Pergamon

SCIENCE DIRECT®

Tetrahedron Letters 44 (2003) 2605–2609

TETRAHEDRON LETTERS

Purine nucleoside synthesis from uridine using immobilised *Enterobacter gergoviae* CECT 875 whole cells

J. A. Trelles,[a] M. Fernández,[b] E. S. Lewkowicz,[a] A. M. Iribarren[a] and J. V. Sinisterra[b,*]

[a]*Universidad Nacional de Quilmes, R.S. Peña 180, (1876) Bernal, Buenos Aires, Argentina*
[b]*Laboratory of Biotransformations, Faculty of Pharmacy, Universidad Complutense de Madrid, E-28040 Madrid, Spain*

Received 3 December 2002; accepted 19 January 2003

Abstract—Biocatalysed purine nucleoside synthesis was carried out using immobilised *Enterobacter gergoviae* CECT 875. Similar yields (80–95%) in adenosine were obtained with both free and immobilised cells though in the last case a long reaction time was necessary. The immobilised cells can be reused at least for more than 30 times without significant loss of enzymatic activity. The immobilised biocatalyst in agarose is active in the synthesis of unnatural nucleosides.

1. Introduction

Modified nucleosides are extensively used as antiviral and antitumor agents.[1] These molecules have been synthesised by different chemical methods.[2] Biotransformations are a promising research process because the reactions take place under very mild conditions and offer environmentally clean technologies.[3] Nucleoside phosphorylases[4,5] are very interesting intracellular enzymes in biotransformations because purine or pyrimidine nucleosides can be obtained, in a one-pot reaction, from other cheap pyrimidine or purine nucleosides, respectively. This synthesis is carried out using—sequentially—both intracellular enzymes. The synthesis of adenosine from uridine has been used as the reaction test (Scheme 1).

In this type of enzymatic synthesis, the reaction is carried out with complete stereocontrol because only the β-isomer is obtained.[4–8] Pentose-1-phosphate is the reaction intermediate.[4,5] Besides, protection de-protection processes are not necessary due to the high specificity of these intracellular enzymes.

The synthesis of nucleosides using intracellular nucleoside phosphorylases is documented.[6–8] If we want to scale up the process, the reuse of the biocatalyst is necessary. This objective can only be achieved using immobilised whole cells because the reaction takes place using two intracellular enzymes. Few articles are in the literature using immobilised whole cells such as the use of *Escherichia coli* immobilised in alginate gel[9–12] to produce some base modified 2′-deoxyribonucleosides or *Xanthomonas campestris* immobilised over glass fibers for virazole synthesis.[13]

Because of its synthetic usefulness we describe a simple immobilisation methodology of *Enterobacter gergoviae* CECT 875 on agar or agarose. We found that it can be usefully applied as a recoverable and reusable biocatalyst for the synthesis of purine nucleoside with high stereospecificity. The bacterium used in this work—*E. gergoviae* CECT 875[14]—was selected after a taxonomic screening from our micro-organism collection.

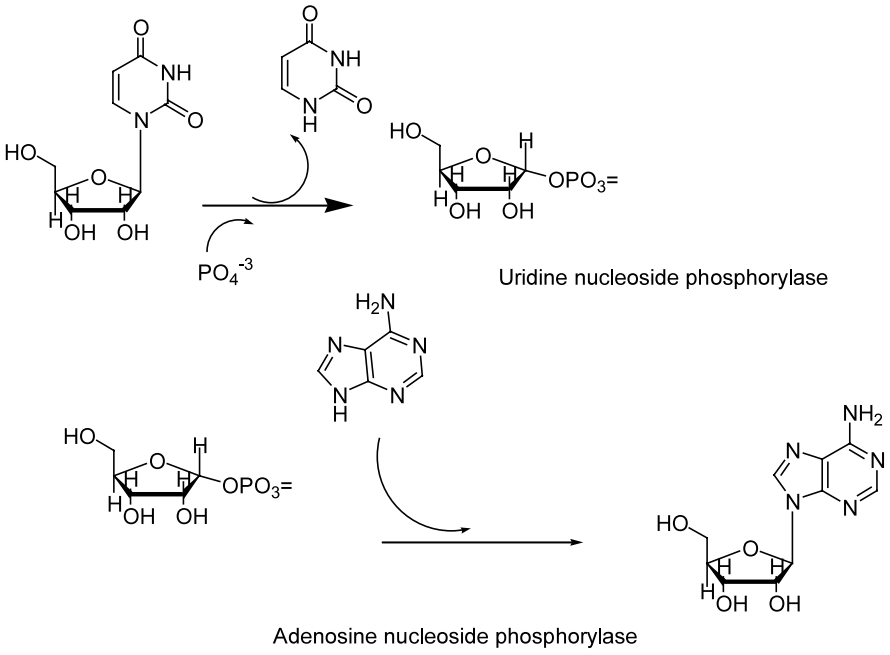
Table 1 shows the best conversions achieved for adenosine using free or immobilised cells as biocatalyst. In the case of the immobilised whole cells high yields have been achieved and similar to that with free cells. The only difference observed was the reaction time, greater for immobilised cells than for free whole cells (1 h) due to intraparticular diffusion restrictions in the immobilised biocatalyst.

