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Journal of Molecular Catalysis B: Enzymatic 30 (2004) 219–227
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Nucleoside synthesis by immobilised bacterial whole cells

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Received 31 March 2003; received in revised form 8 June 2004; accepted 14 June 2004

Abstract

Biocatalysed synthesis of nucleosides was carried out using immobilised whole cells of *Escherichia coli* ATCC 47092, *Enterobacter gergoviae* CECT 857 and *Citrobacter amalonaticus* CECT 863. The synthesis of adenosine from uridine was used as reaction model to test the biocatalysts. Reactions were carried out using non-growing cells. Maximum activity was obtained with cells harvested at the beginning of the stationary phase. Immobilization by whole cell entrapment was employed using different matrix such as alginate, agar, agarose and polyacrylamide. The percentage of monomer, the shaking speed, the catalyst load and nature of the matrix were optimized. In the first reutilization cycle, similar yields (80–95%) were obtained with both free and immobilized cells in the reaction model, although in the last case, longer reaction times were necessary. The immobilized cells can be reused at least for more than 30 times without significant loss of the catalytic activity. The immobilized biocatalysts have been used in the synthesis of different nucleosides.

Keywords: Whole cells immobilization; Nucleoside synthesis; Nucleoside phosphorylases; *Escherichia coli*; *Enterobacter gergoviae*; *Citrobacter amalonaticus*

1. Introduction

Modified nucleosides are extensively used as antiviral and antitumoral agents [1,2]. These molecules have been synthesized by different chemical methods [3] but, biotransformations are a promising synthetic alternative because the reactions take place under very mild conditions and offer environmentally clean chemical technologies [4]. Modified nucleosides can be obtained using free enzymes such as lipases, proteases [5], glycosyltransferases [6] or nucleoside phosphorylases [7,8]. These last enzymes are very interesting because purine or pyrimidine nucleosides can be obtained in one-pot reactions from other cheap pyrimidine or purine nucleosides, respectively. For this synthesis two intracellular enzymes are necessary (Scheme 1). Nucleoside phosphorylases are intracellular enzymes [9,10]. At least, three different kinds of purine nucleoside phosphorylases (PNP) have been described, which display different substrate specificity and optimum pH. Also, two different pyrimidine nucleoside phosphorylases (PyNP) have been reported. One of them recognizes uridine and the other thymidine [9]. Each type is present in different types of microorganisms, and they do not coexist in the same strain. All these enzymes are dimers, homotrimers or homohexamers [9,10].

The mechanism is well established in the literature [8,10]. The phosphorolysis takes place by a SN1-like mechanism—via an oxonium-like intermediate—to give α-ribose-1-phosphate (Scheme 1). The second step is a SN2 mechanism where phosphate is substituted by a base giving the β-nucleoside [8]. The stereochemistry of the α-ribose-1-phosphate has been established by molecular modeling and X-ray analysis of inhibitor–enzyme complexes [9,10]. Both enzymes: one PNP and the other PyNP are necessary to perform the biotransformation (Scheme 1). These enzymes are not monomeric [9,10] and the immobilization of whole cells is the best possibility to carry out the process under preparative conditions.

The synthesis of nucleosides using intracellular nucleoside phosphorylases is well documented [6,7,11–15]. Nevertheless, only a few reports have been published to our

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URL: http://www.ucm.es/info/btg.

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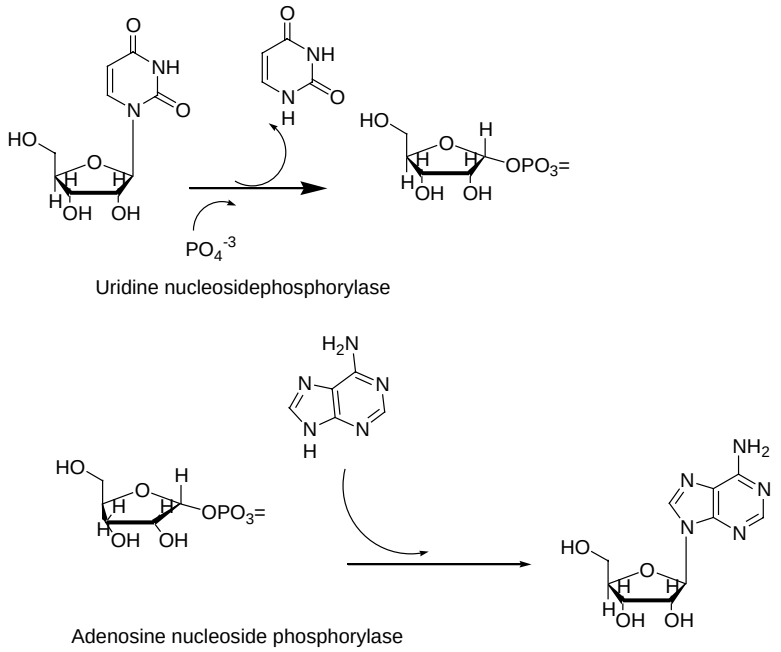
doi:10.1016/j.molcatb.2004.06.001



