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Enhanced adenosine triphosphate production by *Saccharomyces cerevisiae* using an efficient energy regeneration system

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Abstract−The process of ATP biosynthesis from adenosine catalyzed by *Saccharomyces cerevisiae* was studied using an efficient energy regeneration system. A fractional factorial design (2^{9-5}) was used to evaluate the effects of different components in the medium. Magnesium chloride, toluene, and acetaldehyde were found to significantly influence ATP production. The concentrations of the three factors were then optimized using central composition design and response surface analysis. Based on the second-order polynomial model obtained from the experiments, the optimal parameters were obtained as follows: adenosine 20 g/L; glucose 67 g/L; *S. cerevisiae* cells 250 g/L; magnesium chloride 4.37 g/L; potassium dihydrogen phosphate 67 g/L; toluene 1.40 mL/L; acetaldehyde 2.67 mL/L; pH 7.0; and temperature 37.0 °C. Under the condition, the yield and concentration of ATP reached 97.5% and 37 g/L, respectively. The yield was nearly 10% higher than the level before optimization and the concentration increased two-fold. In addition, the utilization efficiency of energy after optimization increased nearly 6%.

Key words: Adenosine Triphosphate, Optimization, Response Surface Methodology, *Saccharomyces cerevisiae*, Utilization Efficiency of Energy

INTRODUCTION

Nucleotide and its derivatives, widely used as taste-enhancing food additives or pharmaceutical intermediates, are an essential material in the synthesis of carbohydrates, which are of intense interest in various scientific fields [1,2]. As a kind of nucleotide, ATP is the focus of many studies and largely synthesized [3-5]. The demand and supply of ATP could affect many complicated physiology processes, such as active transportion [6], peptide folding [7], subunit assembly [8], signal transduction [9,10] and so on. ATP is involved in many metabolic pathways and production of almost all of the metabolites by industrial strains [2]. In clinics, ATP has been widely used in the treatment of heart, brain and vascular diseases as an important energy donor.

Biocatalysis approaches for ATP synthesis include the gene engineering method and the enzymatic method, in which the phosphate groups of ATP and adenosine derivatives are catalyzed by phosphokinase (Fig. 1). Adenosine monophosphate (AMP) or adenosine is used as a substrate and whole-cell yeast is used as the enzyme source in the enzymatic method, which results in a 90% yield and a 20 g/L level of ATP [11-13]. Owing to the multi-enzyme system of the cells and the original activity of the enzymes, the enzymatic method can perform multi-step enzymatic reactions rapidly and effectively. However, its utilization efficiency of energy (UEE) is low, only about 25%. On the other hand, since the 1980s, researchers have used gene cloning and cell fusion technology to improve microbial glucolytic ability and adenylate phosphorylase activity and increase the production capacity [14-17]. The yield of ATP reaches 70-90%, and the ATP concentration reaches 20-100 g/L. These results show that recombinant DNA technology has an effective role in the improvement of glucolytic ability. However, this technology needs further study to develop the different catalytic functions of the multi-enzyme system.

ATP yield and UEE using yeast are still lower according to the above. This may be because the yeast is in a high osmotic environment, which will accumulate glycerol to keep the balance of osmotic pressure inside and outside of the cells [18]. Also, the synthesis of glycerolis is used to eliminate a surplus of reducing power, which means the glycerol pathway will compete for metabolic flux with the alcoholic fermentation pathway [19,20]. As we know, the glycerol pathway does not produce ATP, which leads to a waste of glucose.

Intracellular NADH/NAD^+ and ATP levels have important roles in the glycolytic pathway [5,21]. The low energy state in the cell will increase the activity of key glycolytic enzymes [22], such as phosphofructokinase (PFK) and pyruvate kinase (PK). However, the increase in glycolytic pathway flux needs enough NAD^+, the shortage of which will inhibit the glycolytic pathway [23]. With a lack of oxygen or oxidant factor, NAD^+ will regenerate via the ethanol pathway using acetaldehyde as an electron acceptor [19]. Therefore, to change the metabolic flux, enhance the ATP generation and improve the substrate-level phosphorylation, acetaldehyde and other effectors will be investigated in this study. In addition, under normal physiological conditions of the yeast cells, ATP cannot be secreted across the cell membrane and accumulates in the extracellular region owing to the polarity, so some appropriate membrane penetrants will be added.

