Korean J. Chem. Eng., **30**(4), 913-917 (2013)
DOI: 10.1007/s11814-012-0202-1

Production of cyclic adenosine-3′,5′-monophosphate by whole cell catalysis using recombinant *Escherichia coli* overexpressing adenylate cyclase

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(*Received 16 August 2012 • accepted 16 November 2012*)

Abstract−Adenylate cyclase (EC 4.6.1.1) catalyzes the formation of cyclic adenosine-3′,5′-monophosphate (cAMP) from adenosine 5′-triphosphate (ATP). Recombinant *Escherichia coli* overexpressing adenylate cyclase was used to synthesize cAMP by whole cell catalysis. Some key parameters were examined during the catalytic process, while pH and Mg^{2+} were found to influence cAMP production significantly. Optimum conditions were pH 8.52 and 30 °C with 77.2 mM Mg^{2+} in 100 mM Tris-HCl buffer, including 0.25% Triton-X 100 as detergent and 30 mM pyruvate sodium as enzyme activator for 6 h. 14.93 g/L of cAMP was produced with a conversion rate of 91.5%. The current work provided a potential way for the industrial production of cAMP.

Key words: cAMP, Adenylate Cyclase, Whole Cell Catalysis, Bioconversion

INTRODUCTION

Since the discovery of cyclic adenosine-3′,5′-monophosphate (cAMP) by Sutherland [1] and the pioneering studies scientists thereafter [2-4], biological, physiological and biochemical properties of cAMP have been extensively investigated. This nucleotide is widely distributed in all kinds of organisms, and is well known as the second messenger in cellular signal transduction and an important bioactive substance for numerous biological activities in both prokaryotes and eukaryotes [5,6]. cAMP is also known to act as a mediator of hormone-induced changes in the metabolism of vertebrates and invertebrates [7]. Due to its multiple physiological functions, cAMP has various pharmaceutical effects, including relaxing smooth muscle, expanding blood vessels, improving liver function, and promoting nerve regeneration [8]. Medical application of derivatives of cAMP has also been developed as cardiotonic agents [9] in recent years. cAMP has attracted considerable attention due to its wide clinical applications in treating hyperthyreosis, hepatopathy, as well as cardiovascular, nervous system, cholic, and respiratory diseases [10,11]. Improved production of cAMP would therefore be desirable.

cAMP can be obtained from diverse manufacturing routes from chemical synthesis to fermentation. The chemical synthesis has to go through multiple complicated steps with low yield and high cost which was not commercially available. Several kinds of microorganisms have been discovered to accumulate cAMP to different extent by fermentation, including *Brevibacterium liquefaciens*, *Microbacterium* sp., *Corynebacterium murisepticum* and *Arthrobacter* sp., etc [12-14]. However, the route of cAMP synthesis has been long and the metabolism within the microorganisms is complicated, which are obstacles hindering the improvement of cAMP production. So far as concerned, no whole cell catalysis method has been reported to produce cAMP.

Whole-cell catalysis method has attracted more and more attention since it is simple and economical [15,16]. Compared to the lengthy and laborious procedure during the preparation of purified enzymes, whole-cell catalysts can be prepared easily and inexpensively [17, 18]. In addition to streamlined operation, whole cell catalysis in some cases could reduce the risk of enzyme inactivation during the process of enzyme purification and catalysis [19,20]. Thus, the use of whole-cell catalysts can prolong the use of the biocatalyst [21], which is more favorable in industrial applications.

Recently, we reported the isolation and expression of a bacterial adenylate cyclase that can be applied to the synthesis of cAMP from ATP [22]. In this study, we have developed a whole-cell catalysis system to synthesize cAMP based on the activity of the recombinant adenylate cyclase. Since the substrate and product (ATP and cAMP) are small molecules permeable to the cell membranes [23], whole-cell catalysis might be cost-effective to avoid enzyme isolation and purification steps. In addition, it is known that whole-cell catalysis often supplies a stable and protective environment for enzyme reactions [24]. To use *Escherichia coli* cells overexpressing the recombinant adenylate cyclase as the whole cell catalyst, we determined the relevant conditions giving the maximum whole-cell catalytic activity and cAMP yield.