Scheme 1.

knowledge using immobilized whole cells in this one-pot synthesis. The only available examples involve the use of *E. coli* immobilized in alginate gel [16,17] to produce some base modified 2′deoxyribonucleosides and of *Xanthomonas campestris* immobilized in glass fibres for virazole synthesis [18]. Recently, we published the first results using *Enterobacter. gergoviae* CECT 857 immobilized in agar or agarose [13].

The most widely used technique for whole cell immobilization is cell entrapment [19], in which the cells are enclosed in a polymeric matrix, which is porous enough to allow the diffusion of substrates and products. The main advantages of this methodology are higher operational stability, ease of handling and cell separation and the feasibility of scaling up the process [20–22]. Depending on the hydrophobic/hydrophilic characteristics of substrates and/or reagents, several matrices have been described to reduce the diffusion problems: agar, agarose, κ-karrageenan, alginate, chitosan, polyacrylamide, polyvinyl alcohol and polyurethane foams have been described [23–28].

In order to explore the technological applications of this reaction, we describe the use of immobilized microbial whole cells in alginate, agar, agarose and polyacrylamide, for the production of several nucleosides.

2. Experimental

2.1. Materials

The microorganisms were supplied by the Colección Española de Cultivos Tipo (CECT), Universidad de Valencia (Spain) or purchased from the ATCC (Rockville MD, USA). A tailor-made agar A28/03 and an agarose (L-M3) were a generous gift from Hispanagar S.A. (Spain). All the other chemicals were analytical or HPLC grade quality and were obtained from commercial sources. Virazole was a gift of ICN Iberica (Spain).

2.2. Growth conditions

E. coli ATCC 47092, *E. gergoviae* CECT 857 and *C. amalonaticus* CECT 863 were grown until saturation in 250 ml Erlenmeyer flasks containing 50 ml of 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 shaken at 37 °C for 16 h and then harvested by centrifugation for 10 min at 12,000 × *g*. The supernatant was removed and the bacterial pellet was washed with 30 mM potassium phosphate buffer (pH 7) and re-centrifuged.

2.3. Oxygen electrode assay

An oxygen electrode YSI model 5300A was used in the experiments. A calibration curve was performed using 30 mM phosphate buffer (pH 7), calf spleen catalase solution (4 mg/ml), NADH solution (10 μmol/ml) and an aqueous solution of PMS (200 μg/ml). All the reagents were from Sigma. Hundred microliters of catalase solution and 100 μl of PMS solution were mixed at 28 °C and 25 μl of NADH solution were added each 20 min. The oxygen consumption measured as consumed NADH (μmol) was fitted to a straight line using Sigma Plot calculus program.

Oxygen consumption assay: Three milliliters of culture medium (1% beef extract, 0.5% yeast extract and 0.5 NaCl, pH 7) were added to the electrode cuvette ($T = 28$ or 60 °C). Then 75 μl of cells culture broth were added. The cells were harvested in different growth stages or after the synthesis of nucleosides at 60 °C. The oxygen consumed was followed for 20 min. The amount of oxygen consumed was determined as mmol O_2/million cells or in percentage (low consumption values).

2.4. Immobilisation of cells

Entrapment in alginate: Cells were harvested from the culture broth by centrifugation at 8000 × *g* for 10 min. The supernatant was removed and the pellet mixed with 20 ml of previously autoclaved 2% (w/v) alginic acid. The mixture was then added dropwise to a stirred solution of 0.1 M $CaCl_2$. After vacuum filtration, the resulting gel beads were washed with 0.1 M $CaCl_2$ solution and used as the biocatalyst (catalyst load 15×10^6 cells/g of catalyst).

