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Effect of *pyk* gene location on constructing dATP conversion *E. coli* strain

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ABSTRACT

A dATP conversion *E. coli* strain could be constructed when both *pyk* and *adk*1 gene were expressed successfully. *pyk* gene encodes pyruvate kinase (PK) could be expressed, when inserted it before *adk*1 gene which encodes adenylate kinase (AK) in plasmid pET-*pyk*-*adk*1 after transform into *E. coli* and the recombinant could be used to convert dATP from dAMP. Another plasmid pET-*adk*1-*pyk*, which inserted *pyk* gene behind of *adk*1, the recombinant *E. coli* transformed with this plasmid could not convert dAMP into dATP, *pyk* gene cannot be translated in this recombinant. The different translation levels of *pyk* with gene location switching caused mainly by the different secondary structures formed by the 5'-untranslation regions and the gene sequence of its 5'-terminal. The dATP product *E. coli* strain could be constructed when cloned *pyk* gene at an optimum location.

Keywords: *pyk* Gene Location; dATP Conversion Strain; 5-Untranslation Regions

1. INTRODUCTION

Deoxyadenosine triphosphate (dATP) is one of the necessary precursors used in DNA biosynthesis, especially in the PCR reaction. The commercial dATP products come mainly from chemical synthesis, but the chemical method gives relatively low yield and need the toxic substances issued by the Environmental Protection Agency, like pyridine and dimethylformamide is cited from [1]. The biosynthesis method is environmental friendly one, a couple phosphorylation reactions can be used to synthesis dATP for dAMP. In the first step, adenylate kinase (AMP kinase, AK) was added to convert deoxynucleoside monophosphates (dAMP) into deoxynucleoside diphosphates (dADP), and then pyruvate kinase (PK) participated in subsequentlly to convert deoxynucleoside diphosphates (dADP) into deoxynucleoside triphosphates (dATP) [1]. People should express and purify two enzymes individually, this complicated the procedure and increased the cost.

To clone two or more genes into one host was feasible in recently. In order to simplify the biosynthesis process, two kinases were cloned onto a high copy plasmid pET-17b and performed overexpression in *E. coli* BL21. The recombinants are used to catalyze dATP from dAMP after inducting protein expression of plasmid pET-*pyk*-*adk*1. However, the poor expression spectrum of *pyk* gene on the pET-*adk*1-*pyk* plasmid prevented the conversion of dAMP to dATP, and leading to the accumulation of intermediary dADP produced.

There are some reports on the relationship of gene location and the translation efficiency [2], the transcript level and mRNA stability of the target gene are thought important factors on gene expression efficiency, and the 5' untranslated region is also an important factor on protein expression efficiency [3]. As for a specific gene, the story remains incomplete, the factors mentioned above would have different contributions [2,4].

As for the *pyk* gene, the possible reasons were clarified in this report for its different expression efficiency after changing the location with *adk*1 on plasmid pET-17b. Except the different secondary structures of its mRNA formed with the 5'-UTR, the transcript level and mRNA half-time were remained same level in the two recombinants. The solo difference of *pyk* mRNA secondary structures seems a main reason on PK expression level in *E. coli* BL21 (pET-*adk*1-*pyk*). The different secondary structure afford *pyk* mRNA a higher Minimum Gibbs free energy values (ΔG, –26.7 kJ·mol^{-1}) in *E. coli* BL21 (pET-*adk*1-*pyk*) than that in BL21 (pET-*adk*1-*pyk*) (ΔG, –18.0 kJ·mol^{-1}).

2. MATERIAL AND METHODS

2.1. Reagents and Strains

Restriction endonucleases, T_4 DNA ligase and *Pfu* DNA polymerase were purchased from Fermentas (Hanover,

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USA); Isopropyl-beta-D-thiogalactopyranoside (IPTG) was from Sigma-Aldrich (St Louis, MO, USA); Diaflo Ultrafilter membrane and RNA extracted Kit were from Omega Biotech (Vancouver, Canada); Ampicillin and LB broth were from Sangon Biotech (Shanghai, China).

