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Co-Expression of Recombinant Nucleoside Phosphorylase from *Escherichia coli* and its Application

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Abstract The genes encoding purine nucleoside phosphorylase (PNPase), uridine phosphorylase (UPase), and thymidine phosphorylase (TPase) from *Escherichia coli* K12 were cloned respectively into expression vector pET-11a or pET-28a. The recombinant plasmids were transformed into the host strain *E. coli* BL21(DE3) to construct four co-expression recombinant strains. Two of them had double recombinant plasmids (DUD and DAD) and the other two had tandem recombinant plasmid (TDU and TDA) in them. Under the repression of antibiotic, recombinant plasmids stably existed in host strains. Enzymes were abundantly expressed after induction with IPTG and large amount of target proteins were expressed in soluble form analyzed with SDS-PAGE. Compared with the host strain, enzyme activity of the recombinant strains had been notably improved. In the trans-glycosylation reaction, yield of 2,6-diaminopurine-2'-deoxyriboside (DAPdR) from 2,6-diaminopurine (DAP) and thymidine reached 40.2% and 51.8% catalyzed by DAD and TDA respectively; yield of 2,6-diaminopurine riboside (DAPR) from DAP and uridine reached 88.2% and 58.0% catalyzed by TDU and DUD respectively.

Keywords Nucleoside phosphorylase · Co-expression · *Escherichia coli*

Introduction

Many nucleosides or their derivates have potential therapeutic effect in cancer or viral infection, such as capecitabine as anticancer agent, zidovudine (AZT) as anti-HIV agent, and vidarabine (Ara-A) as anti-HSV agent [1–3]. Generally, nucleoside analogues were chemically synthesized but the process had disadvantages as multiple procedures, harsh reactive conditions, long synthesis period, and separation of the isomers. In the recent years, more researches focus on the biosynthesis of nucleoside analogues [4, 5].

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Nucleoside phosphorylases (NPases) play key roles in the salvage pathway, which provides an alternative to de novo pathways of purine and pyrimidine biosynthesis [6, 7]. Many nucleoside analogues can be synthesized through the reversible phosphorolysis of the ribo- and deoxyribonucleosides catalyzed by NPases in the presence of inorganic orthophosphate (Pi) [6, 8].

Several kinds of NPases exist in *Escherichia coli* including purine nucleoside phosphorylase (EC.2.4.2.1), uridine phosphorylase (EC.2.4.2.3), and thymidine phosphorylase (EC.2.4.2.4) [9–11]. Both of PNPase and UPase are homohexamer with 25.95 kDa [10] and 27.15 kDa [12] of each monomer respectively. TPase, another kind of pyrimidine nucleoside phosphorylase (PyNPase), is homodimeric with monomer of 47.3 kDa [9].

Wild strains express small amount of NPases, which limit their application in the industrial process. Large amount of NPases are expressed in the recombinant strains constructed by means of genetic engineering methods. In our previous study, recombinant strains expressing single NPase (PNPase, TPase, or UPase) were constructed respectively and successfully applied in the nucleoside biosynthesis. However, in the transglycosylation reaction between purine and pyrimidine nucleoside, both of PNPase and PyNpase must participate in the reaction. Thus in our previous research, two kinds of recombinant strains should be cultivated and added in the reaction system. In order to realize the transglycosylation reaction between purine and pyrimidine nucleoside in single bacteria, strains co-expressed PNPase and UPase, or PNPase and TPase were constructed in this study.

Materials and Methods

Materials

The expression vectors of pET-11a and pET-28a and the strain of *E. coli* BL21(DE3) were from Novagen. Xanthine oxidase was purchased from Sigma. Taq DNA polymerase, T4 lignase, and all of restriction enzymes used in the DNA cloning were purchased from Takara, while the GeneClean kit for DNA purification was purchased from Generay Biotech (Shanghai). Inosine, 2,6-diaminopurine, thymidine, and uridine were purchased from Shanghai Biocaxis Chemicals.

