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Optimized Culture Conditions for the Efficient Production of Porcine Adenylate Kinase in Recombinant *Escherichia coli*

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Abstract Temperature shift cultivations with amino acid supplementation were optimized to produce porcine adenylate kinase (ADK) in recombinant *Escherichia coli* harboring a pUC-based recombinant plasmid under the control of the *trp* promoter. With regard to temperature control, the culture condition was initially maintained at 35 °C for cellular growth, but ADK expression was suppressed until the late logarithmic growth phase; subsequently, a temperature shift was applied (from 35 °C to 42 °C), which resulted in maximal ADK production. In addition, supplementation of amino acids, especially valine and leucine, during the temperature shift stimulated ADK expression from 3.5% to 9.2% and 8.6% of the total protein, respectively. After optimization, 1 g ADK per liter was produced within 16 h of cultivation with a dry cell weight of 21.8 g/l. In this system, there was no loss of the recombinant plasmid during cultivation without selective pressure.

Keywords High cell density cultivation · *Escherichia coli* · Porcine adenylate kinase · Temperature shift cultivation

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Introduction

Adenylate kinase (ADK; ATP:AMP phosphotransferase, EC 2.7.4.3) is involved in the reversible transfer of terminal phosphate groups between adenine nucleotides (AMP+ATP= 2ADP), a reaction that is indispensable for energy metabolism in prokaryotic and eukaryotic cells. This enzyme has been regarded as the potential enzyme for ATP regeneration in cell-free protein synthesis [1]. In a previous study, we achieved high expression levels of porcine ADK in recombinant *Escherichia coli* harboring pMKAK1-2 with a pUC18-based replicative origin [2]. However, an efficient production system in a bioreactor was necessary, while there have been no such report for the porcine ADK production.

In order to maximize volumetric productivity, a high-cell-density cultivation (HCDC) technique is important. *E. coli* is most commonly used either as a host for heterologous protein production or as a biocatalyst. In HCDC of recombinant *E. coli* harboring an inductive expression system, separation of the growth phase and expression (production) phase has often resulted in efficient expression and a high cell density in both a chemically [3] and physically induced system. The application of temperature shift is a simple method for achieving this type of two-stage cultivation. For temperature shift cultivation, the $\lambda P_L/P_R$ promoter has been successfully used for the large-scale HCDC of *E. coli* DH5α [4], BL21 [5], and DH1 [6] cells. In our previous study, we found that the expression of hirudin variant 1, encoded by pUC18, was affected by the culture temperature, possibly because of the plasmid copy number controlled by the pUC-encoding factor, as suggested previously [7]. Hence, we decided to apply the temperature shift technique to the cultivation process. Several specific amino acid supplementations during cultivation were also reported to enhance the heterologous protein production in recombinant *E. coli* [8].

In this study, we optimized the culture conditions including temperature shift and amino acid supplementation for efficient production of the porcine ADK in a recombinant strain of *E. coli* harboring the pUC18-based expression vector.

Materials and Methods

Bacterial Strains and Plasmids *E. coli* JM109 (*endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*), *recA1*, F′[*traD36*, *proAB*$^+$, *lac*I^q, *lacZ*ΔM15]) was used as the host strain for pMKAK1-2 [2]. The recombinant plasmid pMKAK1-2 contains a gene encoding the cDNA of porcine muscle ADK under the control of the *E. coli tac* promoter [2] (Fig. 1). This plasmid replicates from a pUC18-based origin. The recombinant *E. coli* strain harboring pMKAK1-2 was ampicillin-resistant, and it constitutively expressed the porcine muscle ADK when cultured in a complete synthetic medium in the absence of tryptophan.