The *E. coli* BL21 (DE3) strain 1 was purchased from *Novagen*, and the plasmid pET-jb was constructed in our lab as reported [1], per liter LB medium contains 100 $mg \cdot ml^{-1}$ of ampicillin.

2.2. Plasmid Construction and Protein Expression

The *pyk* gene was amplified from *Bacillus sp.* ATCC 31821 by PCR according to the gene sequence of NCBI database (accession No: *gi* 255767013). The primers sequences were:
TAGTCGACAAGAAGGAGAACAAATGAGAAAAAC (forward with *Sal* I and ribosome binding site (RBS) region), and
CACCTCGAGTAATTAAA GAACGCT (reverse with *Xho* I) synthesized by Shanghai Biotech (Shanghai, China). pET-*pyk*-*adk*1 and pET-*adk*1-*pyk* was constructed by replacing the amplified fragment at the *Sal* I and *Xho* I sites in pET-*jb*1 with *adk*1 [1]. The endonuclease sequences and the RBS sequence were underlined and framed.

After confirmed by DNA sequence, the plasmids were transformed into *E. coli* BL21 (DE3), and named as *E. coli* BL21 (pET-*adk*1-*pyk*) and *E. coli* BL21 (pET-*pyk*-*adk*1). The co-expression of PK and AK was conducted under optimum inducing conditions, and used for dAMP conversion and protein spectrum analyses on 10% SDS-PAGE. *E. coli* BL21 (DE3) harboring plasmid pET-17b was set as control.

2.3. Conversion Analysis of Recombinants

The conversion activity of the recombinants was conducted in separate but analogous reactions. It containing 100 ml reaction buffer (50 mM Tris buffer pH 8.0 with 500 mM dAMP, 1000 mM PEP, 100 mM KCl, and 10 mM $MgCl_2$), added 2 mg cell pellets of the recombinant (collected from 50 ml culture with OD_{600} at 2.0), the mixtures were kept at 30˚C for 2 hrs. The reactions course was monitored periodically and detected using high performance liquid chromatography (HPLC, LC-9A, Shimadzu). Using Intensil ODS-2 column (4.6 mm × 150 mm, GL Sciences, 254 nm), the elution buffer was 10 mM KH_2PO_4 and 10 mM K_2HPO_4 (pH 7.0) remained an elution rate of 1.0 ml/min at ambient temperature.

2.4. *pyk* mRNA Quantify and Stability Assay

Collected the engineered *E. coli* at OD_{600} reached 0.8, extract and purify the RNA using RNA extracted Kit from Omega Biotech, about 25 μg RNA was extracted in this test. The hybridization and detection procedure were following the method of Ravanshad [5]. The cDNA probe sequence of *pyk* was
GCGAATACGCCAGATACGAG with the 5'-end Dig labeled, according to *pyk* gene sequence of NCBI database (*gi* 255767013). The optical density of the hybrid samples was determined, BL21 (DE3) harboring plasmid pET-17b was set as control.

Actinomycin D was used as transcription inhibitor for mRNA half-life detection. The optimal concentration of Actinomycin D was from 0.05 to 10 $\mu g \cdot ml^{-1}$ added in the culture, after 20 minutes, cells were collected and used for dot blot test. The degradation rate of *pyk* mRNA was tested at different times 0 min - 60 min within an optimal Actinomycin D concentration.

2.5. *pyk* mRNA Secondary Structure Prediction

The secondary structure of the *pyk* mRNA was predicted using online software MFOLD (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form/cgi) based on the principle of minimum free energy [6]. The mRNA sequence calculated was the +25 bp fragment of the 5'-UTR (Untranslated Region) including the RBS sequence and the first 11 codons started from AUG.

