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An improved microbial synthesis of purine nucleosides

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Abstract

E. coli BL21 synthesized purine nucleosides from pyrimidine ones. A 94% yield of adenosine from uridine was reached within 1 h.

Introduction

Nucleoside analogues have been extensively used in cancer and antiviral therapies (Hanrahan & Hutchinson 1992, Perigaud *et al.* 1992) as monomers and, more recently, as building blocks for antisense strategies (Agrawal & Iyer 1995, Iribarren *et al.* 1990). Traditionally, nucleosides were prepared by various chemical methods which often involved difficult, inefficient and time-consuming multistage processes (Pal & Nair 1997).

Several papers and patents have appeared reporting the enzymatic preparations of both natural and unnatural nucleosides (Krenitsky *et al.* 1981, Ling *et al.* 1990). The syntheses were performed as one-pot reactions using a pyrimidine nucleoside as the pentosyl donor and a purine base as the pentosyl acceptor. These processes have the disadvantage of laborious scale-up, in contrast to the whole cell-catalyzed procedure which is very simple.

Though some microorganisms have already been used in the synthesis of purine and purine analogue nucleosides (Murakami *et al.* 1991, Utagawa 1999, Utagawa *et al.* 1985, Yokozeki *et al.* 1990), all the reported conditions required long reaction times in order to achieve good yields. This paper deals with the novel application of a particular *E. coli* strain (BL21) for the fast synthesis of purine nucleosides.

Materials and methods

Materials

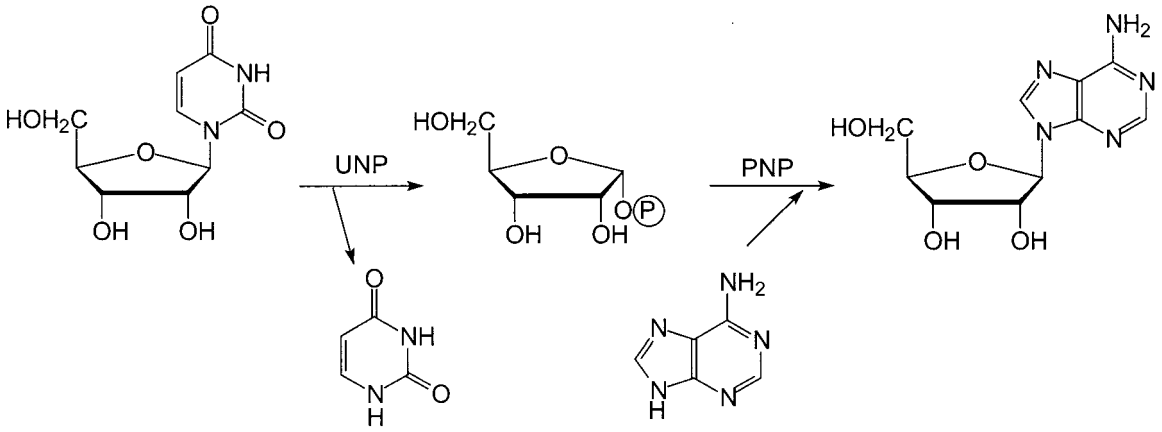
All chemicals employed were of analytical grade. Nucleosides and bases were purchased from Sigma. Culture media chemicals were from Merck and HPLC grade methanol was from Fischer.

Preparation of cells

E. coli ATCC 47092 (BL21) was grown at 30 °C for 16 h with shaking in 250 ml Erlenmeyer flasks containing 50 ml culture medium: 1% (w/v) meat extract, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl in deionized water adjusted to pH 7 with KOH. After growth, cells were harvested by centrifugation for 10 min at 12 000 *g*, washed with 30 mM potassium phosphate buffer (pH 7) and re-centrifuged. The cells were directly used as the biocatalyst.

Synthesis of purine nucleosides

The standard reaction mixture comprising wet cell paste obtained from 15 ml of broth (10.6 mg dry wt ml^{-1}), 30 mM pyrimidine nucleoside, 10 mM purine and 30 mM pH 7 potassium phosphate buffer, in 5 ml, was slowly stirred at 60 °C for 1 h. Samples were centrifuged at 10 000 *g* for 30 s and the supernatants were analysed by both TLC and HPLC.



Scheme 1. Synthesis of adenosine from uridine. UNP: uridine nucleoside phosphorylase; PNP: purine nucleoside phosphorylase.