The yields obtained with immobilised *E. gergoviae* were similar to those described by Yokozeki and Tsuji[15] with free whole cells of *Enterobacter aerogenes* AJ-11125. Nevertheless we could raise the yields described by Prasad et al.[6] using other microorganisms or by Hennen and Wong[16] that need 2–4 days to achieve similar

Keywords: nucleoside synthesis; immobilised whole cells; nucleoside phosphorylases; *Enterobacter gergoviae*.

* Corresponding author. Present address: Departamento de Química Orgánica y Farmacéutica, Facultad de Farmacia, Universidad Complutense de Madrid, Ciudad Universitaria s/n, E-28040 Madrid, Spain. Tel.: 34-91-3941820; fax 34-91-3941822; e-mail: jvsgago@farm.ucm.es

0040-4039/03/$ - see front matter
doi:10.1016/S0040-4039(03)00225-9



Scheme 1.

results to those in Table 1, using free enzymes. Therefore, our immobilised derivatives are interesting biocatalysts for this synthesis.

The immobilised whole cells were used in the synthesis of different adenosine nucleosides as described in Scheme 2. From Table 1 we can deduce that our biocatalysts are active in the synthesis of nucleosides with sugar residues such as ribose, 2′-deoxyribose or 2′,3′-dideoxyribose but not in the case of arabinose nucleosides. These nucleosides can be obtained with *E. coli* BL21.[17] Besides, the ribose nucleoside of thymine (MU) is useful to obtain purine nucleosides. The maximum yields were obtained at different contact time depending on the structure of the substrates. Finally we can see that agarose beads carry more active biocatalysts than agar beads. These differences between both thermogels are more related to diffusion problems than to the specific activity of intracellular enzymes which are—evidently—the same in all the cells. Probably agarose let the end products out of the microenvironment of the cells avoiding the hydrolysis of nucleosides.

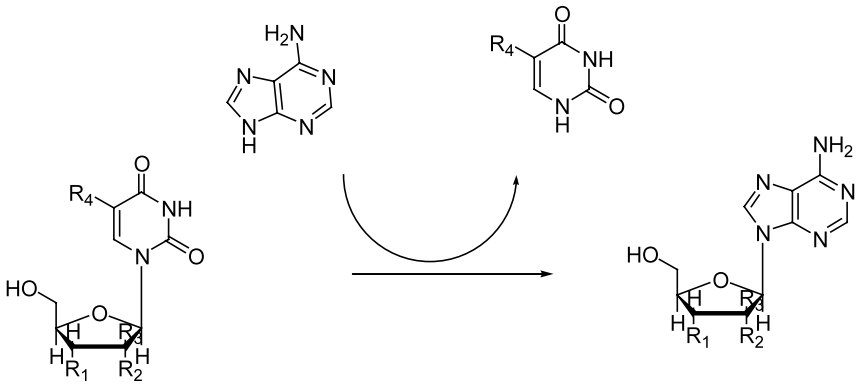
The reactions were performed at 60°C with dead cells. This standard temperature was selected because it is the minimum temperature to deactivate *adenosine deaminase* that transforms adenosine into the by-product inisione.[7] At this temperature nucleoside phosphorylases are still active.[7,8,15]

The potential for the reuse of the immobilised biocatalyst was also investigated. *E. gergoviae* cells were entrapped in 2% agar or 2% agarose. The reactions were carried out subsequently for 30–40 cycles (3 h). Figure 1 shows the comparison between free cells and the immobilised cells in agar (as an example). As expected, immobilisation increases stability of the biocatalyst. This effect is described for many microorganisms.[18,19] The immobilised biocatalysts can be reused at least for 30 cycles of 3 h with a minimum loss of activity

Table 1. Synthesis of adenosine nucleosides using immobilised whole cells of *E. gergioviae* CECT 875 in agar or agarose

Matrix	Pyrimidine nucleoside	Time (h)	Yield in purine nucleoside (%)
None	U	1	89
Agar	U	3	86
	DU	3	68
	DDU	24	0
	MU	6	78
	AU	24	0
Agarose	U	3	89
	DU	1	77
	DDU	21	96
	MU	2	84
	AU	24	0

$T = 60$°C; pyrimidine nucleoside = 0.15 mM; adenine = 0.05 mM; $V = 5$ ml of 30 mM, phosphate buffer (pH 7.0).