MATERIALS AND METHODS

1. Microorganism, Vector and Media

Adenylate cyclase gene (Genbank ID: JN415128) had been cloned from *Arthrobacter* A302 (CGMCC No. 3584, preserved in our laboratory) in previous study [22]. pET-28a vector was used as an expression vector and *E. coli* Rosetta strain was used as a host for protein overexpression. The recombinant *E. coli*, named *E. coli* Rosetta

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(pET28a-*cya*) (CGMCC No. 4706), was maintained on Luria-Bertani (LB) agar slants with yeast extract 5 g/L, tryptone 10.0 g/L, NaCl 10.0 g/L, and agar 20 g/L. Kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml) were added for plasmid maintenance.

2. Induction of Recombinant *E. coli* by Lactose

For inoculation, the strain, *E. coli* Rosetta (pET28a-*cya*), was transferred from a slant culture into an Erlenmeyer flask (500 mL) containing 30 mL seed medium, which was the same as above slant medium without agar. The seed cultures were grown at 37 °C on a rotary shaker incubator (Shanghai Zhicheng, China) at 200 rpm for 12 h. Inoculum (3%, v/v) was transferred into an Erlenmeyer flask (1 L) containing 100 mL of LB medium. For the induction of recombinant adenylate cyclase expression, different concentrations of lactose were added and the cultures were reduced to 30 °C when the OD_{600} reached 0.8. The induction of IPTG (0.8 mM) was carried out as a control to compare the induction effect.

3. Biocatalysis of cAMP from ATP

Biocatalysis of cAMP was carried out by whole cells of *E. coli* Rosetta (pET28a-*cya*) from batch cultures. Recombinant cells were harvested at the end of exponential phase of growth by centrifugation at 8,000 rpm, 10 min, 4 °C (Eppendorf, Germany) and washed twice with 100 mM Tris-HCl buffer (pH 8.0). The biocatalytic reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 0.3 g of wet cells, ATP, $MgCl_2$, nonionic surfactant Triton X-100 and pyruvate. The reactions were performed at 30 °C in 250-mL flasks containing 10 mL of the reaction mixture on a rotary shaker at 200 rpm for 6 h. All measurements were made in triplicate and the error bar indicated the standard deviation of those measurements.

4. Analytical Method

Adenylate cyclase assay was conducted according to the method described by Bellalou [25] with a few modifications. The assay mixture was comprised of 100 mM Tris-HCl buffer (pH 8.0), 5 mM ATP, 50 mM $MgCl_2$, and the purified adenylate cyclase to a final volume of 1 mL. The reaction mixture was incubated at 30 °C for 15 min and then reaction was stopped by increasing the temperature to 95 °C for 5 min. One unit of adenylate cyclase was defined as the amount of enzyme required to produce 1 μmol cAMP per min. The protein concentration was measured by the Bradford method [26]. All tests were in triplicate and the mean value was calculated.

The concentration of cAMP in the reaction mixture was measured by high performance liquid chromatography (HPLC) with an Agilent 1200 system with a UV detector and a C-18 column (4.6 mm×300 mm, 5 μm). Methanol and 0.05 mol/L dipotassium phosphate solution (25 : 75, v/v) was used as the mobile phase at a flow rate of 0.8 mL/min. The detection wavelength was 254 nm.

5. Experimental Design and Data Analysis

The influences of five factors on cAMP production were investigated using a fractional factorial design. Those factors, which were significant at 95% of confidence level ($P<0.05$) from the regression analysis, were considered to have greater effects on cAMP production and were further optimized by a central composite design. The statistical software package STATISTICA 6.0 was used for the experimental design and regression analysis. To validate the optimization of the medium composition, three tests were carried out under the optimized conditions and the results analyzed statistically.