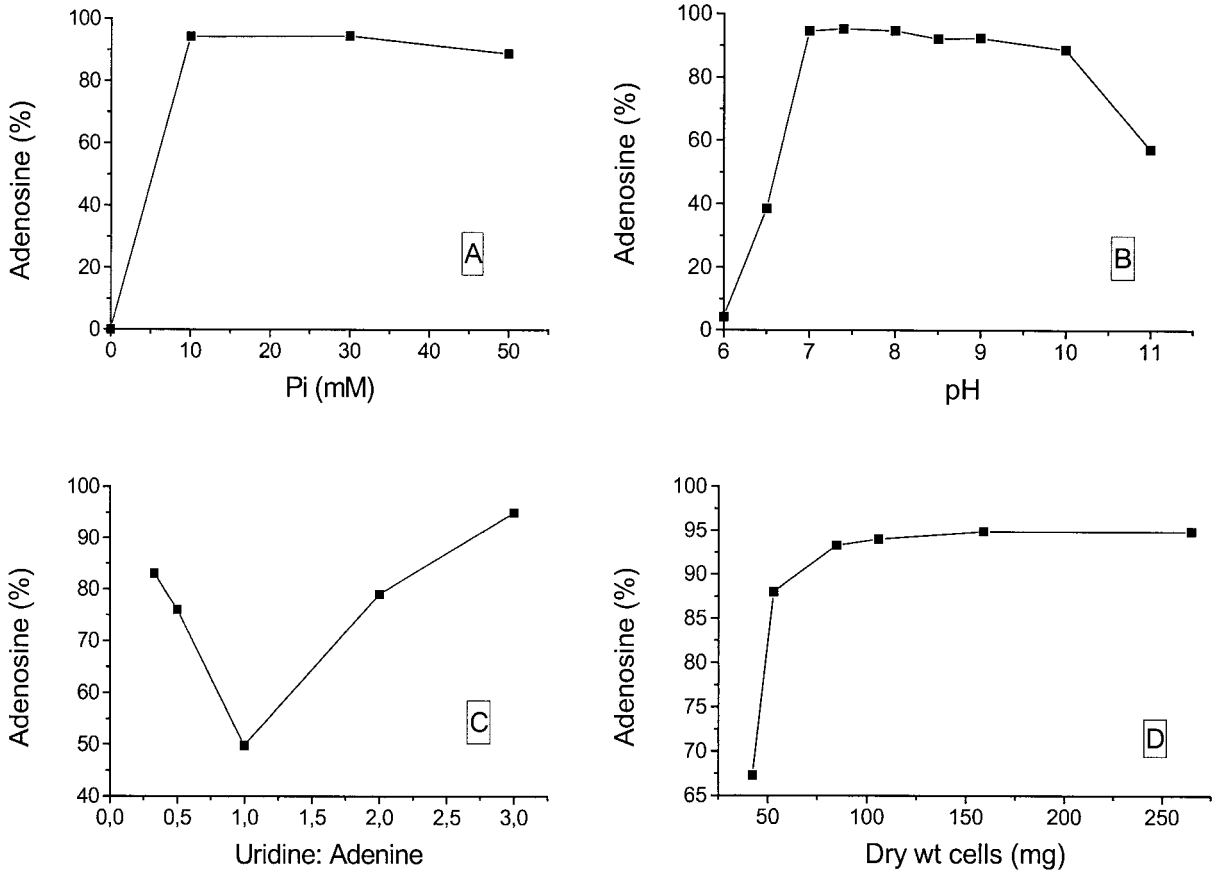


Fig. 1. Effects of Pi concentration (A), pH (B), uridine to adenine ratio (C), mass of dry cells (D) on the production of adenosine by *E. coli* BL21. Reaction mixtures comprising uridine, adenine, phosphate buffer and intact cells all in 5 ml, were incubated at 60 °C with shaking for 1 h. In each case the remaining reaction conditions were standard (see Materials and methods). % $\text{Adenosine}_{\text{formed}} = [\text{adenosine}]_{\text{obtained}} \times 10^2 / [\text{adenosine}]_{\text{theoretic}}$.

Assay of reaction products

Production of purine nucleosides was determined by TLC using chloroform/methanol (8:2, v/v) as the mobile phase. For quantitative analysis an HPLC equipped with an UV detector (254 nm) and a C-18 column was used. The operating conditions were as follows: (1) 10 min water/methanol (95:5, v/v), flow rate 0.5 ml min^{-1}; (2) 7 min gradient to water/methanol (90:10, v/v), flow rate 0.8 ml min^{-1}; (3) 10 min water/methanol (90:10, v/v), flow rate 0.8 ml min^{-1}.

Results and discussion

Optimal conditions for microbial production of adenosine starting from uridine and adenine (Scheme 1) were determined after the analysis of several experimental parameters such as pH, temperature, reaction time, amount of biocatalyst, reagents and buffer concentration.

The most efficient transformation (94.5% yield) occurred when standard conditions were employed (see Materials and methods).

Adenosine was formed only in the presence of inorganic phosphate. As expected, at high phosphate concentration a slightly lower conversion was observed (Figure 1A) because phosphate competes with ribose 1-phosphate in the second reaction step (Scheme 1) (Utagawa *et al.* 1985).

