+1	+1	23.63 ± 0.93
12	−1	−1	−1	−1	−1	−1	−1	−1	61.20 ± 1.79

[a] See Table 1.
[b] The CDP-choline yield means the quotient between the amounts of CDP-choline and CMP.

Table 3
Experimental results of the path of steepest ascent.

Trial	Factors			The CDP-choline yield (%)
	KH_2PO_4 (g/L)	Glycerol (g/L)	$(NH_4)_2SO_4$ (g/L)	
Origin	20.00	12.50	1.50	73.53 ± 0.89
1	20.12	12.30	0.50	84.36 ± 1.76
2	20.24	12.10	0	80.19 ± 0.77
3	20.36	11.90	0	75.37 ± 2.49
4	20.48	11.70	0	70.52 ± 1.14
5	20.60	11.50	0	65.91 ± 0.98
6	20.72	11.30	0	62.03 ± 1.27
7	20.84	11.10	0	59.68 ± 3.82

KH_2PO_4), X_2 (protectant concentration: glycerol), and X_3 (glycolysis stimulator concentration: $(NH_4)_2SO_4$) were simultaneously varied according to a central composite design (CCD) scheme (Table 4). A total of 16 experiments were thus performed (Table 4). The central point of CCD was replicated twice (experiment Nos. 15, 16, 17 and 18) (Table 4). Results from CCD were used for calculating, where Y is the selected response (dependent variable), $X_1 \ldots X_i$ are the independent variables being optimized (X_1 = phosphate source concentration: KH_2PO_4, X_2 = protectant concentration: glycerol and X_3 = glycolysis stimulator concentration: $(NH_4)_2SO_4$) and b_i, b_{ii} and b_{ij} are the linear, quadratic and cross-coefficients, respectively.

$$Y = b_0 + \sum b_i x_i + \sum\sum b_{ij} x_{ij} + \sum b_{ii} x_{ii}^2 \quad (5)$$

The above equation was calculated to estimate the effect of each independent variable ($X_1 \ldots X_i$) on response yield of CDP-choline (dependent variables). This calculation was aimed at predicting the best combination of independent variables for optimization of the reaction conditions. Accordingly, the best combination of X_1 (KH_2PO_4), X_2 (glycerol) and X_3 ($(NH_4)_2SO_4$) to maximize the yield of CDP-choline was obtained by using the above second-order polynomial Eq. (5).

2.6. Data analysis

Data were analyzed using "Statistica" software (Version 6.0.437.0, Statsoft Inc., Tulsa, USA) that includes ANOVA (analysis of variance) to carry out analysis of variance and determination of interactions between variables and responses.

3. Results

3.1. Screening of important reaction components using a Plackett–Burman design

Plackett–Burman experiments (Table 2) showed a wide variation in measured CDP-choline yields. This variation reflected the importance of optimization to attain higher CDP-choline yields. The main effects of the factors on CDP-choline yield are presented in Table 5. When the sign of the concentration effect of the tested variable is positive, the influence of the variable upon CDP-choline yield is greater at a high concentration, and when negative, the influence of the variable is greater at a low concentration. Analyzed by Statistica software, a first-order model was fitted to the data obtained from the experiments. The effects of the eight factors were calculated. The following model was obtained for the coded variables.

Table 4
Optimization of the reaction components for improvements of the CDP-choline production using central composite design.

Trial	Factors[a]			The CDP-choline yield (%)
	X_1 (g/L)	X_2 (g/L)	X_3 (g/L)	
1	20.00	12.00	0.40	42.22 ± 0.97
2	20.00	12.00	0.60	41.42 ± 0.94
3	20.00	12.60	0.40	52.56 ± 1.08
4	20.00	12.60	0.60	53.38 ± 0.58
5	20.24	12.00	0.40	41.23 ± 1.48
6	20.24	12.00	0.60	39.91 ± 0.79
7	20.24	12.60	0.40	63.31 ± 0.83
8	20.24	12.60	0.60	37.62 ± 1.00
9	19.92	12.30	0.50	66.42 ± 0.11
10	20.32	12.30	0.50	68.73 ± 0.58
11	20.12	11.80	0.50	24.45 ± 0.31
12	20.12	12.80	0.50	38.03 ± 0.79
13	20.12	12.30	0.33	42.07 ± 0.44
14	20.12	12.30	0.67	37.24 ± 0.24
15(C)	20.12	12.30	0.50	87.89 ± 0.64
16(C)	20.12	12.30	0.50	88.25 ± 2.71
17(C)	20.12	12.30	0.50	88.15 ± 1.20
18(C)	20.12	12.30	0.50	88.52 ± 0.57

[a] X_1, KH_2PO_4; X_2, Glycerol; X_3, $(NH_4)_2SO_4$.