Entrapment in agar or agarose: Cells were harvested from the culture broth by centrifugation at 8000 × *g* for 10 min. The supernatant was removed and the pellet mixed with 10 ml of previously sterilized 2% (w/v) agar or agarose. The mixture was then added drop wise 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. The beads were used directly as biocatalyst. Catalyst loadings were: agarose beads 3.75 (10^6 cell/g of catalyst) and in agar beads 5000 (10^6 cell/g of catalyst).

Entrapment in polyacrylamide: Cells were harvested from the culture broth by centrifugation at 8000 × *g* for 10 min. The supernatant was removed, the pellet mixed with 10 ml of 30 mM phosphate buffer (pH 7) containing 8% (w/v) acryl amide and 0.2% (w/v) bis-acrylamide. Subsequently, 50 μl of 18% (w/v) $(NH_4)_2S_2O_8$ and 14 μl of TEMED were added to produce the polymerization. The formed gel was cut in little cubic pieces (1.0 cm × 1.0 cm × 0.2 cm, approximately) and used as the biocatalyst (catalyst load 1500×10^6 cell/g of catalyst).

2.5. Biotransformation conditions

Free cells: The standard reaction mixture was composed of wet cell paste, 0.15 mM uridine, 0.05 mM adenine and 30 mM pH 7 potassium phosphate buffer (5 ml). Reactions were incubated with stirring at 200 rpm and 60 °C for 1 h. The cell concentration in the reaction vessel was 3000×10^6 cell/ml.[1] After the reaction, samples were centrifuged at 10,000 × *g* for 30 s and the supernatants were analyzed by both TLC and HPLC.

Immobilized cells: Four grams of agarose gel beads, 3 g of agar gel beads or 10 g of polyacrylamide pieces prepared as above, were used following the previous procedure but the reaction mixture was stirred for 3 h.

2.6. Biocatalyst reuse

Free cells: After 1 h reaction, the suspension was centrifuged at 10,000 × *g* for 30 s, the supernatant was discarded and the microbial pellets were washed with phosphate buffer and re-centrifuged. This wet cell paste was used as biocatalyst for a new biotransformation as indicated above.

Immobilized cells: After 3 h, the gel beads or the small cubes were filtered, washed with phosphate buffer and used as biocatalyst for a new biotransformation as indicated above.

2.7. Synthesis of other nucleosides

The synthesis of other nucleosides was performed under the same conditions as described above for the synthesis of adenosine from uridine, but changing either the base or the nucleoside. The equimolecular ratio (0.05 mM each reagent) was maintained in all cases.

2.8. Analytical methods

The synthesis was qualitatively followed by TLC analysis. Silica gel plates (Merck) were used with Cl_3CH/MeOH, 80:20 (v/v) as development solvent.

The production of the nucleoside 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 analyzed with a C-18 column, 5 μm, (250 mm × 4 mm) The UV detector was set at 254 nm and the Waters T42354 column was operated at 30 °C. The chromatographic conditions were:

(1) Adenosine synthesis from uridine and the reverse reaction. Mobile phase: isocratic, water/MeOH (95/5, v/v); flux 1.5 ml/min.
(2) 2′-Deoxyadenosine synthesis. Mobile phase: (i) 4 min (water/MeOH (95/5, v/v) (ii) then a lineal gradient till water/MeOH (80/20, v/v), gradient time 5 min (iii) 1 min. at water/MeOH (80/20, v/v).
(3) 2′,3′-Dideoxyadenosine synthesis. C-18 Wonders Bondapak column, 5 μm (300 mm × 3.9 mm). Mobile phase: ammonium acetate buffer/MeOH (95/5, v/v) flux = 0.6 ml/min, λ = 254 nm.

The quantification of the nucleosides (Tables 4 and 5) was performed in the same HPLC conditions that those described for the synthesis of the adenine nucleosides with the same sugar: uridine (U), 2′-deoxyuridine (DU), 2′,3′-dideoxyuridine (DDU) or ara-uridine (AU).

The retention times of each compound were compared to those of commercial samples or with the chemically

[1] The adsorption at 660 nm was used to measure the concentration of cells in the reaction flask.