RESULTS AND DISCUSSION

1. Induction of the Recombinant Rosetta (pET28a-*cya*)

To save cost and meet the requirements of industrial production, lactose was used for the induction of the whole cell recombinant adenylate cycalse overexpression instead of IPTG. Different concentrations (0.1-1.2%, w/v) of lactose had little influence on cell growth (Fig. 1(a)), but the proportion of target protein was not the same. The results showed that 0.9% lactose was the most beneficial to the expression of the recombinant enzyme (0.255 mg/mL of recombinant adenylate cyclase constituting 10.2% of the total protein).

According to the time course shown in Fig. 1(b), the specific activity of adenylate cyclase in whole cells reached the maximum at 20 h (0.034 U/mg protein), which was almost consistent with that of IPTG induction (0.031 U/mg protein). The cells were harvested at 20 h cultivation and used for subsequent experiments.

2. The Effect of pH Control on the Catalytic Process

As shown in Fig. 2, the reaction profile was totally different with or without pH control. When the pH of reaction mixture was controlled by the reaction buffer alone, the pH value dropped sharply in the previous 2 hours, especially the beginning 2 h of the catalytic process and 5.16 g/L of cAMP was obtained after 6 h with the

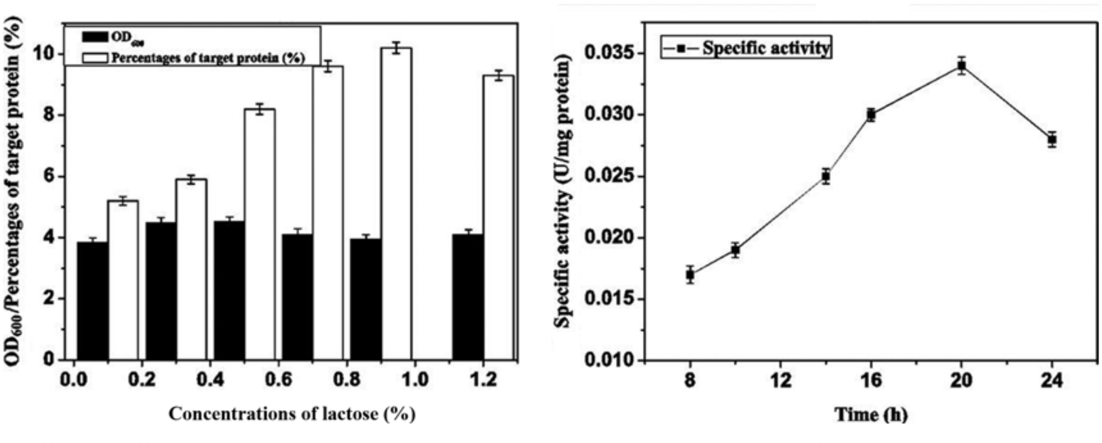

Fig. 1. (a) The effect of different concentrations of lactose on the cell growth and expression of recombinant adenylate cyclase after the induction of 16 h; (b) The time course with 0.9% of lactose induction. All measures were in triplicate samples and the error bar indicates the standard deviation of those measures.

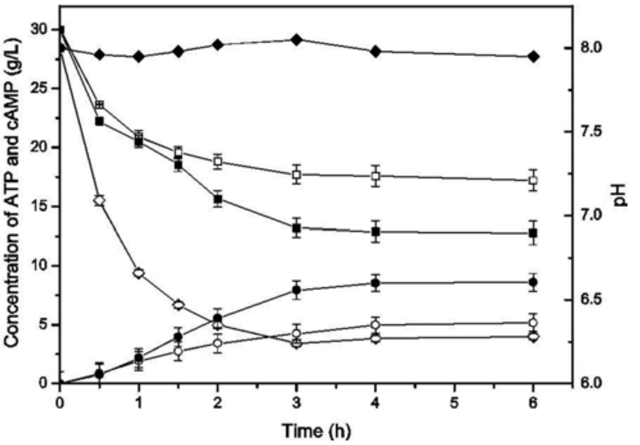

Fig. 2. Time course of ATP consumption (square), cAMP production (circle) and pH fluctuation (rhombus) with (filled symbols) and without (hollow symbols) pH control during the catalytic process.

molar conversion rate of 31.6% from 30 g/L of ATP. However, 9.15 g/L of cAMP was yielded within the same time when the pH was maintained at 8.0 by feeding 1 N NaOH.