Table 5
T-test, coefficients and significance levels calculated from the CDP-choline yield obtained in the screening experiments.

Factor	Effect	S.E.	Coefficient	*t*	Pr > |*t*|
A	0.073	0.843	0.02	0.087	0.9448
B	−1.975	0.730	−0.30	−2.704	0.2255
C	17.943	0.843	2.24	21.277	0.0299*
D	−0.427	0.843	−0.07	−0.506	0.7018
E	0.357	0.843	0.18	0.423	0.7452
F	−2.415	0.730	−0.02	−3.307	0.1869
G	−19.322	0.730	−3.86	−26.455	0.0241*
H	−19.838	0.730	−19.84	−27.163	0.0234*

R^2 = 0.9995.
* Statistically significant at 95% probability level.

3.2. First-order model equation

$$Y = 103.014 + 0.0183A - 0.299B + 2.243C - 0.071D + 0.178E - 0.024F - 3.864G - 19.838H \quad (6)$$

This fit of the model was checked by *t*-test. For CDP-choline production, the effect of variables *C* (KH_2PO_4), *G* (glycerol), and *H* ($(NH_4)_2SO_4$) are 17.943, −19.322 and −19.838, respectively (Table 5). These variables had confidence levels above 95% and were considered to influence CDP-choline production by *S. cerevisiae* As 2.398 significantly.

3.3. Path of steepest ascent

Based on the first-order model equation obtained and the three important effect factors (KH_2PO_4, glycerol and $(NH_4)_2SO_4$), the path of steepest ascent was determined to find the correct direction of the changing variables increasing or decreasing the concentration according to the sign of the main effects to improve CDP-choline production. The path of steepest ascent started from the center of the Plackett–Burman design and moved along the path in which the concentration of KH_2PO_4 increased, while glycerol and $(NH_4)_2SO_4$ decreased. The design and results of the path of steepest ascent experiments are shown in Table 3. It was shown that the highest response was 84% when the reaction medium contained: KH_2PO_4 20.12 g/L, glycerol 12.30 g/L and $(NH_4)_2SO_4$ 0.50 g/L. This suggested that this point was near the region of maximum yield response.

3.4. Central composite design and response surface analysis

The level of CDP-choline yield after application of the CCD scheme is shown in Table 4. An average value of the central point of CCD was 88.20%. According to the second-order polynomial Eq. (5), the data reported in Table 6 were converted into the second-order polynomial equation, shown below:

$$\text{Yield} = -229321 + 19127X1 + 5661X2 + 8423X3 - 469X12 - 220X22 - 1618X32 - 9X1X2 - 282X1X3 - 95X2X3 \quad (7)$$

where X_1, KH_2PO_4; X_2, glycerol; and X_3, $(NH_4)_2SO_4$. The statistical significance of Eq. (7) was checked by *F*-test, and the analysis of variance (ANOVA) for response surface quadratic model is summarized in Table 6. It is evident from Table 6 that the model is highly significant, as is evident from the model *F* value and a very low probability value (*P* model, $F < 0.00019$). The *P*-values were used

Table 6
Results of ANOVA for the central composite design.

Factor[a]	Degree of freedom	Sum of squares	Mean square	*F* value	Pr > *F*
X_1	1	0.988	0.988	0.0569	0.00046*
X_1^2	1	561.273	561.273	32.2988	0.00046*
X_2	1	309.033	309.033	17.7835	0.00021*
X_2^2	1	4837.943	4837.943	278.4029	0.00000*
X_3	1	89.921	89.921	5.1746	0.01074*
X_3^2	1	3436.179	3436.179	197.7374	0.00000*
X_1X_2	1	0.788	0.788	0.0453	0.83675
X_1X_3	1	91.328	91.328	5.2555	0.05107
X_2X_3	1	64.695	64.695	3.7229	0.08979
Model	9	7431.167	825.685	47.5160	0.00019*
Error	6	139.020	17.377		
Total	15	7570.187			

R^2 = 0.9816; Adj-R^2 = 0.9610; R = 0.9908.
[a] X_1 = KH_2PO_4, g/L; X_2 = glycerol, g/L; X_3 = $(NH_4)_2SO_4$, g/L.
* Statistically significant at 95% probability level.

as a tool to check the significance of each coefficient, which also indicated the interaction strength between each independent variable. The smaller the *P*-values, the bigger the significance of the corresponding coefficient (Liu et al., 2003). Table 6 shows that, the regression coefficients of all the quadratic coefficients and linear term (X_2) were significant ($P < 0.05$).