Scheme 2.

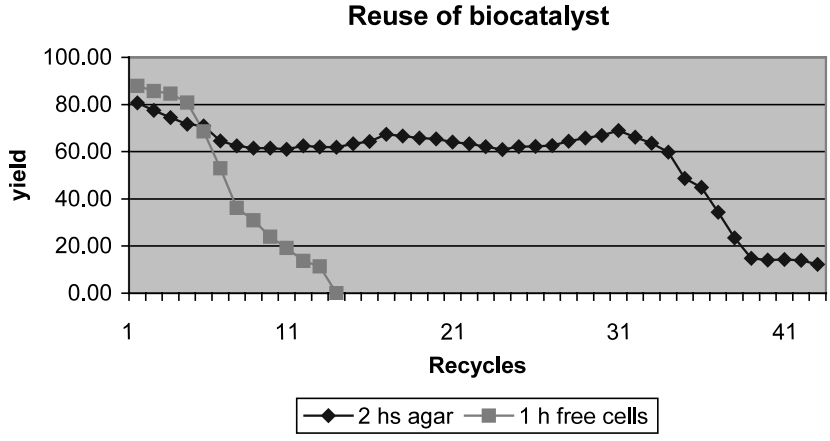


Figure 1. Biocatalyst reuse—free and immobilised *E. gergoviae* whole cells (2% agar support). Reaction conditions: see Section 2. % Adenosine = $[\text{adenosine}]_{\text{obtained}} \times 10^2 / [\text{adenosine}]_{\text{theoretic}}$.

(>60% yield) but the free cells carry to<30%, after 8 cycles. These results suggest that the biocatalysts described here fulfill the requirements for low cost production in view of an industrial application due to their high activity, their large half-life, the absence of secondary products and the high stereocontrol.

The deactivation of intracellular enzymes could be responsible for the decrease of the catalytic activity after several cycles, because of catalyst weight loss having not been observed after recycles.[20]

In Table 2 we show some results obtained in the synthesis of other nucleosides using the biocatalyst prepared from the agarose. The maximum yields were obtained at different contact temperatures, depending on the sugar or base structures.

We can see in Table 2 that U is a better reagent than DU or DDU, as in Table 1. Besides very different structures of bases can be used to obtain unnatural nucleosides with *E. gergoviae* immobilised in agarose. The yields obtained are similar to that described by Prasad et al.[6] with whole cells but they are lower that those described by Murakami et al.[21] in the case of 2′3-dideoxyribonucleoside (DDU).

Table 2. Synthesis of some nucleosides using *E. gergoviae* immobilised in agar (2%)

Py nucleoside	Base	Yield in nucleoside (%)
U	6-Mercaptopurine	56
DU	6-Mercaptopurine	18
U	3-Carboxyamido1,2,4-triazol	45
DU	6-Methoxypurine	27 (24 h)
U	Purine	80
DU	Purine	40 (5 h)
DDU	Purine	38 (21 h)

$T = 60°C$; $t = 3$ h; pyrimidine nucleoside = 0.15 mM; base = 0.05 mM.

2. Experimental

2.1. Materials

Agar and agarose from Glacillaria were a generous gift from HISPANAGAR S.A. (Spain). *E. gergoviae* CECT 875 was kindly supplied by the 'Colección Española de Cultivos Tipo (CECT)', Universidad de Valencia (Spain). All chemicals were analytical or HPLC grade quality and were obtained from commercial sources.

3. Growth conditions

E. gergoviae CECT 875 was cultured 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 deionised water adjusted to pH 7 with KOH. The cells were shacked at 37°C for 16 h and then harvested by centrifugation for 10 min at 12 000 g.

3.1. Immobilisation of cells

3.1.1. Entrapment in agar or agarose. The whole cell pellet was mixed with 10 ml of previously sterilised 2% (w/v) agar or agarose. The homogeneous mixture was then added dropwise to stirred sunflower oil (20 ml) at 25°C for 5 min. The resulting gel beads (mean diameter: 4 mm, load) were cooled, filtered, washed with hexane and then with physiological solution to obtain free solvent beads.

3.2. Biotransformation conditions

3.2.1. Free cells. The standard reaction mixture comprising: wet cell paste, 0.15 mmol of uridine, 0.05 mmol of adenine and 30 mM pH 7 potassium phosphate buffer (5 ml), was stirred at 200 rpm in a orbital shaker and 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.

3.2.2. Immobilised cells. 4 g of agarose gel beads or 3 g of agar gel beads, were used following the previous procedure but the reaction mixture was stirred for 3 h. The reactions were performed in a Kunner orbital shaker (200 r.p.m.) to reduce the mechanic abrasion of the beads.