The transformation from ATP to cAMP catalyzed by adenylate cyclase was a dephosphorylation process. The pH of reaction mixture dropped quickly at the initial phase due to the rapid reaction rate and the resultant diphosphate. The reaction buffer, 100 mM Tris-HCl (pH 8.0), has a certain capacity to maintain the pH within a range. However, the recombinant adenylate cyclase was sensitive to a slight pH decrease when pH was below 8.0 (data not shown). The results indicated that the control of pH during the catalytic process was necessary and the strategy was used for subsequent experiments.

3. Different Concentrations of Substrate on the Production of cAMP

With the addition of nonionic surfactant Triton X-100, the cytoplasmic membrane was partially solubilized and the cells were permeabilized for molecules, such as ATP and cAMP, to penetrate [29]. So the substrate uptake and product release were no longer obstacles in this whole cell catalysis system. In the range of 5-30 g/L of ATP, the yield of cAMP increased with the concentration of the substrate (shown in Fig. 3). However, suppression of the substrate appeared when the concentration of ATP exceeded 30 g/L, which was five times of the pure enzyme (the pure enzyme would be restrained when the concentration of ATP surpassed 6 g/L). Therefore, the limit concentration of substrate in batch reaction is 30 g/L.

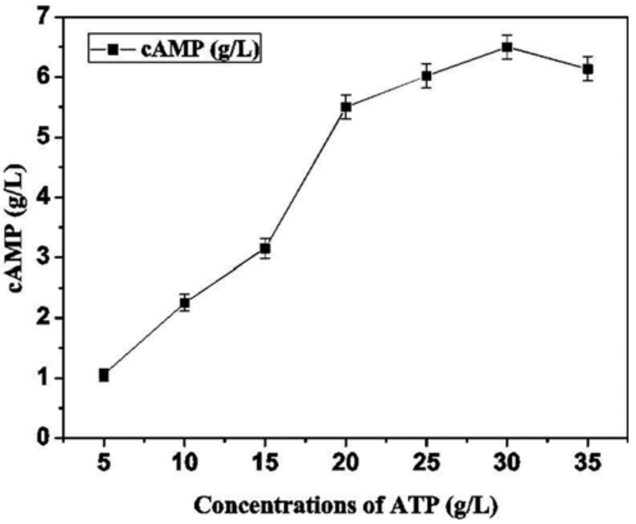

Fig. 3. The effect of substrate concentration on the production of cAMP.

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

Factor	Levels		
	−1	0	+1
pH (X_1)	7.5	8.0	8.5
Temperature (X_2, °C)	25	30	35
Mg^{2+} (X_3, mM)	20	60	100
Triton X-100 (X_4, %)	0.1	0.25	0.4
Pyruvate (X_5, mM)	10	30	50

4. Screening Key Factors with Fractional Factorial Design

Since the production of cAMP by whole cell catalysis system was first reported here, it is necessary to screen key factors that significantly influenced the cAMP production. The fractional factorial design has proven to be an effective tool for screening important medium components [27]. The levels of the key factors tested in the experimental design are shown in Table 1. In our previous study [22], adenylate cyclase exhibited sensitivity to pH and temperature change. Mg^{2+} and pyruvate were the cofactor and activator to this catalyst, respectively. The concentration of Triton X-100 determined the permeability of whole cell catalyst. The experimental results with the fractional factorial design are shown in Table 2. According to the analysis by Statistica 6.0, a linear regression equation could be obtained from the regression results of fractional factorial experiments:

$$Y=9.90+1.03X_1+0.16X_2+2.87X_3+0.02X_4-0.07X_5 \qquad (1)$$

The regression coefficients and determination coefficient (R^2) for the linear regression model of cAMP production are presented in Table 3. The model was significant ($P<0.05$) and $R^2=0.9850$, indicating that 98.50% of the variability in the response could be