The goodness of the model can be checked by the determination coefficient R^2 and the multiple correlation coefficient R. The value of adjusted R^2 (0.9610) for Eq. (7) suggests that the total variation of 96% for CDP-choline yields is attributed to the independent variables and only about 4% of the total variation cannot be explained by the model. The closer the values of R to 1, the better the correlation between the experimental and predicted values. Here, the value of R (0.9908) indicates good agreement between the experimental and predicted value of CDP-choline yields.

As a result, response surface diagrams for yield of CDP-choline were plotted as a function of the variables X_1 (KH_2PO_4), X_2 (glycerol) and X_3 ($(NH_4)_2SO_4$) (Figs. 2–4).

The best combination of variables X_1 (KH_2PO_4), X_2 (glycerol) and X_3 ($(NH_4)_2SO_4$) for maximizing the response were found (X_1 = 20.13 g/L, X_2 = 12.35 g/L and X_3 = 0.49 g/L, respectively).

3.5. Validation of the model

In order to confirm the optimization results, the yield of CDP-choline was studied using predicted reaction components. The result from three replications (i.e., 87.12%, 88.11% and 87.58%) was consistent with the predicted value and the model was proven to be adequate. Fig. 5 shows the profile of glucose concentration, glycerol concentration, CDP-choline concentration, ethanol concentration, choline chloride conversion yield and energy utilization efficiency of the reaction in the 7.5-L bioreactor before and after optimization. Under the optimal conditions, CMP conversion yield and CDP-choline concentration reached 85.55% and 13.50 g/L, respectively. The energy utilization efficiency and choline conversion yield reached 15.72% and 42.78%, respectively. The final medium composition optimized with response surface methodology was (g/L): CMP, 10; choline chloride, 8.64; glucose·H_2O, 30; KH_2PO_4, 20.13; $MgSO_4$, 13; acetaldehyde, 7; wet yeast cells, 250; glycerol, 12.35; $(NH_4)_2SO_4$, 0.49.

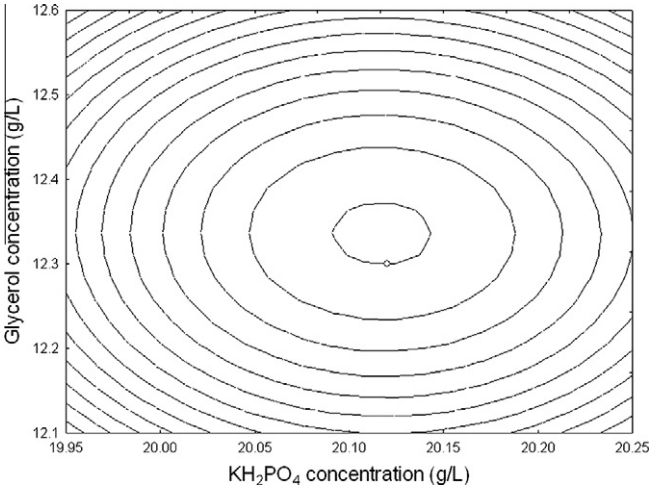

Fig. 2. Response surface contour for CDP-choline production by *S. cerevisiae* As 2.398 as a function of KH_2PO_4 and glycerol concentration, when the $(NH_4)_2SO_4$ concentration was 0.49 g/L.

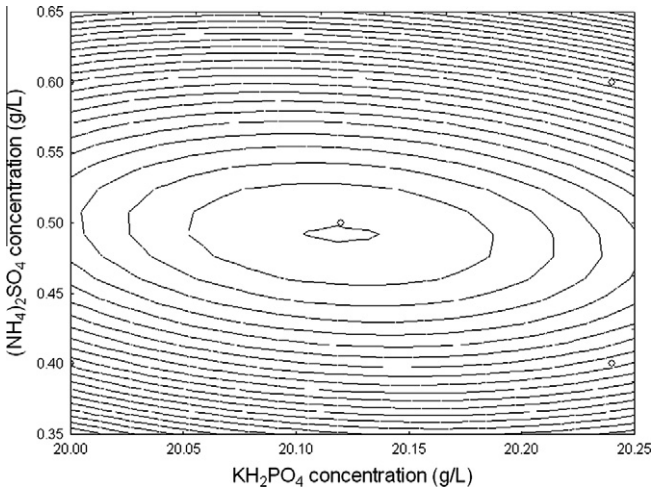

Fig. 3. Response surface contour for CDP-choline production by *S. cerevisiae* As 2.398 as a function of KH_2PO_4 and $(NH_4)_2SO_4$ concentration, when the glycerol concentration was 12.35 g/L.