3. RESULTS AND DISCUSSION

3.1. Recombinant Plasmid Construction and PK Expression Analysis

There are two castles of *adk*1 and *pyk* gene with location changing under the same operon controlling, one is 5'-*adk*1-*pyk*-3' and another is 5'-*pyk*-*adk*1-3'. Two plasmids pET-*pyk*-*adk*1 and pET-*adk*1-*pyk* were constructed accordingly, as **Figures 1(a)** and **(b)** show. After being transformed into BL21 (DE3), two recombinants BL21 (pET-*pyk*-*adk*1) and BL21 (pET-*adk*1-*pyk*) were named.

The protein expression was conducted under different IPTG induction concentration (0, 0.2, 0.4, 1.0 mM) (**Figure 2(a)**) and time course (0, 2, 4, 8 hrs) (**Figure 2(b)**). The SDS-PAGE for *E. coli* BL21 (pET-*pyk*-*adk*1) showed two protein bands at 26 kD and 58 kD positions [1], AK and PK were expressed successfully in this strain. However, the lysate of *E. coli* BL21 (pET-*adk*1-*pyk*) has only a 26 kD band of AK, though under the same induction conditions as *E. coli* BL21 (pET-*pyk*-*adk*1) (**Figures 2(a)** and **(b)**).

3.2. dATP Conversion Activity of the Two Recombinants

Under the given reaction conditions, *E. coli* BL21 (pET-*pyk*-*adk*1) 500 mM dAMP substrates could be converted into dATP within 90 minutes and no by-product as

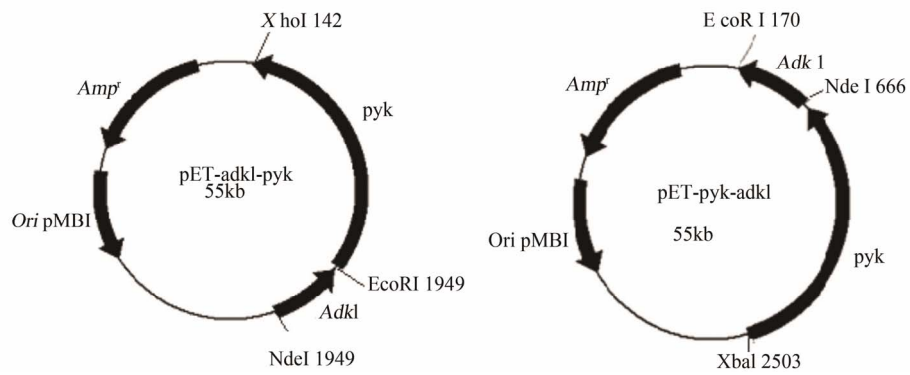


Figure 1. Physical map of the co-expression plasmids pET-*adk*1-*pyk* (a) and pET-*pyk*-*adk*1(b) Denote: pBR322 ori, origin of replication; *Amp*^{r}, ampicilinresistant marker.