Construction of Strains Hosting the Double Plasmids Co-expression System

Plasmids with different antibiotic resistance were transformed into one host strain to construct the double plasmids co-expression system. Three NPases genes were amplified from *E. coli* K-12 through PCR. All PCR primers used in this step were listed in Table 1. PCR procedure was as follows, initial denaturation at 95 °C for 5 min, then denaturation at 92 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 90 s, total 30 cycles. The final cycle was followed by additional 10 min elongation at 72 °C. The PCR products were detected and separated on a 0.9% agarose gels. The target strand was recovered and purified, then inserted into pMD18-T vector. The nucleotide sequence was determined by Invitrogen (Shanghai). Assembly and analysis of DNA sequences were done by Lasergene (Version 7.1.0, DNASTAR). The basis local alignment tool (BLAST) from the National Center for Biotechnology Information BLAST website was used for database searches. Determined UPase gene *udp* and TPase gene *deoA* were cloned to the expression vector pET-11a (ampicillin resistance) to create recombinant plasmids p11U (pET11a-*udp*) and

Table 1 Primers used in this work for the construction of co-expression strains hosted double plasmids.

Primer	Sequences 5'→3', restriction enzyme site (underlined)	GenBank accession no.
udp(+)	GGGAATTCCATATGTCCAAGTCTGATG, *Nde I*	CP 000948
udp(−)	GCGGATCCTTACAGCAGACGACGCGCC, *BamH I*	
deoA(+)	GGGAATTCCATATGTTTCTCGCACAAG, Nde I	NC 000913
deoA(−)	GCGGATCCTTATTCGCTGATACGGCGATAG, *BamH I*	
deoD(+)	GGTACCCATATGGCTACCCCACACATTAATGC, *Nde I*	NC 000913
deoD(−)	GCGGATCCTTACTCTTTATCGCCCAGCAGAAC, *BamH* I	

p11A (pET11a-*deoA*) respectively, PNPase gene *deoD* was coloned to the multi-cloning site of pET-28a (kanamycin resistance) to construct recombinant plasmid p28D (pET28a-*deoD*). Both of p28D and p11U were transformed into *E. coli* BL21(DE3) to construct the double plasmids stain DUD, which expressed PNPase and UPase simultaneity. The p28D and p11A were transformed into *E. coli* BL21(DE3) to construct strain DAD which simultaneously expressed PNPase and TPase [13]. Plasmids and strains used in this study were listed in Table 2.

Construction of the Strains Hosting Tandem Plasmid Co-Expression System

In order to construct tandem plasmids, *udp* and *deoA,* which were amplified again and restriction enzyme sites were changed, were inserted into the downstream of *deoD* on p28D respectively. Otherwise, ribosome binding site (rbs, parentheses in Table 3) were added to the upstream of the matching sequences in the 5'-end primer (Table 3), thus, every inserted NPase gene had its own rbs to ensure transcription. PCR procedure were similar to the double plasmids system. Fragments of *udp* and *deoA* recovered from agarose gels and

Table 2 Plasmids and *Escherichia coli* strains used in this study.

Strains/ plasmids	Description	Reference
Plasmids		
pMD-18T	Cloning vector, pUC18 derivative, Amp^r	Takara, Japan
pET-11a	pBR322-origin vector for expression NPases, T7 promotor, Amp^r,	Studier et al.
pET-28a	pBR322-origin vector for expression NPases, T7 promotor, Kan^r	Studier et al.
p11U	pET-11a carrying *udp*, Amp^r	Previous study
p11A	pET-11a carrying *deoA*, Amp^r	Previous study
p11D	pET-11a carrying *deoD*, Amp^r	Previous study
p28D	pET-28a carrying *deoD*, Kan^r	This study
pDA	pET-28a carrying *deoD* and *deoA*, Kan^r	This study
pDU	pET-28a carrying *deoD* and *udp*, Kan^r	This study
Strains		
JM109	F′ *traD36 proA*$^{++q-}$ *gyrA96 recA1 relA1 endA1 thi hsdR17*	New England Biolabs
BL21(DE3)	F-*ompT hsdS*B (rB-mB-) *gal dcm* (DE3)	Novagen
DAD	BL21(DE3) (p11A, p28D),	This study
DUD	BL21(DE3) (p11U, p28D)	This study
TDA	BL21(DE3) (pDA)	This study
TDU	BL21(DE3) (pDU)	This study

Amp^r Apramycin resistance, Kan^r kanamycin resistence

Table 3 Primers used in this work for the construction of tandem co-expression plasmids.