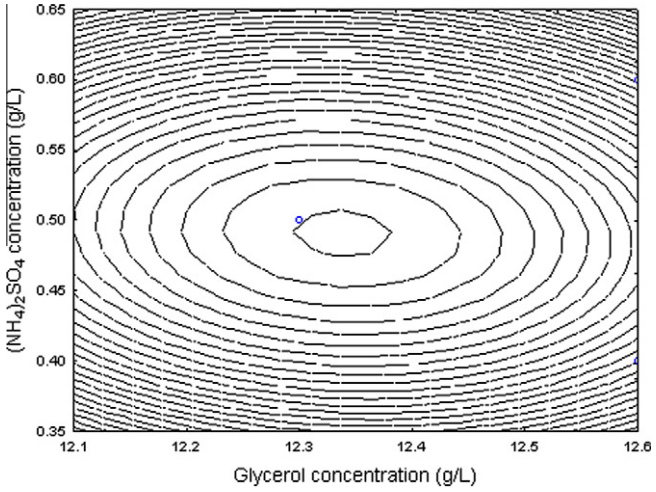

Fig. 4. Response surface contour for CDP-choline production by *S. cerevisiae* As 2.398 as a function of glycerol and $(NH_4)_2SO_4$ concentration, when the KH_2PO_4 concentration was 20.13 g/L.

4. Discussion

The statistical experimental results may suggest that the importance of $(NH_4)_2SO_4$ in modulating CDP-choline production could probably be explained in terms of the activation/inhibition of spe-

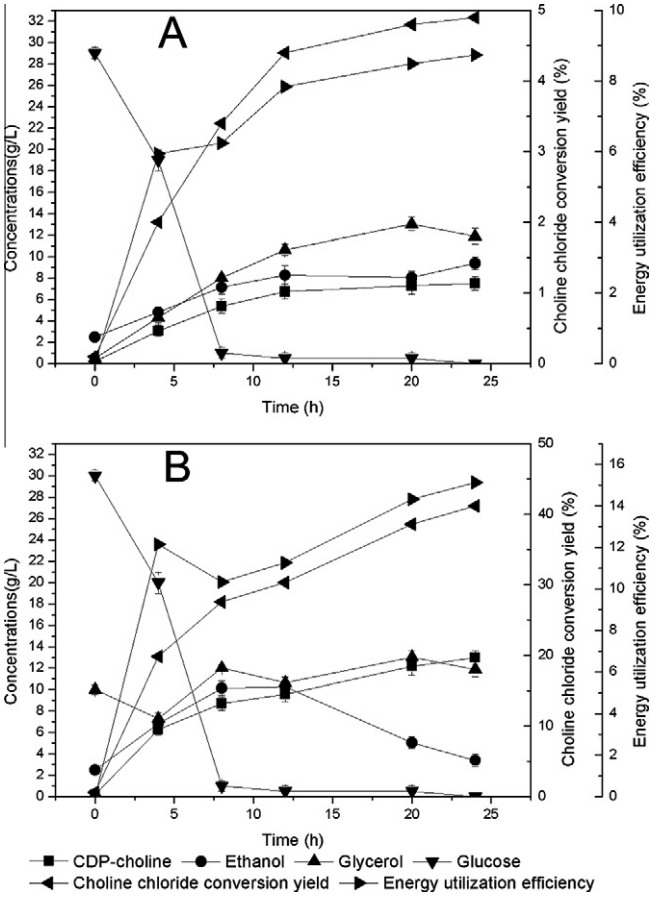

Fig. 5. Profile of CDP-choline concentration, ethanol concentration, glycerol concentration, glucose concentration, choline chloride conversion yield and energy utilization efficiency before and after optimization. (A) Before optimization. (B) After optimization.