Similar yields in adenosine were obtained between pH 7 and 10 (Figure 1B). We selected pH 7 as the working pH since this gives better reagent solubility. The temperature dependence was also optimized (data not shown). Our results were in agreement with those of Utagawa *et al.* (1985). If the reaction was carried out below 55 °C, adenine and adenosine were deaminated by adenosine deaminase to hypoxanthine and inosine respectively, detected by HPLC. No adenosine was obtained above 65 °C due to inactivation of nucleoside phosphorylases (Krenitzky & Koszalka 1981).

Varying the uridine to adenine ratio (Figure 1C), best yields were obtained with excess of either of the reactants. Therefore, this system seems also suitable for the synthesis of sugar modified nucleosides using the pentosyl donor as the limiting reagent.

Reaction time was dependent on the amount of biocatalyst (Table 1). Long reaction times were required in order to achieve high yields when small quantities

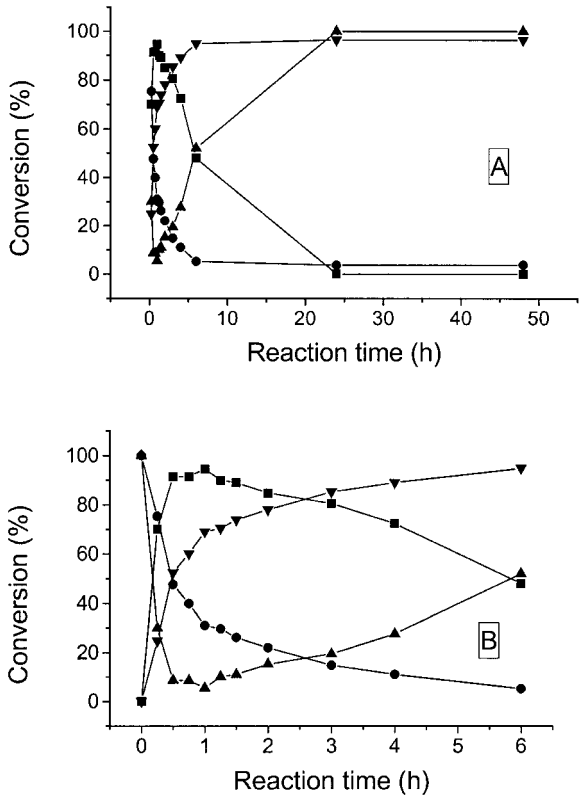


Fig. 2. Time course of adenosine production by *E. coli* BL21. Reaction mixture comprising 30 mM uridine, 10 mM adenine, 30 mM phosphate buffer (pH 7) and intact cells in a total volume of 5 ml, was incubated at 60 °C for 48 h (A). (B) 0-6 h expanded graphic A. % conversion = $\text{concentration}_{\text{measured}} \times 10^2/\text{concentration}_{\text{theoretic}}$. (■) adenosine, (●) uridine, (▲) adenine, (▼) uracil.

Table 1. Influence of the amount of biocatalyst on reaction time to achieve adenosine maximum yield[a].

Dry wt of cells (mg)	Adenosine[b] (%)	Reaction time[c] (h)
265	93.7	0.5
159	94.5	1.0
80	89.1	3.0

[a]Reaction conditions: 60 °C, 30 mM uridine, 10 mM adenine, 30 mM phosphate buffer (pH 7). Total volume: 5 ml.
[b]% Adenosine = $[\text{adenosine}]_{\text{obtained}} \times 10^2/[\text{adenosine}]_{\text{theoretic}}$.
[c]Required time to obtain maximum yield.

of the biocatalyst were employed. Adenosine yields over 90% were achieved in 1 h using cell paste equivalent to 106 mg dry cells (Figure 1D). When more than 212 mg dry cells were employed, the reaction took place faster but by-products were formed due to the action of deaminases (data not shown); moreover, the work-up of this reaction slurry was more difficult. Taking into account these results, we selected 15 ml of broth of a concentration of 10.6 mg dry wt ml^{-1} as the optimum volume.

Analysis of time dependence showed that the reversal of the second step of the biotransformation took place, and after 24 h incubation only adenine and uracil were present (Figure 2). To explain these results, cell viability was studied. A negative Bradford assay showed that cells were not lysed during the reaction; moreover, at very long reaction times (100 h) the inoculation of reaction mixture into solid media demonstrated that cells were still viable. Therefore, we conclude that possibly ribose 1-phosphate has been used as an energy source. An additional evidence to support this hypothesis is that the reaction proceeded slowly when carried out with sonicated cell wet paste and adenosine was not consumed even after 24 h incubation. Therefore reaction time is an important experimental parameter which must be optimized.

Using hypoxanthine as purine base under standard conditions, similar yields of inosine were obtained, but when thymidine was used as sugar donor the reaction did not proceed at 60 °C because thymidine nucleoside phosphorylase is not active at this temperature (Krenitzky & Koszalka 1981). At 40 °C, deoxyinosine could be obtained (50% yield) in 2 h. Optimization of these reaction conditions as well as the synthesis of dideoxynucleosides and other unnatural purine nucleosides are in progress.

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