Primer	Sequences 5'→3', restriction enzyme site (underlined)	GenBank accession no.
udp(+)	GGAGCTC(GAAGGAG)TTGTTTCTCG, *Sac I*	CP 000948
udp(−)	TTAAGCTTTTATTCGCTGATACGGG, *Hind III*	
deoA(+)	GGAGCTC(GAAGGAG)ATGTCCAAGTC, *Sac I*	NC 000913
deoA(−)	TTAAGCTTTTACAGCAGACGACGCGC, Hind III	

digested by *SalI-HindIII* were inserted into the vector p28D respectively to obtain two tandem co-expression plasmids: pDU (pET-28a-*deoD-udp*) and pDA (pET-28a-*deoD-deoA*) (Fig. 1). The plasmids were transformed respectively into *E. coli* BL21(DE3) to construct two recombinant strains TDA and TDU [13].

Evaluation the Stability of Double Plasmids System

The four recombinant strains were serially subcultivated respectively in Luria-Bertani (LB) media with antibiotics at 37 °C for every 12 h. Strains were recovered from the culture per 24 h and incubated on LB agar plate for 12 h at 37 °C, individual colony was selected randomly and replica plated on selective LB agar plates with antibiotics and cultivated for 12 h at 37 °C. The ratio of the number of colony forming units (CFU, plasmid-carring cells) on the selective agar plate to that (non-plasmid-carring cells) on the non-selective agar was used to evaluated the stability of double plasmids system [14].

Induced Expression of Nucleoside Phosphorylase

Strains of DUD and DAD were cultured in LB medium at 37 °C supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. And the other two of TDU and TDA were incubated in the LB medium with 50 μg/ml kanamycin. Until $OD_{600\ nm}$ reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium to a final concentration of 0.5 mmol/L. The cultures were grown further at 37 °C for 4 h and harvested by centrifugation. Cells washed three times and suspended with 10 mmol/L TE buffer (pH 8.0) were disintegrated by sonication. The proteins were indentified by SDS-PAGE (12% acrylamide).

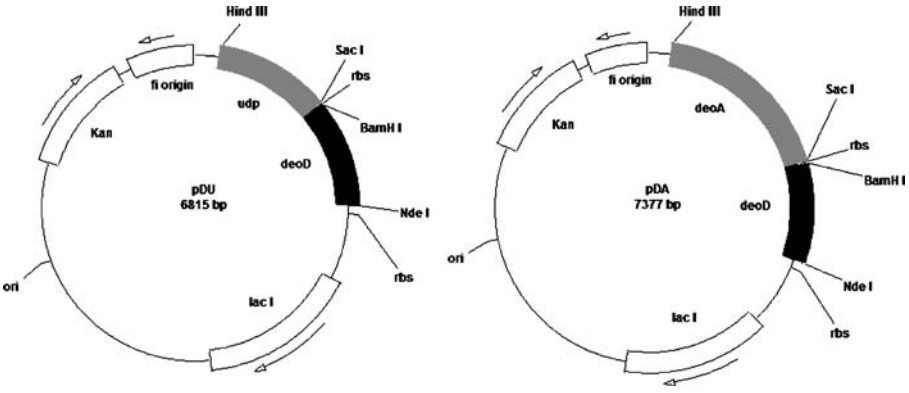

Fig. 1 Gene maps of recombinant plasmids of pDU and pDA

Assay of Enzyme Activity

PNPase activity was determined according to Kalckar's method [15]. Two-milliliter reaction mixture (10 mmol/L inosine, 1 mmol/L EDTA, 50 mmol/L potassium phosphate buffer pH 7.0, proper amount of wet bacteria) was incubated at 60 °C for several minutes, then heated to 100 °C in water bath for 5 min to terminate the reaction. After centrifugation, 30 μl supernatant was diluted to 3 ml with 33 mmol/L potassium phosphate buffer and proper amount of xanthine oxidase was added [16]. The oxidizing reaction lasted for 1 h at 25 °C and stopped in 100 °C water bath for 5 min. Then the absorbance at 290 nm was recorded.

UPase and TPase activity were determined referring to Thomas et al. [17]. Two-milliliter reaction mixture (25 mmol/L uridine/thymidine, 1 mmol/L EDTA, 50 mmol/L potassium phosphate buffer of pH 7.0, and proper wet bacteria) was water bathed at 50 °C and stopped by adding 0.01 mol/L NaOH. The mixture absorbance at 290 nm were monitored.