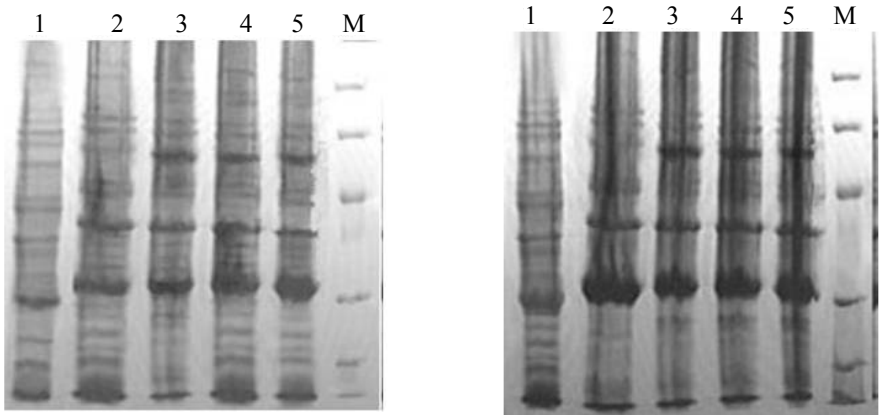


Figure 2. SDS-PAGE of AK and PK in the recombinant strains *E. coli* BL21 (pET-*adk*1-*pyk*) and *E. coli* BL21(pET-*pyk*-*adk*1) under different IPTG concentration at 37 ˚C for 6 hours (a) and induction time with 0.4 mM IPTG in the medium at 37˚C (b). (a) Lane 1: Negative control; Lane 2 to 5 and Lane 6 to 9: protein spectrum with 0, 0.2, 0.4, 1.0 mM IPTG in *E. coli* BL21 (pET-*pyk*-*adk*1) and *E. coli* BL21 (pET-*adk*1-*pyk*) respectively; Lane 10: Protein MW markers. (b) Lane 1: Negative control; Lane 2 to 5 and Land 6 to 9: protein spectrum after inducing for 0, 2, 4, 8 hours with 0.4 mM IPTG for E.coli BL21 (pET-*pyk*-*adk*1) and *E. coli* BL21 (pET-*adk*1-*pyk*) respectively; Lane 10: protein MW markers.

(**Figure 3(a)**) shows. When use the recombinant *E. coli* BL21 (pET-*adk*1-*pyk*) as catalyst, a bound up of dADP accumulated in the reactor (**Figure 3(b)**) , the conversion reaction did not go to completion.

3.3. *pyk* mRNA Transcript Level and Stability Analysis in the Two Strains

The optical density of *pyk* mRNA hybrid blot with the cDNA probe in *E. coli* BL21 (pET-*adk*1-*pyk*) (dot 2) and *E. coli* BL21 (pET-*pyk*-*adk*1) (dot 3) are comparable, both stronger than in *E. coli* BL21 (pET-17b) (dot 1) as **Figure 4** shows.

As for the stability analysis of *pyk* mRNA, with Actinomycin D concentration increasing the optical intensity of the hybrid blots decreasing in both recombinants accordingly. The intensity of the hybrid dot was only about 15% with 0.5 μg·ml^{-1} Actinomycin D in the medium, the transcript activity of *pyk* was almost inhibit completely in both strains (**Figure 5(a)**). 10 minutes later with 0.5 μg·ml^{-1} Actinomycin D in the medium, the intensity of *pyk* hybrid blot in the two recombinants was decreased to 16% of the zero point (**Figure 5(b)**) and kept at the same level, it was very weak in the two strains. The results indicated that the *pyk* gene has the same transcript level and mRNA stability in the two recombinants.

3.4. *pyk* mRNA Secondary Structure Prediction

The sequence of of *pyk* mRNA which used for secondary structures prediction including the 25 nucleotides of