Cultivation TK-1 medium, derived by modifying TK-25 medium [9], was used for the HCDC of both the recombinant and non-recombinant strains of *E. coli* JM109. This medium contained 40 g glucose, 5 g $(NH_4)_2HPO_4$, 1.65 g K_2SO_4, 1.63 g NaCl, 1.65 g $MgSO_4{\cdot}7H_2O$, 3.58 g $FeSO_4{\cdot}7H_2O$, 34 mg $ZnSO_4{\cdot}7H_2O$, 0.87 mg $CuSO_4{\cdot}5H_2O$, 2.4 mg $MnSO_4{\cdot}4\text{-}5H_2O$, 1.88 g $CaCl_2{\cdot}2H_2O$, 3.4 mg $Na_2B_4O_7{\cdot}10H_2O$, 1.5 mg $(NH_4)_6Mo_7O_{24}$, 10 g citric acid, and 20 mg thiamine–HCl in 1 L deionized water (pH 7.0). HCDC was performed as described in a previous report using a 2.6-L laboratory fermentor (initial working volume, 1 L) [10]. A preculture was prepared by growing the cells at 37 °C in BC medium [11], and 2% of the seed culture was used as the inoculum. Briefly, the culture was

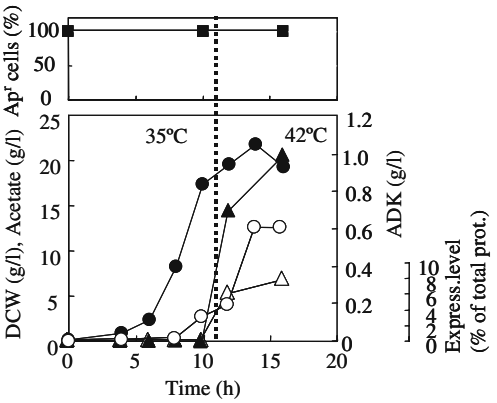

Fig. 1 Cultivation of *E. coli* JM109/pMKAK1-2 by combining temperature shift and valine supplementation in ADK production. *Closed circles*, dry cell weight (g/L); *closed triangles*, volumetric concentration of the ADK product (g/L); *open circles*, acetate concentration (g/L); *open triangles*, expression level of ADK in the total protein (%); *closed squares*, segregational plasmid stability expressed as the ratio of ampicillin-resistant cells in the total viable cells. Temperature of the fermentor was shifted from 35 °C to 42 °C at the *dotted line*

maintained at a desired temperature and stirred at 1,000 rpm. The pH was maintained at 7.0 and adjusted with 15 N aqueous ammonium hydroxide. The glucose concentration was maintained in the range of 1–6 g/L by intermittently feeding the cells with 40% sterilized glucose solution, as described in a previous report [10]. The dissolved oxygen (DO) concentration was monitored by a Garvani-type oxygen probe (DX-26; Marubishi Bioeng. Co., Tokyo, Japan) and maintained at the desired level by controlling the oxygen ratio using a DO controller (Marubishi Bioeng. Co.).

Analysis Cellular growth was estimated by measuring the optical density (OD) at 660 nm and calculating the dry cell weight (DCW) per unit volume. At 660 nm, the OD correlated well with the DCW: DCW/OD=0.568 (R^2=0.99). The acetate concentration was determined using the F-kit (JK International Co., Tokyo, Japan) according to the manufacturer's instructions. Segregational plasmid stability was determined by counting the number of ampicillin-resistant cells according to a method described in a previous report [10].

Porcine muscle ADK was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a cell-free extract prepared according to a previously described method [12]. The expression level of the protein was analyzed using a gel densitometer (LK-B Ultra ScanXL; Pharmacia, USA) and was expressed as a percentage of the total protein according to a previously described method [11]. The amount of soluble protein was determined by the Bradford method [13] using bovine serum albumin as the standard.

Results and Discussion

Optimization of Cultivation Temperature for Cellular Growth and Expression

For achieving temperature shift cultivation under optimum conditions, it is necessary to separate the cultivation phase from the optimal growth phase but suppress protein

Table 1 Cellular growth and ADK production under constant temperature using *E.coli*/pMKAK1-2.