One unit of NPases was defined as the amount of enzyme which under the above conditions caused an increase in optical density of 0.01 per minute at 290 nm [18].

Synthesis of Nucleosides

The reaction mixture, containing 30 mmol/L uridine or thymidine, 30 mmol/L 2,6-diaminopurine (DAP), 50 mmol/L potassium phosphate buffer (pH 7.5), and 1% (*w*/*v*) wet bacteria in a total volume of 2 ml was incubated at 50 °C for 1 h. Nucleosides synthesized was analyzed by high performance liquid chromatography (HPLC)[19] with ultraviolet (UV) detection at 254 nm using a Hypersil ODS2 5 μm column (4.6×250 mm) with a solvent of 5% (*v*/*v*) acetonitrile and 95% (*v*/*v*) water and a flow rate of 0.70 ml/min.

The reactions were as follows:

$$\mathrm{DAP} + \mathrm{uridine} \xrightleftharpoons{\mathrm{PNPase,\ UPase}} \mathrm{DAPR} + \mathrm{uracil}$$

$$\mathrm{DAP} + \mathrm{thymidine} \xrightleftharpoons{\mathrm{PNPase,\ TPase}} \mathrm{DAPdR} + \mathrm{thymine}$$

Results

Expression of Nucleoside Phosphorylase in *E. coli*

Analyzed by SDS-PAGE, the recombinant NPases were notable co-overexpressed in the host strain of *E. coli* BL21(DE3) after induced by IPTG (Figs. 2 and 3). Target proteins were expressed inside bacteria cells, no secretive protein detected in the culture media after centrifugation (Lane 1, 2 in Fig. 2b and Lane 3, 8 in Fig. 3). The NPases were denatured to monomers in SDS-PAGE, as monomeric PNPase(~26 kDa) and TPase (~47 kDa) were shown in the Lane 3 in Fig. 2a and Lane 7 in Fig. 3. There were also massive co-overexpression products of PNPase and UPase as the darkest band, however, the two monomeric proteins could not be visibly separated through SDS-PAGE (Lane 4 of Fig. 2a and Lane 2 of Fig. 3) owing to the molecular weight of the two monomers were nearly equivalent (molecular weight of monomeric PNPase was ~ 26 kDa and monomeric UPase was ~27 kDa). Both of the double-plasmid and the tandem co-expression plasmid recombinant strains had remarkable quantity of soluble target proteins (Lane 3, 4 of Fig. 2b;

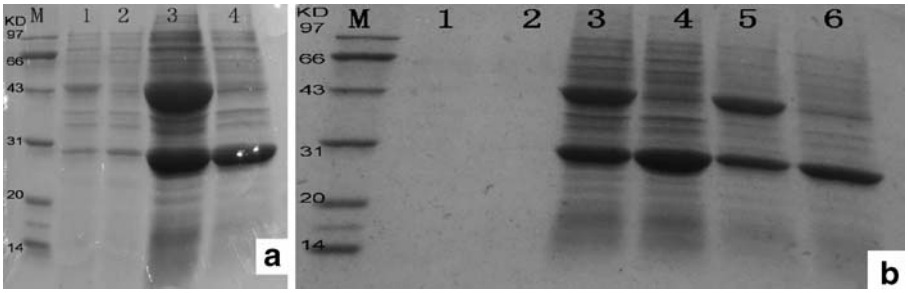

Fig. 2 SDS-PAGE of recombinant strains with double plasmids (DAD and DUD) SDS-PAGE was performed in a 12% PAG. **a** *M* protein marker; lane *1* and *2* total proteins of DAD or DUD before adding IPTG; lane *3* total proteins of DAD after inducing; lane *4* total proteins of DUD after inducing; **b** *M* protein marker; lane *1* and *2* culture media of DAD or DUD after centrifugation; lane *3* supernatant after sonication of DAD; lane *4* supernatant after sonication of DUD; lane *5* precipitate after sonication of DAD; lane *6* precipitate after sonication of DUD

Lane 4, 9 of Fig. 3). There expressed more inclusion bodies in DUD and DAD than that in TDU and TDA (Lane 5, 6 of Fig. 2b and Lane 5, 10 of Fig. 3).