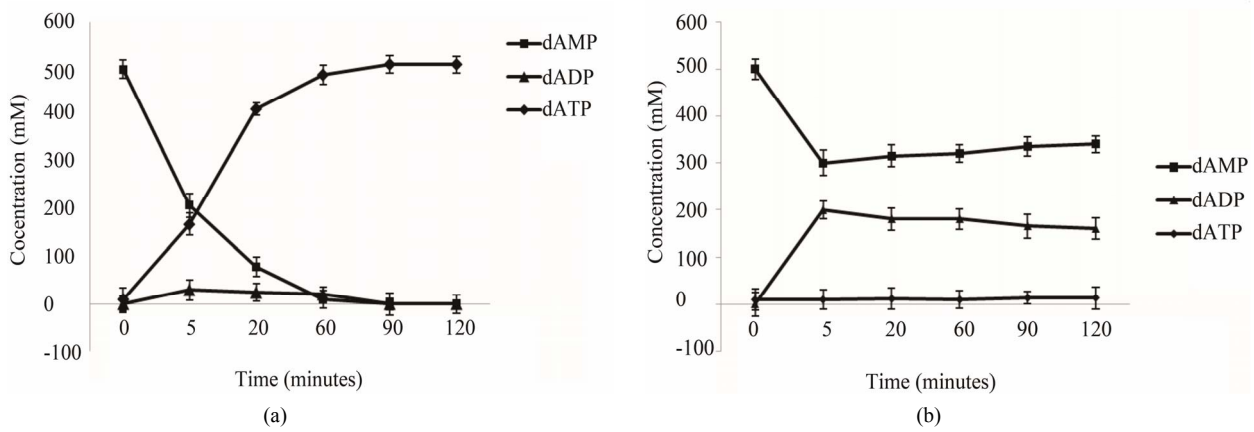


Figure 3. dATP conversion time course of the engineering strains. The catalyst is 2 ml cell lysate (collected from 50 ml culture at OD_{600} 2.0) of the *E. coli* BL 21 (pET-*pyk*-*adk*1) (a) *E. coli* BL 21 (pET-*adk*1-*pyk*) (b), the reaction mixed with 500 mM of dAMP, 1000 mM PEP at 30˚C in 50 mM Tris at pH of 8.0, with 100 mM potassium chloride and 10 mM magnesium chloride.

Figure 4. *pyk* gene transcript level assay in *E. coli* BL21 (pET-*adk*1-*pyk*) and *E. coli* BL21 (pET-*pyk*-*adk*1) using Dot-blot tests. Hybridized blot with *pyk* cDNA probe in *E. coli* pET-17b Dot 1, in *E. coli* BL21 (pET-*adk*1-*pyk*) Dot 2 and in *E. coli* BL21 (pET-*pyk*-*adk*1) Dot 3.

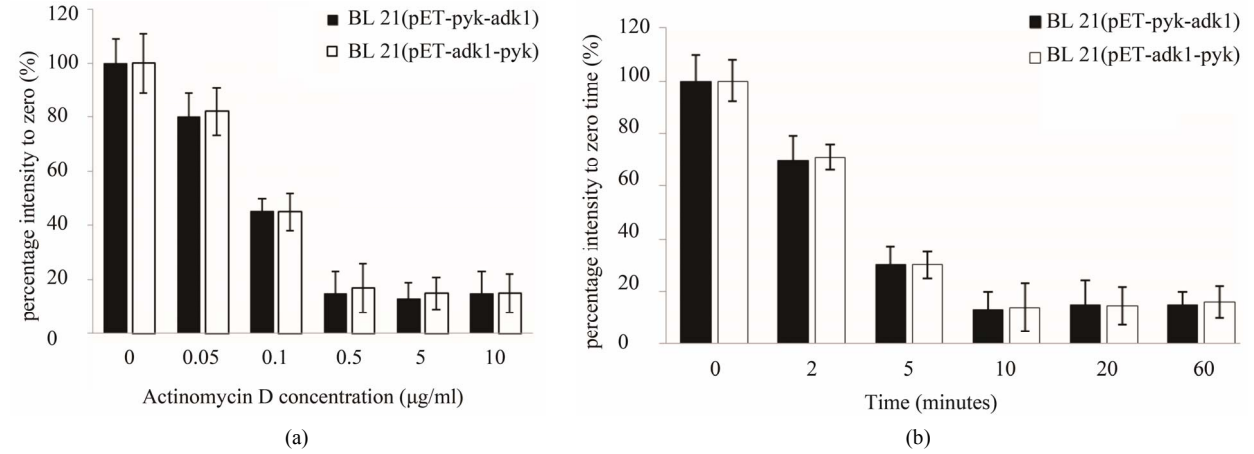


Figure 5. *pyk* mRNA stability assay in *E. coli* BL21 (pET-*adk*1-*pyk*) and E.coli BL21 (pET-*pyk*-*adk*1). (a) add 0; 0.05; 0.1; 0.5; 5.0; 10.0 μg ml^{-1} Actinomycin D for 20 minutes before collected for Dot blot test; 0 μg ml^{-1} Actinomycin D inhibition rate was set as 100%. (b) *pyk* mRNA degradation rate at different time point (0; 2; 5; 10; 20; 40; and 60 min) with 0.5 μg·ml^{-1} Actinomycin D in the medium. 0 point was set as 100%.