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

Run	X_1	X_2	X_3	X_4	X_5	cAMP g/L	
						Observed	Predicted
1	−1	−1	−1	1	1	5.62	5.79
2	1	−1	−1	−1	−1	7.77	7.96
3	−1	1	−1	−1	1	6.46	6.07
4	1	1	−1	1	−1	8.72	8.31
5	−1	−1	1	1	−1	12.05	11.66
6	1	−1	1	−1	1	13.96	13.55
7	−1	1	1	−1	−1	11.77	11.94
8	1	1	1	1	1	13.72	13.91
9	0	0	0	0	0	9.51	9.90
10	0	0	0	0	0	9.43	9.90

Table 3. Regression results of the fractional factorial design

Factor	Coefficient	T-value	P-value
Intercept	9.901000	58.77161	0.000001
X_1	1.033750	5.48844	0.005369*
X_2	0.158750	0.84284	0.446765
X_3	2.866250	15.21765	0.000109*
X_4	0.018750	0.09955	0.925492
X_5	−0.068750	−0.36501	0.733584

$R^2=0.9850$
*Statistically significant at 95% confidence level

Table 6. Regression results of the central composite design

Factor	Coefficient	P-value
Intercept	12.56000	0.000023
A	2.55347	0.000787*
A^2	−1.02188	0.050189
B	1.84298	0.002707*
B^2	−0.89688	0.071703
AB	−1.04750	0.056466

$R^2=0.9731$
*Statistically significant at 95% confidence level

explained by the model. Statistical analysis of the data showed that in the concentration range tested, only Mg^{2+} and pH influenced cAMP production significantly, which had confidence levels above 95% ($P<0.05$) and were considered to influence cAMP production significantly. The other factors had confidence levels below 95% and hence were considered insignificant.

Mg^{2+} was considered as a cofactor to adenylate cyclase during the transformation from ATP to cAMP. In our previous study, divalent magnesium exhibited indispensable effect to the activity of recombinant adenylate cyclase (data not shown). pH was another key factor that influenced the production of cAMP to a large extent. The effects of pH and Mg^{2+} were investigated for further optimization to achieve a maximum response.

5. Optimization of pH and Mg^{2+} Content Using Central Composite Design

Following screening, response surface methodology using central composite design was employed to determine the optimal levels of the two selected factors that affected cAMP production. The respective low and high levels with the coded levels for the factors are defined in Table 4. The concentrations of the other factors were fixed at zero level as shown in Table 1. Experimental design and results are shown in Table 5. The experimental results were fitted with the second-order polynomial:

$$Y=12.56+2.55A+1.84B-1.02A^2-0.90B^2 \qquad (2)$$

where Y is the predicted response, and A and B are coded values of Mg^{2+} and pH, respectively.

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

Factor	Levels				
	−1.41	−1	0	+1	+1.41
pH (A)	7.3	7.5	8.0	8.5	8.7
Mg^{2+} (B, mM)	3.6	20	60	100	116.4

Table 5. Experimental design and results of the central composite design

Run	pH	Mg^{2+}	cAMP g/L	
			Observed	Predicted
1	−1	−1	6.06	5.20
2	−1	1	10.86	10.98
3	1	−1	12.91	12.40
4	1	1	13.52	13.99
5	−1.41	0	6.46	6.91
6	1.41	0	14.18	14.13
7	0	−1.41	7.27	8.16
8	0	1.41	13.87	13.37
9	0	0	12.6	12.56
10	0	0	12.52	12.56

The analysis of variance (ANOVA) of the regression model given in Table 6 gives a satisfactory value for the coefficient of determination R^2, which was calculated as 0.9731, indicating that 97.31% of variability in the response could be explained by the model. Normally, a regression model having an R^2-value higher than 0.9 is considered as having a very high correlation [28]. The present R^2-value, therefore, reflected a very good fit between the observed and predicted responses, and it was considered reasonable to use the regression model to analyze trends of the responses.