Temp (°C)	30	33	35	37	39	42
μ_{max}(1/h)	0.38	0.46	0.61	0.56	0.52	0.29
Time (h)	15	23	15	14	15	20
DCW (g/L)	26.8	32.1	32.2	31.7	30.3	6.3
Expression level (%)	0	0	0	0.14	0.14	6.2
Product concentration (g/L)	0	0	0	0.026	0.036	0.21

expression; subsequent application of a temperature shift will result in optimal production. Table 1 shows the results of the HCDC of JM109/pMKAK1-2 at constant temperatures. In these recombinant cells, both the specific growth rate (0.61 L/h) and final cell concentration (32.2 g DCW per liter) was the highest at 35 °C. ADK expression was not observed when the cells were cultivated at temperatures lower than 35 °C, while ADK was efficiently expressed at 42 °C (total protein, 6.2%). However, no growth was observed at temperatures higher than 42 °C (data not shown). These data confirmed that the ADK expression level in our strain depends on the cultivation temperature. On the basis of the above results, we concluded that the optimal temperatures for cellular growth and ADK expression are 35 °C and 42 °C, respectively.

Temperature Shift Cultivation

To further investigate the optimal temperature shift for ADK production, the temperature was shifted from 35 °C to 42 °C at various growth phases during the same fed-batch cultivation (Table 2). Induction during the early growth phase (DCW, 0.57 g/L) resulted in low cell concentration and expression level, while induction during the stationary growth phase (DCW, 28.4 g/L) resulted in low expression level but the highest cell concentration as compared to under other conditions. Induction during the mid-exponential phase when the cell concentration was 5.7 g/L (OD at 660 nm of 10) resulted in maximal ADK production of 0.39 g/L with a cell concentration of 14.5 g/L and expression level of 4.5%. On the other hand, there have been few reports, including one by Ansorge et al. [14], regarding the optimization of temperature shift cultivation using recombinant *E. coli* harboring a heat-inducing replicating plasmid for bacterial-derived protein production.

Table 2 Effect of temperature shift timing on ADK production in *E.coli*/pMKAK1-2.

Induced DCW[a]	0.57	2.8	5.7	11.4	28.4
(Growth phase)[b]	exp.	exp.	exp.	exp.	station.
Time (h)	14	18	16	16	18
DCW (g/L)	8	11.5	14.5	17.8	24.8
Expression level (%)	0.15	4.2	4.5	3.1	1.4
Product concentration (g/L)	0.072	0.29	0.39	0.33	0.21

[a] Temperature was shifted from 35 °C to 42 °C at the indicated DCW (g/L)

[b] Growth phase of the induced DCW: *exp.* exponential phase, *station.* stationary phase

Table 3 Effect of amino acid supplementation during the cultivation of *E.coli*/pMKAK1-2 on cellular growth and ADK expression in flask cultures.

Added amino acid	Expression level (% of total protein)	Biomass (OD at 660 nm)
None	3.5	5.4
Casamino acid	4.3	5.4
Glu (9.8)[a]	3.8	5.4
Gly (9.8)	3.9	5.4
Leu (9.3)	8.6	4.6
Lys (10.3)	4.5	5.4
Val (8.8)	9.2	1.2

ADK expression in flask cultures initially cultivated at 35 °C and, subsequently, at 42 °C (OD, 0.7) with 120 mg/L amino acid supplementation

[a] Amino acid number ratio in the ADK polypeptide (expressed as %)

Effect of Amino Acid Supplementation on ADK Production in *E. coli*

ADK contains glutamic acid, glycine, valine, leucine, and arginine amino acid residues. Since it has been reported that the addition of such amino acids in large amounts in a heterologous protein affects cultivation [7], the effect of amino acids on ADK production was further examined to improve ADK expression. Interestingly, only leucine and valine, branched amino acids, significantly enhanced the expression level from 3.5% to 8.6% and 9.2%, respectively, while the addition of valine inhibited cellular growth (Table 3). ADK was not expressed when leucine or valine was added without applying temperature shift (data not shown). This may suggest that these amino acids were required only in the case of heterologous protein expression. Metabolic response of these amino acid biosynthesis (such as acetohydroxy synthase, a key enzyme in branched chain amino acid synthesis) under the temperature shift conditions would be necessary to elucidate the mechanism.

