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Two-step efficient synthesis of 5-methyluridine via two thermostable nucleoside phosphorylase from *Aeropyrum pernix*

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ABSTRACT

5-Methyluridine has been synthesized in high yield using guanosine and thymine as starting materials in the presence of highly thermostable recombinant purine nucleoside phosphorylase (PNP) and uridine phosphorylase (UP) obtained from hyperthermophilic aerobic crenarchaeon *Aeropyrum pernix*. Key reaction parameters such as pH, temperature, concentration of buffer and substrates were investigated. At the optimal conditions, 5-methyluridine was achieved in yield 85% with a guanosine conversion of 96% in 10 ml scale. The process can be performed at high temperature, which will highly increase the solubility of substrates, therefore, this process is suitable for the industry application.

5-Methyluridine is a unique unnatural nucleoside. It is a key intermediate in the synthesis of anti-AIDS drug such as stavudine (d4T)[1,2] and zidovudine (AZT).[3]

Conventional chemical methods for the synthesis of 5-methyluridine always suffer from the complex protection and deprotection steps, low stereoselectivity and the environmental concerns of the process.[4] Biosynthesis of nucleoside analogues has been proven to be a practicable and favorable option, which are regiospecific, stereospecific and environmentally friendly.

Several processes for enzymatic synthesis of 5-methyluridine have been developed employing different kinds of enzymes. Using 2′-deoxyribose-5′-phosphate aldolase (DERA) and phosphopentomutase (PPM), pyrimidine nucleosides were synthesized from inexpensive sugars, such as glucose in the presence of acetaldehyde and nucleobase.[5,6]

An alternative way of obtaining 5-methyluridine is the biosynthetic process catalyzed by nucleoside phosphorylases from microorganisms.[7] The process of the enzymatic synthesis of 5-methyluridine is depicted in Figure 1.

Inosine was first used as starting material to synthesize 5-methyluridine. Hori et al. used immobilized purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859 to synthesize 5-methyluridine in about 32% yield on a large scale.[8] To improve the conversion yield, further research was investigated by Hori et al. Various experimental conditions were conducted to improve the conversion of the starting material. However, such improvement did not result due to the existing equilibrium in the reaction.[9,10] In order to push the equilibrium forward, increasing the solubility of starting material, reducing the concentration of released base and carrying out the reaction at higher temperature were the better choices. For example, in the presence of xanthine oxidase which reduced the released base through oxidation the yield of 5-methyluridine increased to 76%.[11,12]

In the past, we had successfully cloned a purine nucleoside phosphorylase and a uridine phosphorylase from *Escherichia coli* K-12. The two nucleoside phosphorylases were abundantly expressed after the induction by isopropyl-thio-β-D-galactoside (IPTG) in *E. coli* BL21 and large amount of target proteins were expressed in a soluble form. Using an auxiliary enzyme such as xanthine oxidase from *Arthrobacter globiformis*, the reaction system produces 5-methyluridine from inosine and thymine in yield 70%.

Compared with inosine, guanosine is a better start material to synthesize 5-methyluridine because the released guanine in the reaction (Fig. 1) is less soluble in water than hypoxanthine. Ishii et al. reported that 5-methyluridine was synthesized in 74% yield from guanosine and thymine using the whole cell of *Erwinia carotovora* AJ-2992 with high nucleoside phosphorylase activity.[13] Bode and co-workers developed a high yielding coupled enzymatic reaction using *Bacillus halodurans* purine nucleoside phosphorylase and *E. coli* uridine phosphorylase for synthesis of 5-methyluridine.[14] The drawback of the process using guanosine as the starting material was the low solubility of the substrates.[15]

One of the key points of the present work was that two promising highly thermostable nucleoside phosphorylases (PNP and UP) from hyperthermophilic aerobic crenarchaeon *Aeropyrum*