In this study, an efficient energy regeneration system was designed and the optimal conditions of ATP synthesis from adenosine by *Saccharomyces cerevisiae* were investigated. As the factors in the pro-

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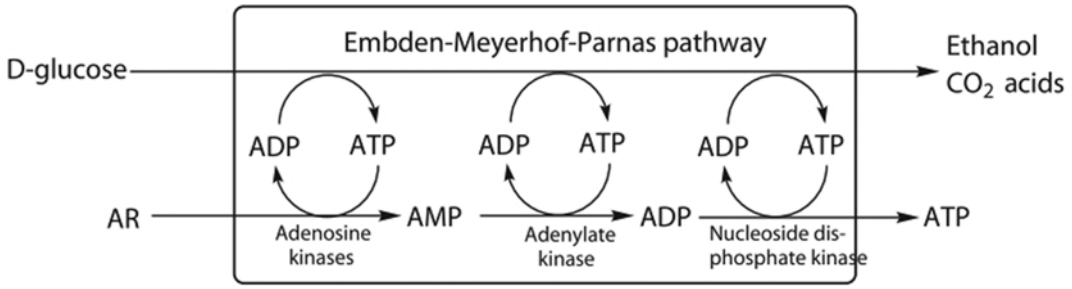

Fig. 1. The biosynthesis of adenosine triphosphate from adenosine.

cess of ATP production have interactions and complex relationships, fractional factorial design and response surface method were used to determine the optimal conditions for ATP synthesis and improve the yield and UEE. And the application of this technique on optimizing conditions of ATP production with *Saccharomyces cerevisiae* has never been reported before in the literature. This method would allow production of other active substances that need ATP as energy, such as S-adenosyl-L-methionine (SAM), glutathione [24], penicillin and its derivatives [25], poly-amino acids [26], and polysaccharides [27].

MATERIALS AND METHODS

1. Strain and Medium

Saccharomyces cerevisiae As2.398 preserved in the Nanjing University of Technology Lab was used for production of ATP from adenosine in this study. The growth medium contained 5% glucose, 0.5% peptone, 0.2% yeast extract, 0.2% $NH_4H_2PO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.2% KH_2PO_4 at an initial pH of 5.8. The culture medium was maintained at 30 °C for 72 h.

2. Preparation of Yeast Cells

The yeast cells were harvested aseptically by the vacuum filtration method at 4 °C, and washed twice with deionized water. The wet cells were frozen and stored at −20 °C.

3. Biocatalytic Reaction

The biocatalytic reaction mixture contained *Saccharomyces cerevisiae*, glucose, inorganic salt, and other required components; precise compositions are described in the "Results and discussion" section. The reactions were performed in 500-mL flasks containing 300 mL of the reaction mixture with shaking on a thermostat-controlled water bath at 60 rpm and 37 °C for 7 h.

4. Analytical Method

Aliquots of the reaction were centrifuged at 10,000 rpm for 10 min and the supernatant was used for determination of ATP. High-performance liquid chromatography (HPLC, Agilent 1100 system with a UV detector) was performed using a Lichrospher C18 column (4.6 mm×300 mm, 5 μm), methanol 0.05 mol/L dipotassium phosphate solution (3 : 97, v/v) as the mobile phase and a flow rate of 1.0 ml/min^{-1} at room temperature. The detection wavelength was 254 nm.

5. Fractional Factorial Design (FFD)

It has been shown that FFD can substantially reduce test times and estimate the main effect of each fraction and fractional interactions [28,29]. In our study, a 2^{9-5} FFD with nine factors at two levels was required. Each factor was studied at two different levels (−1, +1) and a set of 16 experiments were performed (Tables 1 and 2).