5'-UTR and the first 11 codon from ATG start codon, the secondary structures in *E. coli* BL21 (pET-*adk*1-*pyk*) and *E. coli* BL21 (pET-*pyk*-*adk*1) are shown in **Figure 6**. The *pyk* mRNA 5'-end formed more hairpin structures (**Figure 6(a)**) in *E. coli* BL21 (pET-*adk*1-*pyk*) than it did in *E. coli* BL21 (pET-*pyk*-*adk*1) (**Figure 6(b)**). And the calculated Gibbs free energy values (ΔG) of *pyk* mRNA secondary structure based on the principle of minimum free energy in *E. coli* BL21 (pET-*pyk*-*adk*1) was –26.7 kJ·mol^{-1}, it was –18.0 kJ·mol^{-1} in *E. coli* BL21 (pET-*adk*1-*pyk*). There were 31.8% energy barriers increased in BL21 (pET-*pyk*-*adk*1) than in BL21 (pET-*adk*1-*pyk*) as **Table 1** show.

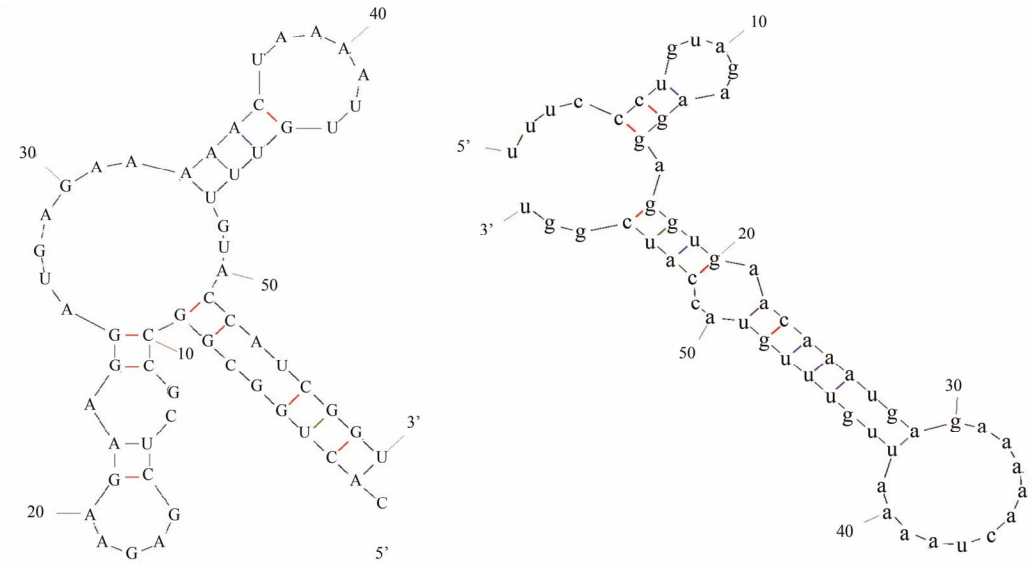
4. DISCUSSION

To simplify the biosynthesis procedure of dATP from dAMP, an efficient method was reported here. Two phosphorylate kinases were coexpressed in the same engineered bacteria, adenylate kinase (AK) which converted deoxynucleoside monophosphates (dAMP) to deoxynucleoside diphosphates (dADP) encoded by *adk*1-gene, and pyruvate kinase (PK) participating in the sequential step to convert the intermediate product deoxynucleoside diphosphates (dADP) to deoxynucleoside triphosphates (dATP) encoded by the *pyk* gene.

Though there have two kinds of arranging castle for *pyk* gene and *adk*1 gene, only the recombinant *E. coli* BL21 (pET-*pyk*-*adk*1) could be used to catalyze 500 mM of dAMP converting to dATP. Another recombinant *E. coli* BL 21(pET-*adk*1-*pyk*) could not complete this coupling phosphorylation reaction, for PK could not be expressed in this strain, the difference existing in the two strains was *pyk* gene and *adk*1 gene exchanged the relevant location on plasmid pET-17b.