The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables and interactive effects of each independent variable on the response variables. The response surface based on the independent variables pH

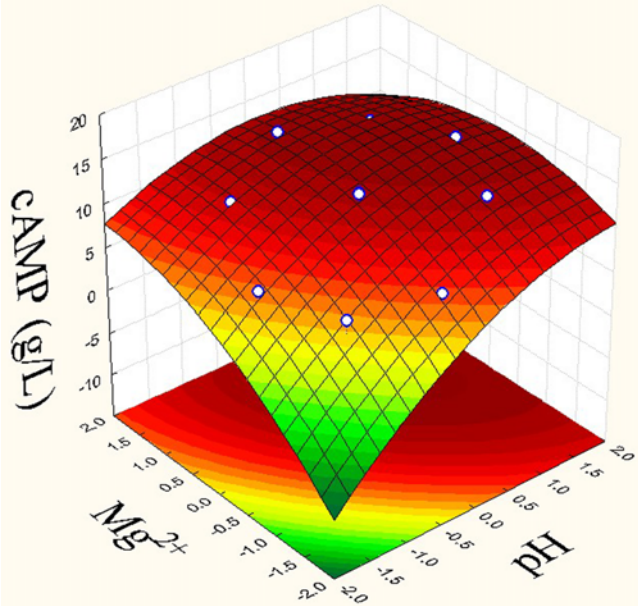

Fig. 4. Response surface plot of the combined effects of pH and Mg^{2+} on cAMP production by whole cell catalysis in recombinant *E. coli.*

and Mg^{2+} is shown in Fig. 4. It is clear that cAMP production was sensitive to even small changes of pH and Mg^{2+} concentration. The model predicted that the optimal values of test factors in the coded units were A=1.03, B=0.43. At these values, the values of pH and Mg^{2+} were 8.52 and 77.2 mM, respectively. The maximum predicted value of cAMP concentration was 14.27 g/L.

6. Experimental Validation of the Optimized Condition

To confirm the model adequacy, three additional experiments were performed in shake flasks in the optimum conditions. The mean value of cAMP concentration was 14.93 g/L, which was close to the predicted value (14.27 g/L). Hence, the model was proved to be adequate. The final medium composition optimized with response surface methodology was (g/L): 30 g/L ATP, 30 g/L wet cells, pH 8.52, 30 °C, 77.2 mM Mg^{2+}, 0.25% Triton X-100 and 30 mM pyruvate.

CONCLUSION

A gene encoding adenylate cyclase from *Arthrobacter* was overexpressed in *E. coli* Rosetta by the induction of inexpensive lactose instead of IPTG. The recombinant *E. coli* Rosetta (pET28a-*cya*) was permeabilized by the addition of nonionic detergent Triton-X 100 and used as whole cell catalyst for the biotransformation from ATP to cAMP. Some key parameters of the biocatalytic process, including pH, temperature, Mg^{2+}, Triton X-100 and pyruvate, were optimized. Utilizing whole cell catalysis instead of conventional enzyme catalysis is a potential way to reduce the cost for the preparation of biocatalyst and to increase the substrate tolerance. Compared to fermentation, the production of cAMP by whole cell catalysis greatly shortened the production cycle from 66 h to 6 h and improved the productivity from 0.17 g/h to 2.49 g/h. The current work provided a potential way for the industrial production of cAMP.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Program of China (973) (Grant No.: 2012CB721100); the National Science Fund for Distinguished Young Scholars (Grant No.: 21025625); Program for New Century Excellent Talents at the University of Ministry of Education of China (Grant No.: NCET-11-0987); the Research Fund for the Doctoral Program of Higher Education of China (RFDP) (Grant No.: 20113221120007); Program for Changjiang Scholars and Innovative Research Team in the University (Grant No.: IRT1066); The Natural Science Foundation of Jiangsu Province (Grant No.: BK2011031) and the Priority Academic Program from Development of Jiangsu Higher Education Institutions.

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