Table 1. Levels of the variables tested in the experimental design

Factor	Levels of factors	
	+1	−1
X_1	30	10
X_2	92	42
X_3	300	200
X_4	6	3
X_5	92	42
X_6	2	1
X_7	4	2
X_8	8	6
X_9	39	35

X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, and X_9 represent coded variales of adensine (g/L), glucose (g/L), yeast (g/L), magnesium chloride (g/L), potassium dihydrogen phosphate (g/L), toluene (mL/L), acetaldehyde (mL/L), pH and temperature (°C), respectively

The first-order model used to fit the results of fractional factorial design was represented as:

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

where Y is the predicted response; β_0 is the intercept; β_i is the linear coefficient and x_i is the coded independent factor.

6. Central Composite Design and Response Surface Methodology

Response surface methodology is a statistical method that can be used to study the optimum conditions under which the interactions between factors reach the maximum response value [30-32]. In this study, FFD experiments determined three key factors with important effects on response value. The three independent factors were studied in one block and a set of 16 experiments were performed. The behavior of the system was explained by the following second-order polynomial equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \qquad i=1, 2, \ldots, k \quad (2)$$

where Y is the predicted response, β_0 is the intercept, x_i and x_j are the coded independent factors, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

Design expert version 6.0 (STATEASE Inc., Minneapolis, USA) was used for experimental designs and regression analysis of the experimental data obtained.

7. Utilization Efficiency of Energy (UEE)

Utilization efficiency of energy is defined as the ratio between

Table 2. Experimental design and results of the 2^{9-5} fractional factorial design

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	ATP yield (%)	
										Observed	Predicted
1	−1	−1	−1	−1	−1	−1	−1	−1	1	39.34	40.92
2	1	−1	−1	−1	1	−1	1	1	−1	18.78	21.01
3	−1	1	−1	−1	1	1	−1	1	−1	70.66	63.01
4	1	1	−1	−1	−1	1	1	−1	1	42.11	43.42
5	−1	−1	1	−1	1	1	1	−1	−1	38.33	36.46
6	1	−1	1	−1	−1	1	−1	1	1	72.50	70.00
7	−1	1	1	−1	−1	−1	1	1	1	33.95	33.56
8	1	1	1	−1	1	−1	−1	−1	−1	41.90	49.20
9	−1	−1	−1	1	−1	1	1	1	−1	44.51	51.81
10	1	−1	−1	1	1	1	−1	−1	1	80.11	79.72
11	−1	1	−1	1	1	−1	1	−1	1	45.79	43.29
12	1	1	−1	1	−1	−1	−1	1	−1	66.43	64.56
13	−1	−1	1	1	1	−1	−1	1	1	68.56	69.87
14	1	−1	1	1	−1	−1	1	−1	−1	45.65	38.00
15	−1	1	1	1	−1	1	−1	−1	−1	77.78	80.01
16	1	1	1	1	1	1	1	1	1	70.78	72.36

the ATP consumed by product synthesis and the ATP produced when all of the glucose is converted to ethanol.

$$UEE = \frac{3n_{ATP}}{2n_{glucose}} \quad (3)$$

where n_{ATP}, the amount of ATP produced; $n_{glucose}$, the amount of glucose used in the process. One glucose molecule could produce two ATP molecules via glycolysis under anaerobic conditions, and the biosynthesis of one ATP from adenosine required three ATP molecules.

RESULTS AND DISCUSSION

1. Fractional Factorial Design

The effects of the following factors on ATP production were determined: adenosine, glucose, yeast, magnesium chloride, potassium dihydrogen phosphate, toluene, acetaldehyde, pH, and temperature. These nine components were selected on the basis of preliminary experiments and literature. L16 (2^9) orthogonal was used to arrange fractional factorial experiments with nine factors at two levels, and the results are shown in Table 2.