The possible reasons which would affect PK expression efficiency in strain *E. coli* BL 21(pET-*adk*1-*pyk*) were analyzed, the transcription level, mRNA stability and the gene-dependent sequence were not the crucial reasons responsible for PK translation efficiency, though they were thought important factors on heterogenous gene expression efficiency in the engineering bacteria [7, 8].

The 5'-UTR +25 bp fragment of the upstream and the gene 5'-terminal sequence of the first 11 codons from the start codon ATG was important on gene translation



(a) (b)

Figure 6. Predicted Secondary structure of *pyk* mRNA 5'-end the first 11 codons and the fragment of 5'UTR + 25 bp, totally the 58 nucleotides in BL21 (pET-*adk*1-*pyk*) and BL21 (pET-*pyk*-*adk*1) as (a) and (b).

Table 1. Comparing Free energy of the anlysis *pyk* mRNA secondary structure sequence in two recombinants.

Plasmid name	Gibbs free energy (ΔG kJ·mol^{-1})	Increased energy barrier (%)
pET-*adk*1-*pyk*	–18.0	-
pET-*pyk*-*adk*1	–26.4	31.8

initiation. The hairpin or loops formed in this region could afford the ribosomes different binding efficiency, and affected the formation of the initiation complex [4, 9]. *pyk* mRNA in the *E. coli* BL21 (pET-*adk*1-*pyk*) stain formed more hairpin structures than it were in *E. coli* BL21 (pET-*pyk*-*adk*1) predicted using the software MFOLD [7]. The calculated Gibbs free energy values (ΔG) of this region was –26.7 kJ·mol^{-1} in *E. coli* BL21 (pET-*adk*1-*pyk*) and –18.0 kJ·mol^{-1} *E. coli* BL21 (pET-*pyk*-*adk*1), there were 31.8% higher energy barriers in *E. coli* BL21 (pET-*pyk*-*adk*1) than in *E. coli* BL21 (pET-*adk*1-*pyk*). Zuker has reported that the –25.1 kJ·mol^{-1} Minimum Gibbs free energy of a gene mRNA secondary structure was the "on or off" switch for gene translation initiation [6]. The higher energy barrier of *pyk* mRNA in *E. coli* BL21(pET-*adk*1-*pyk*) will afford more energy barrier for initiating the protein translation as Pfleger [9] reported, and thus blocked the translation efficiency of *pyk* in this strain. However, it was not happening in *E. coli* BL21 (pET-*pyk*-*adk*1), both PK and AK could be expressed efficiently and used to catalyze dAMP to dATP.

Usually, a stronger mRNA secondary structure would enhance its stability and prolong the half-time of the mRNA. As for *pyk* mRNA in *E. coli* BL21 (pET-*adk*1-*pyk*), the stronger secondary structure only inhibited its translation efficiency, have no effect on its half-time, the reasons might because of there have no endoribonuclease recognizing sequences in this region [10].

This study constructed a dATP conversion strain, and made it feasible to convert dATP from dAMP directly. The relative location has important effect on *pyk* gene translation efficiency. Only cloned *pyk* gene at an optimum location in the expression vector, the dATP conversion strain could be constructed. The recombinants could be used directly to convert 500 mM dAMP into dATP, after a simple purification, above 95% purity dATP could be produced, and the yield was above 89%, it was much higher than the chemical method used recently.

5. CONCLUSIONS

The gene location has very important effects on for *pyk* gene expression level in pET-17b plasmid. The 5'-UTR sequence including the upstream +25 bp fragment, and the first 11 codons from the start codon ATG are sensitive factors on PK translation initiation. Only the *pyk* gene was cloned ahead of *adk*1, the adenylate kinase (AK) and pyruvate kinase (PK) can be co-expressed in one recombinant strain and used directly to complete the two-step phosphorylation reaction, 500 mM dAMP substrates can be converted into dATP directly by one cell.

Using this simple, economically and environmentally friendly method the dATP yield was above 89%.

6. ACKNOWLEDGEMENTS

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