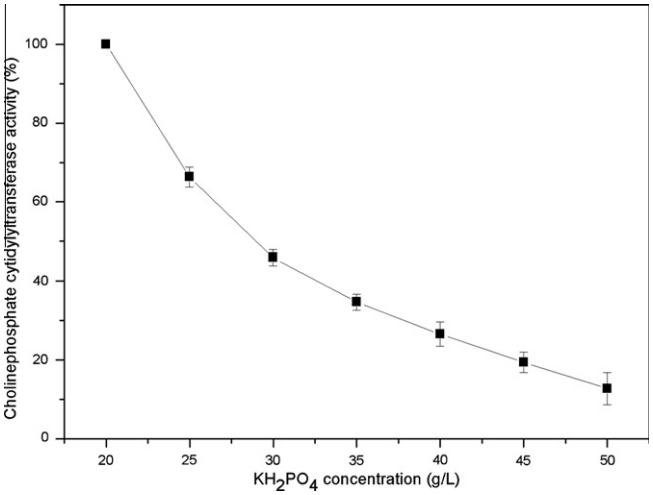

Fig. 6. Effect of KH_2PO_4 concentration on the CCT activity.

cific enzymes involved in this biochemical pathway. Ammonium ion not only strengthened the glycolytic pathway (Papagianni et al., 2005), but also coordinated the reaction rate between the glycolytic pathway and the Kennedy pathway (Tang et al., 2009). Intracellular acetaldehyde can leave the cell resulting in slower regeneration of NAD^+ and slower the rate of glycolysis. The stimulatory effect of exogenous acetaldehyde may therefore lie in its ability to replenish the intracellular acetaldehyde pool and restore the cellular redox balance (Stanley et al., 1997). Therefore, ammonium ions and acetaldehyde increased the glycolytic flux. As shown in Fig. 1, the improvement of glycolytic flux enhanced ATP regeneration. An increase of ATP supply could activate the activity of choline kinase to strengthen the phosphorylation of choline (Kim et al., 1998). The phosphorylation of choline matched that of CMP to promote CDP-choline production and energy utilization efficiency.

In contrast, the ability to optimize CDP-choline yield as a response to KH_2PO_4 (from 20 to 20.13 g/L), could be justified by its well-documented role as a phosphate source for ATP regeneration and CDP-choline synthesis. It has been shown that one CDP-choline molecule needed three inorganic phosphates, and the supply of inorganic phosphate directly influenced the yield of CDP-choline (Watanabe et al., 1981). Furthermore, phosphate can activate phosphofructokinase and accelerate the glycolytic flux (Hofmann and Kopperschläger, 1982); however, inorganic phosphate greatly inhibited the activity of CCT (Weinhold et al., 1986; Weinhold and Feldman, 1992). As shown in Fig. 6, CCT activity decreased with increasing concentrations of phosphate salts. Accordingly, the CDP-choline yield was highly sensitive to the concentration of inorganic phosphate. Considering to the positive (Hofmann and Kopperschläger, 1982) and negative effects (Weinhold et al., 1986; Weinhold and Feldman, 1992) of phosphate, the optimal concentration of KH_2PO_4 was 20.13 g/L.

Of the three primary factors influencing CDP-choline yield, glycerol was the most significant (Table 6). The Kennedy research group described CCT as lipid-activated and this enzymatic step is rate-limiting and regulatory in CDP-choline biosynthesis (Fiscus and Schneider, 1966; Sleight and Kent, 1980; Vance et al., 1980). Therefore, the plasma membrane, which CCT was bound to, was damaged by the high osmolarity (Stefan et al., 2007); however, excess glycerol increases the viscosity of the reaction system and decreases the mass transfer efficiency (Arvia et al., 1966), which leads to the low CDP-choline yield. An appropriate amount of glycerol in the reaction mixture could balance the extra- and intracellular osmotic pressure (Ben-Amotz and Avron, 1973; Ohshiro and Yagi, 1996), protect the spatial configuration of the membrane and CCT, and maintain the activity of the enzyme (Tang et al., 2009).

The efficient CDP-choline production depended on the system which quickly supplied enough ATP. If the rate of ATP regeneration system could match that of the phosphorylated compounds production system, a high efficiency ATP regeneration system directly would enhance the product yield and the productivity. The addition of small molecule effectors (ammonium ion, glycerol and acetaldehyde) improved the efficiency of ATP regeneration and enhanced the activity of CDP-choline production system. Thus, high-utilization efficiency of energy and a high choline conversion yield could be achieved.

5. Conclusions

A high-utilization ATP regeneration system in whole cells of *S. cerevisiae* was successfully employed to improve the production of cytidine 5′-diphosphorylcholine. The increase of the EMP flux, the acceleration of NAD^+ regeneration and key enzymes protection in the native state together increased energy utilization efficiency and choline chloride conversion yields. Therefore, the methods which could be used to match the rate of ATP regeneration system with ATP utilization system, can be applied in an attempt to the field of the biosynthesis of high-energy phosphorylated compounds and their derivatives in a whole-cell catalyst reaction, as well as oligosaccharide, cell-free protein and glutathione, which

need to use high-energy phosphorylated compounds as energy donors.

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