Stability of Recombinant Plasmids

Since strains of DAD and DUD had two incompatible recombinant pET vectors, any one or both of the recombinant plasmids may lose in the serial cultivation. So the stability of double plasmids system should be considered. After serial cultivation for 5 days, 75% cells of DAD retained double plasmids and so did 70% cells of DUD (Fig. 4). Two recombinant plasmids demonstrated relatively stable coexistence in cells under the pressure of double antibiotics (Amp^r and Kan^r).

There was only one recombinant plasmid in the cell of TDA or TDU, the stability was higher than that of DAD or DUD (Fig. 5). After 5 days serial subcultivation, cells carrying plasmid maintained above 90%, TDA was 91.2% and TDU was 90.4%.

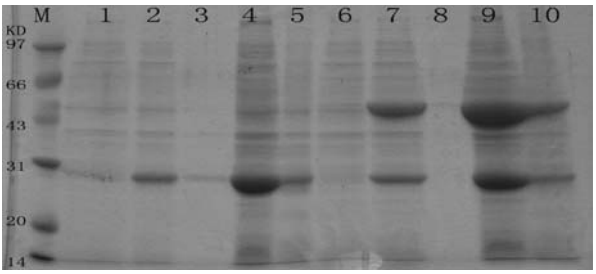

Fig. 3 SDS-PAGE of recombinant strains with tandem co-expression plasmid (TDA and TDU). Cells were grown in LB culture media. SDS-PAGE was performed in a 12% PAG. *M* protein marker; lane *1* and *6* total proteins of TDU or TDA before adding IPTG; lane *2* total proteins of TDU after inducing; lane *3* and *8* culture media of TDU or TDU after centrifugation; lane *4* supernatant after sonication of TDU; lane *5* precipitate after sonication of TDU; lane *7* total proteins of TDA after inducing; lane 9: supernatant after sonication of TDA; lane 10: precipitate after sonication of TDA

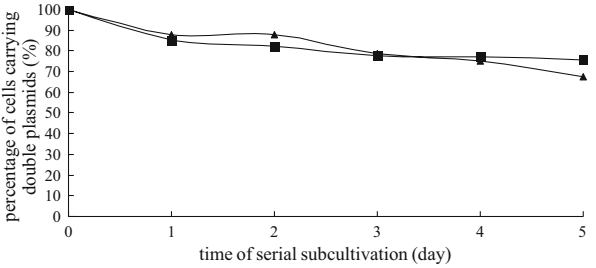

Fig. 4 Cells carrying double plasmids in 5 days serial subcultivation, DAD (*filled square*) and DUD (*filled triangle*)

Activity of NPases

Each of NPases activity in the recombinant co-expression strains was detected respectively. Wet cells centrifugated from cultivation liquid were used as the crude enzyme in activity measurement. The activity of each NPase in recombinant strains was shown in Table 4. All recombinant strains exhibited much higher enzyme activity than the control *E. coli* strain. Activity of PNPase or TPase in DAD was much higher than that in TDA, however activity of PNPase or TPase in DUD was a little higher than that in TDU and strains containing double plasmids perform better than the strains with tandem co-expression plasmid.

Biosynthesis of Nucleosides with Recombinant Bacteria

DAP and pyrimidine nucleoside as uridine or thymidine were used as substrates to estimate the ability of conversion between purine and pyrimidine nucleoside, the conversion yield was listed in Table 5. All the recombinant strains showed good catalysis potentiality. Little uridine or thymidine was converted to purine nucleosides in the presence of *E. coli* BL21 (DE3), the conversion yield was only 1.4% or 4.3%. Catalyzed by DUD or DAD, DAP could easily obtained pentose from pyrimidine nucleoside and then was transformed to 2,6-diaminopurine riboside (DAPR) or 2,6-diaminopurine-2'-deoxyriboside (DAPdR), and the yield reached 58.0 and 40.2% respectively. Also, compared with two strains above, TDU and TDA had higher converted ability, which transformed 88.2% uridine to DAPR and 51.8% thymidine to DAPdR.