3.3. Biocatalyst reuse

3.3.1. Free cells. After 1 h of reaction, the suspension was centrifuged at 10 000 g for 30 s, the supernatant was removed and the microbial pellets reused as indicated above.

3.3.2. Immobilised cells. After 3 h reaction, the gel beads or pieces were filtered, washed with phosphate buffer and used as biocatalyst for a new biotransformation as indicated above.

3.4. Analytical methods

The synthesis of nucleosides was qualitatively followed by TLC analysis. Silica gel plates were used with Cl_3CH/methanol 80:20 (v/v) as development solvent.[17]

The production of purine nucleosides was quantitatively measured by HPLC from LDC Analytical, model CM 4000. Samples from the supernatants were diluted 10 times with 30 mM phosphate buffer and analysed with a C-18 column (250×4 mm) at a flow rate of 0.9 ml min^{-1}. The mobile phase was water/methanol 85/15 (v/v). The UV detector was set at 254 nm and the column was operated at room temperature.

The H NMR spectra of isolated compounds were compared to those of commercial nucleosides and of the *ribo* and 2′-doxyribo adenosines obtained in the laboratory, in order to confirm the β-configuration previously described for nucleosides obtained with whole cells.[5,7]

Acknowledgements

This work was supported by Project BQU 2001-1301 from Ministerio de Educación y Ciencia of Spain and by the Agencia Española de Cooperación Internacional (Project PR158/01-100 65) and by Universidad Nacional de Quilmes, Secretaría de Ciencia y Técnica de la Nación, Argentina. AMI is research member of CONICET, Argentina.

References

1. Garg, R.; Gupta, S.; Gao, H.; Babu, M.; Debnath, A.; Hansch, C. *Chem. Rev.* **1999**, *99*, 3523–3601.
2. Ichikawa, E.; Kato, K. *Curr. Med. Chem.* **2001**, *8*, 385–423.
3. Cheetham, P. S. J. *J. Biotechnol.* **1998**, *66*, 3–10.
4. Pugniere, M. J.; Ealick, S. E. *Biochem. J.* **2002**, *361*, 1–25.
5. Bzowska, A.; Kulikowska, E.-; Shugar, D. *Pharmacol. Ther.* **2000**, *88*, 349–425.
6. Prasad, A. K.; Trikha, S.; Parmar, V. S. *Bioorg. Chem.* **1999**, *27*, 135–154.
7. Utagawa, T. *J. Mol. Catal. B: Enzym.* **1999**, *6*, 215–222.
8. Lewkowicz, E.; Martínez, N.; Rogert, M. C.; Porro, S.; Iribarren, A. M. *Biotechnol. Lett.* **2000**, *22*, 1277–1280.
9. Gaevaia, L. V.; Zhukov, V. N. *Prikl. Biokhim. Mikrobiol.* **1987**, *23*, 309–316.
10. Hori, N.; Watanabe, M.; Sunagawa, K.; Uehara, K.; Mikami, Y. *J. Biotechnol.* **1991**, *17*, 121–131.
11. Holy, A.; Votruba, I. *Nucleic Acids Symp. Ser.* **1987**, *18*, 69–72.
12. Ewing, D. F.; Holy, A.; Votruba, I.; Humble, R. W.; Mackenzie, G.; Hewedi, F.; Shaw, G. *Carbohydr. Res.* **1991**, *21*, 109–118.
13. Iaskovich, G. A.; Iakovleva, E. P. *Prikl. Biokhim. Mikrobiol.* **1999**, *35*, 146–149.
14. *Enterobacter gergoviae* CECT 875, was kindly supplied by 'Colección Española de Cultivos Tipo (CECT)', Universidad de Valencia, Spain.

15. Yokozeki, K.; Tsuji, T. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 207–213.
16. Hennen, W. J.; Wong, Ch. H. *J. Org. Chem.* **1989**, *54*, 4692–4695.
17. Rogert, M. C.; Trelles, J. A.; Porro, S.; Lewkowicz, E. S.; Iribarren, A. M. *Biocatal. Biotransform.* **2002**, *20*, 347–351.
18. *Methods in Enzymology*; Mosbach, K., Ed.; Academic Press: New York, 1987; Vol. 135.
19. *Immobilised Cells: Basics and Applications*; Progress in Biotechnology Vol. 11; Wijffels, R. H.; Buitelaar, R. M.; Bucke, C.; Tramper, J., Eds.; Elsevier: Amsterdam, 1996.
20. Free cells from the disintegration of the biocatalyst beads were not observed using the optic microscopy.
21. Murakami, K.; Shirasaka, T.; Yoshioka, H.; Kojima, E.; Aoki, Sh.; Ford, H. J., Jr.; Driscoll, J. S.; Kelley, J. A.; Mitsuya, H. *J. Org. Chem.* **1991**, *34*, 1606–1612.