The regression coefficients and determination coefficients (R^2) for the linear regression model of ATP yield are presented in Table 3. In addition, the function of the coded levels of all factors was obtained:

$$Y=53.57+1.21X_1+2.60X_2+2.61X_3+8.88X_4 +0.79X_5+8.52X_6-11.08X_7+2.20X_8+3.07X_9 \quad (4)$$

The determination coefficient (R^2) for the regression model of ATP production was 0.949, indicating that the analysis results were reliable. Table 3 shows that magnesium chloride, toluene and acetaldehyde had a significant effect on ATP production ($p \leq 0.05$). When X_4 and X_6 were near maximal levels and X_7 was near minimal level, it was beneficial to improve the ATP yield. According to the effect trends of X_4, X_6 and X_7, other factors that had little influences took fixed values at zero level as follows, respectively: X_1, 2; X_2, 67; X_3, 250; X_5, 67; X_8, 7.0; X_9, 37.0. Response surface design was then applied using X_4, X_6 and X_7 as variables.

Table 3. Results of the regression analysis of the 2^{9-5} experimental design

Term	Regression analysis		
	Coefficient	*T*-value	*P*-value
Intercept	53.5738	32.44099	0.000000
X_1	1.2088	0.73195	0.491790
X_2	2.6013	1.57516	0.166288
X_3	2.6075	1.57894	0.165428
X_4	8.8775	5.37567	0.001703
X_5	0.7900	0.47838	0.649315
X_6	8.5238	5.16146	0.002092
X_7	−11.0863	−6.71316	0.000531
X_8	2.1975	1.33067	0.231632
X_9	3.0688	1.85825	0.112497

2. Central Composite Design and Response Surface Methodology

According to the factorial design results, magnesium chloride, toluene, and acetaldehyde had significant effects, and a response surface design with three factors at five levels was used to determine the optimal levels. Experimental design and results are shown in Tables 4 and 5.

The results of the regression analysis are shown in Table 6. The fitted second-order polynomial had the following form:

$$Y=93.70-1.61A-10.04A^2+2.17B-12.17B^2-6.28C -15.85C^2+3.06AB-1.46AC+20.83BC \quad (5)$$

where Y is the predicted response, and A, B and C are coded values of magnesium chloride, toluene and acetaldehyde concentrations, respectively.

Table 4. Levels of the factors tested in the central composite design

Factor	Levels of factors				
	+1.68	+1	0	−1	−1.68
$MgCl_2$ (A, g/L)	7.02	6	4.50	3	1.98
Toluene (B, mL/L)	2.34	2	1.50	1	0. 66
Acetaldehyde (C, mL/L)	4.68	4	3	2	1.32

Table 5. Experimental design and results of the RSM together with predicted yields from the model equation

Run	A ($MgCl_2$)	B (Toluene)	C (Acetaldehyde)	ATP yield (%)	
				Observed	Predicted
1	−1	−1	−1	78.52	83.79
2	−1	−1	1	39.89	32.51
3	−1	1	−1	46.03	40.36
4	−1	1	1	70.12	72.38
5	1	−1	−1	80.03	77.36
6	1	−1	1	14.98	20.24
7	1	1	−1	39.21	46.18
8	1	1	1	78.05	72.37
9	−1.68	0	0	64.98	68.07
10	1.68	0	0	65.18	62.67
11	0	−1.68	0	56.19	55.71
12	0	1.68	0	61.94	63.01
13	0	0	−1.68	62.04	59.52
14	0	0	1.68	35.33	38.43
15	0	0	0	93.57	93.70
16	0	0	0	93.94	93.70

Table 6. Regression results of the central composite design

Factor	Regression coefficient	Standard error	*T*-value	*P*-value
Intercept	93.7033	4.716527	19.86701	0.000001[a]
A	−1.6090	1.811430	−0.88823	0.408611
B	2.1730	1.811430	1.19960	0.275512
C	−6.2751	1.811430	−3.46418	0.013399[a]
A^2	−10.0383	2.201053	−4.56068	0.003848[a]
B^2	−12.1695	2.201053	−5.52893	0.001475[a]
C^2	−15.8472	2.201053	−7.19982	0.000363[a]
AB	3.0638	2.365703	1.29507	0.242893
AC	−1.4588	2.365703	−0.61662	0.560136
BC	20.8263	2.365703	8.80341	0.000119[a]