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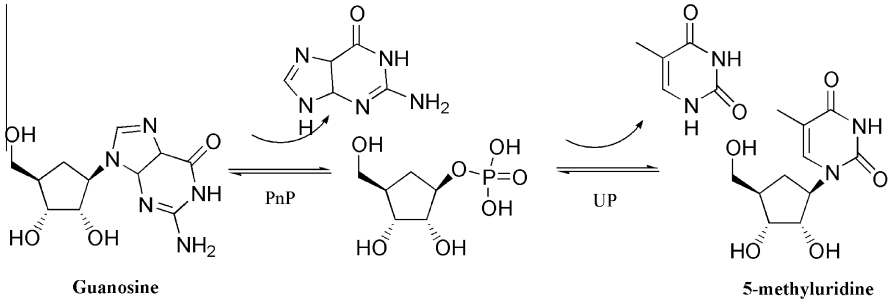

Figure 1. Mechanism for synthesis of 5-methyluridine from guanosine.

pernix were found for the first time by our group.[16] The two enzymes were expressed in *E. coli* BL21 as shown in Figure 2.

Both PNP and UP were purified by one single heat treatment step. Cells from the expression culture (total 1000 ml) were suspended in phosphate buffer and sonicated. The cell-free extract was then heated at 80 °C for 30 min and centrifuged 12000*g* for 15 min. Approximately 100 mg of the recombinant protein was purified.

Small-scale assays were first performed to optimize the reaction conditions. As the reaction temperature had significant influences on the solubility of substrates and productivity of the 5-methyluridine. We first investigated the effect of temperature on the productivity of 5-methyluridine. Reactions were initialed by adding 100 μg PNP and 100 μg UP into 500 μl reaction medium containing 60 mM guanosine and 120 mM thymine, with shaking at different temperature. As expected, even at 90 °C, the 5-methyluridne yield still maintained at a high level of 85% yield. The results showed that the increasing reaction temperature had a negligible impact on the productivity from 60 to 90 °C (Fig. 3). This reaction which is benefited from the increasing solubility of guanosine and thymine, therefore, can highly improve the productivity.

Next, the optimum reaction pH was studied at 90 °C. As shown in Table 1, the reaction was carried out at pH 6.0–9.0. We found that the optimum pH value of the reaction was 9.0. While UP shows the optimum pH value at 9.0 past studies showed that PNP exhibited an optimum pH value at 5.0 with a wide range of solubility at different values of pH. These results suggest that UP is a key enzyme in the reaction and the solubility of the substrates increase at the optimum pH value.

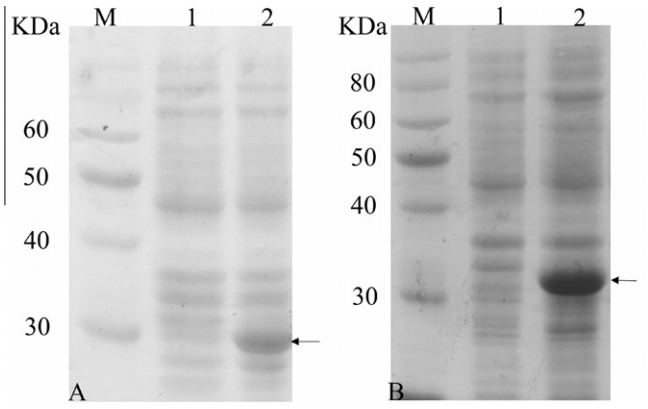

Figure 2. Coomassie-stained gel after SDS–PAGE of expression proteins from transformed bacteria by gentle lysis (A). M. protein marker; lane 1 *E. coli* BL21 (DE3) cells transformed with empty pET30a vector; lane 2. *E. coli* BL21 (DE3) cells transformed with pET30a-PnP vector (B). M. protein marker; lane 1. *E. coli* BL21 (DE3) cells transformed with empty pET30a vector; lane 2. *E. coli* BL21 (DE3) cells transformed with pET30a-UP vector.

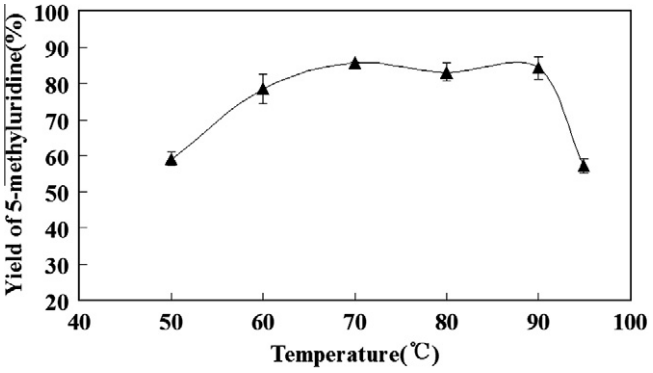

Figure 3. Effect of temperature on productivity of 5-methyuridine.