Effect of valine supplementation was further examined to improve the total ADK production under various valine concentration and temperature shift timing (Table 4). Under the same condition (temperature shifted OD 0.7 with valine concentration of 120 mg/L) as in Table 3, the ADK expression level was approximately as high as 10% with final OD of 1.1. The cellular growth was improved by changing the timing of induction from OD=0.7 (early

Table 4 Effect of valine addition and temperature shift timing on ADK production by *E. coli* JM109/pMKAK1-2 in flask cultures.

Val conc. (mg/L)	Shifted OD[a] (−)	Final OD (−)	Expression level (% of total protein)	Total ADK (g/l)
2.4	2.5	4.6	3.4	0.096
6	2.5	4.6	3.6	0.096
12	2.5	4.6	9.2	0.25
24	2.5	4.6	12.7	0.34
120	0.1	0.19	12.0	0.013
120	0.7	1.1	11.4	0.073
120	2.5	4.5	13.1	0.34
120	4.5	4.6	0	0

[a] Temperature was shifted from 35 °C to 42 °C at the indicated OD

logarithmic growth phase) to 2.5 (late logarithmic growth phase). Increased expression level was also observed by increasing the valine supplementation. Based on the results as above, we concluded to induce ADK expression at late logarithmic growth phase with valine supplementation at 25 mg/L OD (corresponding to 120 mg/L for the flask experiment in Table 2), optimal both for ADK expression and the cellular growth.

Combination of Temperature Shift and Amino Acid Supplementation

From the above results, we concluded that application of the temperature shift (from 35 °C to 42 °C) combined with the addition of valine during the late logarithmic stage results in an increase in the ADK expression by approximately 24 mg valine per OD and leads to the efficient expression of ADK without inhibition of cellular growth. These conditions were further applied to a jar fermentor with glucose fed-batch culture as described in "Materials and Methods," as shown in Fig. 1. In addition to the temperature shift from 35 °C to 42 °C at 11 h of cultivation, 2.4 g valine per liter was added to the fermentor because we expected the biomass production to be higher by approximately 20-fold (OD=100) as compared to the results obtained in the flask experiment (Table 3). Production of ADK rapidly increased to 1 g/L after the temperature shift was applied; 7.9% of ADK was expressed in the total protein fraction within 16 h of cultivation. Under the same condition except for temperature induction and amino acid supplementation, cells grew up to 21.8 g/l, approximately ten times higher than the flask cultivation due to the pH control, sufficient supply of oxygen, and intermittent carbon source supplementation. The amount of acetate, known to be a growth inhibitory metabolite derived from glucose in an aerobic culture of *E. coli* [15], significantly increased from 2.8 to 12.8 g/L after induction and possibly led to the inhibition of cellular growth. Although relationships between the metabolic response and recombinant protein production in *E. coli* remain unclear, recent progress in proteome analysis focusing on acetate production [16] in recombinant *E. coli* may provide further insight, such as the effect of valine or other amino acids tested on the specific enzymes responsible for acetate production from glucose.

In this paper, we combined temperature shift induction and amino acid supplementation for enhancing protein production in recombinant *E. coli* and thus demonstrated production of high levels of an animal-derived heterologous protein.

Conclusion

There have been few reports to produce porcine-derived protein in recombinant *E. coli* [17, 18]. In this study, separation of growth phase and the heterologous protein production in a recombinant *E. coli* led to the efficient production of recombinant porcine ADK in *E. coli* up to 1 g/L, which is comparable to the reported value for other recombinant porcine-derived protein production in *E. coli*. In addition to the studies on further optimization such as determining the optimal host cell and other cultivation conditions, construction of the bioprocess by the combination of this study and refolding process [19] would be necessary.

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