R^2=0.963

[a]Statistically significant at 95% probability level

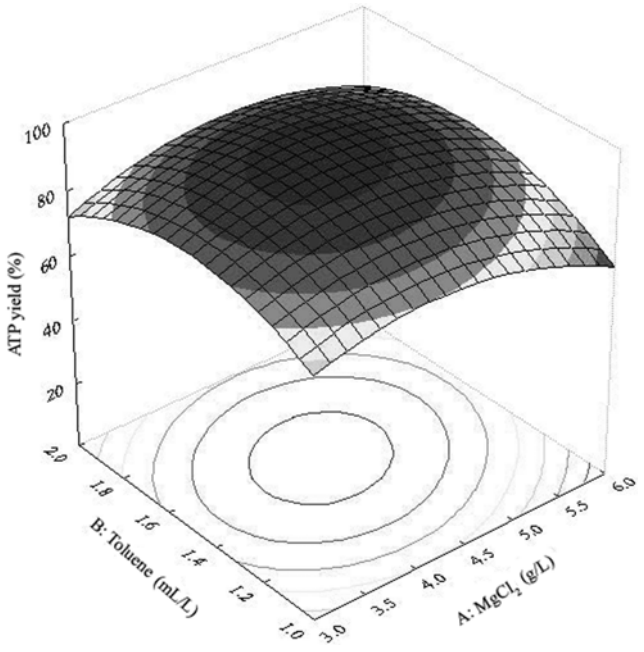

Fig. 2. Response surface curve for ATP production by *S. cerevisiae* as a function of $MgCl_2$ and toluene concentrations, when acetaldehyde concentration was maintained at 3 mL/L.

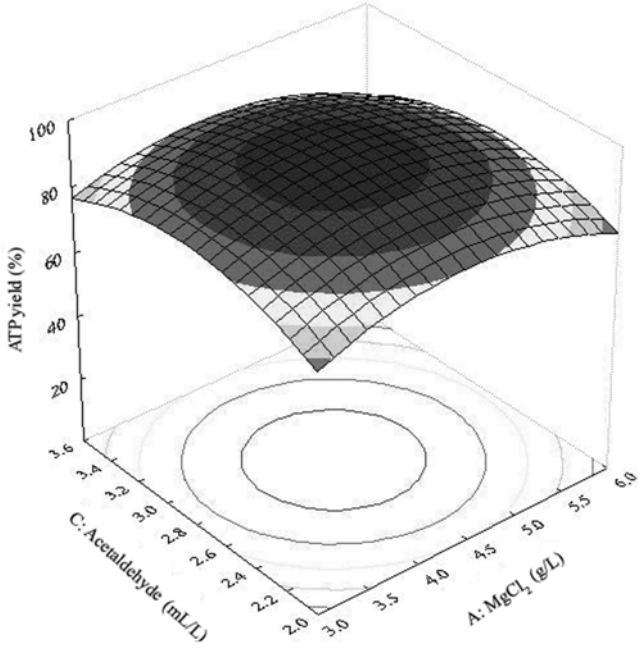

Fig. 3. Response surface curve for ATP production by *S. cerevisiae* as a function of $MgCl_2$ and acetaldehyde concentrations, when toluene concentration was maintained at 1.5 mL/L.

The determination coefficient (R^2) for the equation was 0.963. From equations derived by differentiation of Eq. (5), the model predicted the optimal values of test factors in the coded units were A= −0.087, B=−0.201 and C=−0.326. At these values, the concentrations of magnesium chloride, toluene and acetaldehyde were 4.37 g/L, 1.40 mL/L and 2.67 mL/L, respectively. The model predicted a maximum response of 94.58% with these conditions. Also, the 3D response surface curves were then plotted to present the effect of two factors while the other factor was held at zero level (Figs. 2, 3 and 4). Under the optimal conditions, the practical ATP yield was 97.5%, which was an increase compared with the initial 87.5% and also showed that the second-order mathematical model was credible and effective. In addition, the utilization efficiency of energy after optimization was 32.35%, which was an increase of nearly 6%.