Table 1
Effect of pH on the yields of 5-methyluridine at 90 °C

pH	Guanosine conversion (%)	5-methyluridine yield (%)
6	96.9	71.7
7	97.3	78.7
8	98.0	84.1
9	98.3	85
10	97.0	19
11	97.1	17

Table 2
Summary of the optimum reaction conditions

Parameters	Optimum
Temperature	90 °C
pH	9.0
Time	48 h
Substrate concentration	50 g/L
Phosphate buffer concentration	20 mmol/L
Mole ratio of substrate	Thymine to guanosine (2:1)
Enzyme loading	UP to PNP (1:2)

The optimum phosphate buffer concentration was investigated which showed that optimal concentration was 20 mmol L^{-1}. The parameters such as the mole ratio of guanosine and thymine loading, mole ratio of UP and PNP were also investigated. The results were summarized in the Table 2.

To demonstrate the overall reaction at a larger scale, an experiment was conducted at 10 ml using an enzyme loaded with 10 mg PNP and 5 mg UP and a thymine to guanosine mole ratio of 2:1 (177 mM guanosine and 397 mM thymine) performed at 90 °C over 48 h, with sodium phosphate buffer (20 mM) at pH 9.0. The result was analyzed by HPLC, showing a high conversion of 85%.

5-methyluridine was synthesized[17](Fig. 4).

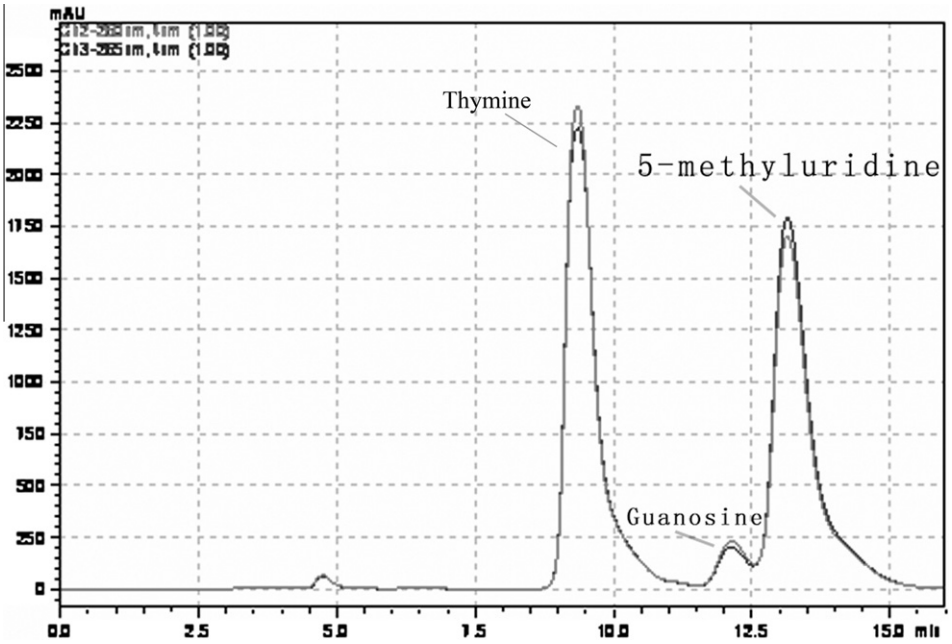

Figure 4. HPLC analysis of the results, 5-methyluridine was synthesized with high yield.