Discussion

Esipov et al. cloned three *E. coli* NPases to the expression vector pET20b respectively and expressed active proteins in the host strain BL21(DE3) [20]. And in our previous study, we

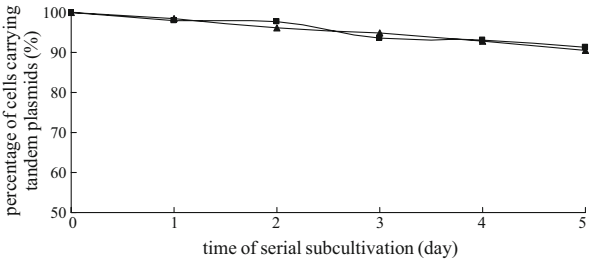

Fig. 5 Cells carrying tandem plasmids in 5 days serial subcultivation, TDA (*filled square*) and TDU (*filled triangle*)

Table 4 Enzyme activity of recombinant *E.coli*[a].

	DAD (U/mg)	DUD (U/mg)	TDA (U/mg)	TDU (U/mg)	Control *E.Coli* strain[b](U/mg)
PNPase	544	235	292	209	31
Tpase	15,600	–	6,742	–	425
Upase	–	275	–	221	21

[a] Dates in Table 2 were obtained by assaying the activity of free integrity cells

[b] *E. coli* BL21(DE3)

had cloned the same three NPases genes into pET11a, after induced by IPTG, the genes were highly expressed as well. As mentioned above, in the conversion between purine nucleoside and pyrimidine nucleoside, both of PNPase and PyNPase should take part in the reaction, so recombinant strains with single gene expression had limitation in practice. In this study, co-expression strains hosting double recombinant plasmids or tandem recombinant plasmid was constructed according to pattern of PNPase+PyNPase.

Owing to the strong T7*lac* promoter and translational enhancer in pET expression system, after induced, nearly all of cell resources were used to express target protein. In most situations, many exogenous genes were prone to be expressed as inclusion bodies. Generally soluble protein could be obtained from denaturation of inclusion body. However in most industrial enzymatic reaction, whole cells were usded as catalyst directly, it couldn't work if the inclusion body was the major expression. On the other hand, expression at lower temperature or reducing the amount of inducer was also good way to increase the soluble target protein, but the expression time would be prolonged or insuffient protein was acquired. In this research, genes of NPases from E. *coli* K-12 were chosen, as intrinsic genes, to be cloned into pET/BL21(DE3) system to express soluble protein easily [21]. From analysis of SDS-PAGE, abundant of soluble protein was expressed after induced at 37 °C in the recombinant strains, and low amount of inclusion body was detected.

In this work, wet recombinant bacteria were chosen as the crude enzyme for the enzyme activity determination and biosynthesis of nucleosides. Co-expression recombinant strains showed remarkably higher specific activity of NPase than the control. And in the nucleoside conversion reactions, co-expression recombinant strains exhibited favorable bioconversion ability. Uridine and DAP, 88.2%, were converted into DAPR by TDU. In the synthesis of DAPdR, 51.8% thymidine was convertered by TDA, 12-fold higher than the control strain. M´edici [22] had screened several wild strains which had the potential to biosynthesis 2,6-diaminopurine nucleosides. It was reported that 90% DAP was converted into DAPR by *Proteus vulgaris*, *Aeromonas salmonicida*, and *Achromobacter cycloclastes* and 85% DAP was converted into DAPdR by *Chromobacterium violaceum* and *Serratia marescens*. Compared with the wild strains above, recombinant strains had a fair DAPR biosynthesis ability, and the yield of DAPdR was a little lower. But in M'edici research, the

Table 5 Yield of different products produced by recombinant strains.

Substrate	Product	Strain	Yield (%)
DAP+ Uridine	DAPR	DUD	58.0
		TDU	88.2
		BL21(DE3)	1.4
DAP+ Thymidine	DAPdR	DAD	40.2
		TDA	51.8
		BL21(DE3)	4.3

reaction needed 4 h to complete and large amount of bacteria cells were required. In this work, the reaction finished in 1 h and less wet cells were used.

In the recombinant plasmids stability investigation, the tandem plasmid co-expression bacteria had higher stability, 90% cells retained the plasmids under pressure of antibiotic. But in double-plasmid system, only about 70% cells retained double plasmids.

The recombinant co-expression strains displayed great potency and provided a valuable way to produce nucleoside and analogues. Fermention conditions of recombinant strains and reaction conditions in nucleosides biosynthesis were optimized in further study. More nucleoside or derivates would be produced easily with our recombinant.

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