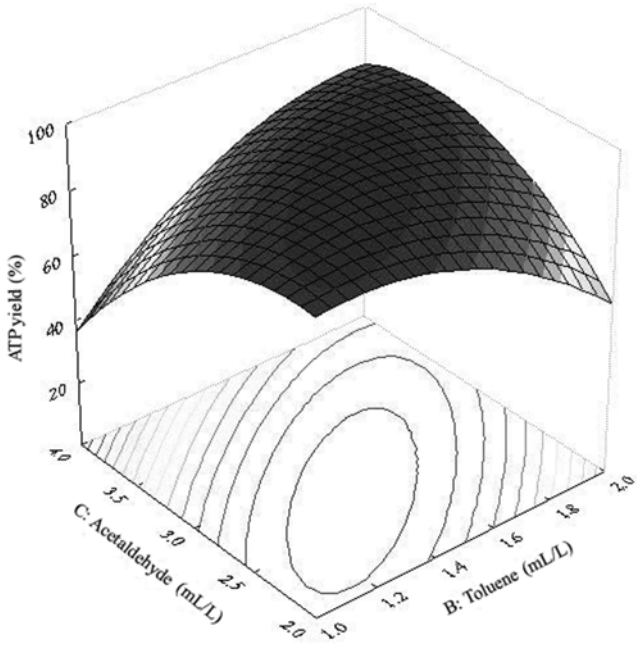

Fig. 4. Response surface curve for ATP production by *S. cerevisiae* as a function of toluene and acetaldehyde concentrations, when $MgCl_2$ concentration was maintained at 4.5 g/L.

The second-order polynomial model indicated that magnesium chloride had a major effect on ATP yield. Magnesium ion was an essential co-factor for ATP production [33] and necessary for the activity of a series of enzymes, such as phosphofructokinase and pyruvate kinase, which catalyzed the key reactions in glycolysis. Depletion of magnesium ion would inhibit the activities of phosphofructokinase and pyruvate kinase, reducing the glycolytic flux and inhibiting the ATP systhesis. However, an excess of magnesium ion had an inhibitory effect [34]. As the addition of more magnesium did not increase the soluble magnesium ion concentration, it would produce insoluble phosphate precipitation with phosphate ion in the reaction mixture, thereby lowering the phosphate concentration and reducing the ATP yield and UEE. Through optimization, it showed that the optimal concentration of magnesium chloride was 4.37 g/L.

Among the three primary factors influencing ATP yield, toluene had the greatest effect (Table 6). When using yeast cells as the enzyme source to synthesize ATP, the cell membrane prevented the transportation of polar substances, thereby increasing mass transfer resistance in the reaction [35]. The addition of toluene to the reaction system could cause permeabilization of the plasma membrane [36]. After permeation treatment, the transportation rates of substrates, products and energy resource (glucose) were improved, increasing the conversion of substrates and the accumulation of products. However, when the concentration of toluene was excessively high, enzymes would be deactivated. Through optimization, it showed that the optimal concentration of toluene was 1.40 mL/L.

On the other hand, excessive intracellular NADH would slow the regeneration of NAD^+, resulting in redox imbalance and a slower glycolysis rate [37,38]. Under low oxygen conditions, a certain concentration of acetaldehyde was added as an exogenous electron acceptor, which could oxide NADH to NAD^+ by alcohol dehydrogenase [39]. By this method, the regeneration of NAD^+ was more rapid, the level of NADH/NAD^+ was maintained and the cellular redox equilibrium recovered. Therefore, acetaldehyde could redirect NADH oxidation to the ethanol pathway from the glycerol pathway and accelerate the regeneration of NAD^+, increasing ATP regeneration effeciency and ATP production yield. By optimization, the optimal concentration of acetaldehyde was shown to be 2.67 mL/L.

CONCLUSION

High biosynthesis of ATP by *Saccharomyces cerevisiae* was achieved based on an efficient energy regeneration system. A second-order polynomial model was used to describe the biosynthesis of ATP by *Saccharomyces cerevisiae*. Through fractional factorial design and response surface optimization, the yield and concentration of ATP reached 97.5%, and 37 g/L, respectively. The yield was nearly 10% greater than the level before optimization, and the concentration increased nearly two-fold. In addition, the UEE after optimization was 32.35%, which was an increase of nearly 6%.

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