In summary, an efficient process to synthesize 5-methyluridine was developed which employed a combination of two thermostable nucleoside phosphorylases in high yields of 85%. Easily enzyme preparations could be applied directly to a two-step transglycosylation reaction. The most important part of our work was that for the first time we have successfully employed highly thermostable enzymes for the preparation of 5-methyluridine. This approach is promising. By virtue of the excellent thermostability of nucleoside phosphorylases, this reaction is amenable for industrial preparation. In fact, the higher the reaction temperature, the higher solubility of the substrates and the higher speed of the reaction. As a result, the reaction offers higher yield. To our best knowledge, this two-step reaction employing highly thermostable nucleoside phosphorylases was not disclosed in the literature.

References and notes

1. Chen, B.-C.; Quinlan, S. L.; Stark, D. R.; Gregory Reid, J.; Audia, V. H.; George, J. G.; Eisenreich, E.; Brundidge, S. P.; Racha, S.; Spector, R. H. *Tetrahedron Lett.* **1995**, *36*, 7957.
2. Chen, B.-C.; Quinlan, S. L.; Reid, J. G.; Spector, R. H. *Tetrahedron Lett.* **1998**, *39*, 729.
3. Chen, B.-C.; Quinlan, S. L.; Gregory Reid, J. *Tetrahedron Lett.* **1995**, *36*, 7961.
4. Wilson, L. J.; Hager, M. W.; El-Kattan, Y. A.; Liotta, D. C. *Synthesis* **1995**, *1995*, 1465.
5. Horinouchi, N.; Kawano, T.; Sakai, T.; Matsumoto, S.; Sasaki, M.; Mikami, Y.; Ogawa, J.; Shimizu, S. *New Biotechnol.* **2009**, *26*, 75.
6. Horinouchi, N.; Ogawa, J.; Kawano, T.; Sakai, T.; Saito, K.; Matsumoto, S.; Sasaki, M.; Mikami, Y.; Shimizu, S. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 615.
7. Hanrahan, J. R.; Hutchinson, D. W. *J. Biotechnol.* **1992**, *23*, 193.
8. Hori, N.; Watanabe, M.; Sunagawa, K.; Uehara, K.; Mikami, Y. *J. Biotechnol.* **1991**, *17*, 121.
9. Hori, N.; Watanabe, M.; Sunagawa, K.; Uehara, K.; Mikami, Y. *J. Ferment. Bioeng.* **1992**, *73*, 326.
10. Hori, N.; Watanabe, M.; Mikami, Y. *Biocatalysis Biotransformation* **1991**, *4*, 297.
11. Nobuaki, H.; Kyouko, U.; Yoichi, M. *Agric. Biol. Chem.* **1991**, *55*, 1071.
12. Pal, S.; Nair, V. *Biocatalysis Biotransformation* **1997**, *15*, 147.
13. Ishii; M.; Shirae; H.; Yokozeki; K. Enzymatic production of 5-methyluridine from purine nucleosides and thymine by Erwinia carotovora AJ-2992; Tokyo, JAPON: Agricultural Chemical Society of Japan, 1989.
14. Gordon, G. E.; Visser, D. F.; Brady, D.; Raseroka, N.; Bode, M. L. *J. Biotechnol* **2011**, *151*, 108.
15. Visser, D. F.; Hennessy, F.; Rashamuse, J.; Pletschke, B.; Brady, D. *Journal of Molecular Catalysis B: Enzymatic* **2011**, *68*, 279.
16. The PNP and UP were amplified by PCR. for PNP the primers used were: The sense primer APPufwd was5′-gggaattccatatggaggaa gccggttca-3′ and the antisense primer APPu rwd was 5′-cccaagcttgactcctcctgtgaggac-3′ the NdeI and HindIII sites (underlined) were added to the forward and reverse primers, respectively for UP the primers used were: The sense primer Appyfwd was 5′-GGGAATTCCATATGGGAGACGAGAGTCT -3′, the NdeI site was underlined. The antisense primer Appyrwd was 5'-CCCAAGCTTTCATGTGCGTCTGCAC GCCAGGCTC -3′, the HindIII site was underlined.
17. HPLC analysis: sample were analyzed by HPLC on a Shimadzu HPLC (interfaced with Shimadzu LC solution Software) equipped with a detector at 260 nm and a Phenomenex Synergi 4 μ Max-RP 80A column (150 × 4.60 mm) at 22 °C. The mobile phase was 92% water: 8% methanol, at a flow rate of 1.0 ml/min.