Table 1
Adenosine production from different free and immobilised biocatalysts

Entry	Microorganism	Support	Adenosine yield (%)[a]
1	*Escherichia coli*	None[b]	94
2	*Escherichia coli*	Alginate (2%)	95
3	*Escherichia coli*	Agar (2%)	78.5
4	*Escherichia coli*	Agarose (2%)	89
5	*Escherichia oli*	Polyacrylamide (8%)	87
6	*Enterobacter gergoviae*	None[b]	89
7	*Enterobacter gergoviae*	Agar (2%)	87
8	*Enterobacter gergoviae*	Agarose (2%)	86
9	*Enterobacter gergoviae*	Polyacrylamide (8%)	99
10	*Citrobacter amalonaticus*	None[b]	93
11	*Citrobacter amalonaticus*	Agar (2%)	93
12	*Citrobacter amalonaticus*	Agarose (2%)	92
13	*Citrobacter amalonaticus*	Polyacrylamide (8%)	91.5

$T = 60\,^{\circ}C$; $t = 3$ h; uridine = 0.15 mM; adenine = 0.05 mM. Stirring speed = 200 rpm.
[a] % Adenosine = $[\text{adenosine}]_{\text{obtained}} \times 10^2/[\text{adenosine}]$ theoretic.
[b] Reaction time = 1 h.

synthesized compounds: 6-mercaptopurine ribonucleoside *o*-2′-deoxyribonucleoside and the nucleosides of the purine heterocycle.

2.8.1. Chemical synthesis of purine nucleosides

The purine nucleosides used as the reference for HPLC analysis were prepared by direct reaction of ribose, 2′deoxyribose or 2′,3′-dideoxyribose-1-tosylate with the purine in CH_2Cl_2 and purified by adsorption column chromatography in water/MeOH (3/1, v/v).

2.9. Structural analysis

H NMR spectra of nucleoside obtained in the biotransformations were compared with H NMR from commercial nucleosides or from ribo- and 2′-doxyribopurine nucleosides chemically obtained in the laboratory, in order to confirm the β-stereochemistry previously described for nucleosides obtained with whole cells [7]. Virazole obtained using our biocatalysts was isolated and its H NMR compared with commercial product.

3. Results and discussion

3.1. Synthesis of adenosine from uridine

The bacteria used in this work (*E. coli* ATCC 47092, *E. gergoviae* CECT 857 and *C. amalonaticus* CECT863) were selected by means of a taxonomic screening using 176 microorganisms[2]. These selected strains were those that afforded the highest yields in adenosine from uridine and reproducible large-scale fermentation conditions. These whole cells have an active uridine nucleoside phosphorylase and an active purine nucleoside phosphorylase as demostrated in the screening. This was confirmed in the reverse synthesis (uridine from adenosine) where low yields of uridine were achieved[3]. The cells were harvested at different culture times and the maximum activity was achieved at the beginning of the stationary phase as described by Lewkowicz et al. [11] for *E. coli*.

Table 2
Biocatalyst cell load optimisation for the adenosine synthesis from uridine by *E. coli* BL21 on agarose

Entry	*E. coli* ($\times 10^6$ cell/g)	Reaction time (h)	Adenosine yield (%)[a]
1	937	1	8
2	937	2	7
3	937	3	9
4	1875	1	42
5	1875	2	83
6	1875	3	89
7	3750	1	70
8	3750	2	89
9	3750	3	87
10	7500	1	57.5
11	7500	2	84
12	7500	3	81.5

$T = 60\,^{\circ}C$; $t = 3$ h; uridine = 0.15 mM; adenine = 0.05 mM. Shacking speed = 200 rpm.
[a] % Adenosine = $[\text{adenosine}]_{\text{obtained}} \times 10^2/[\text{adenosine}]_{\text{theoretic}}$.

Table 1 shows the adenosine yields obtained using the immobilized biocatalysts. All the experiments were carried out with the same amount of total cells ($(15{,}000 \pm 500) \times 10^6$ cells) for free or for immobilized whole cells. In the first cycle, almost all of immobilized biocatalysts produced similar yields to those obtained from the corresponding free

[2] The synthesis of adenosine from uridine plus adenine or the synthesis of 2′-deoxyadenosine from 2′-deoxyuridine was used as screening reaction test.

[3] Two new screening are in progress. One screening using the pair Inosine + uracyl as the reaction test and another using adenosine + uracil, to explore the presence of the different enzymes and the relative catalytic activity of each nucleoside phosphorylase.

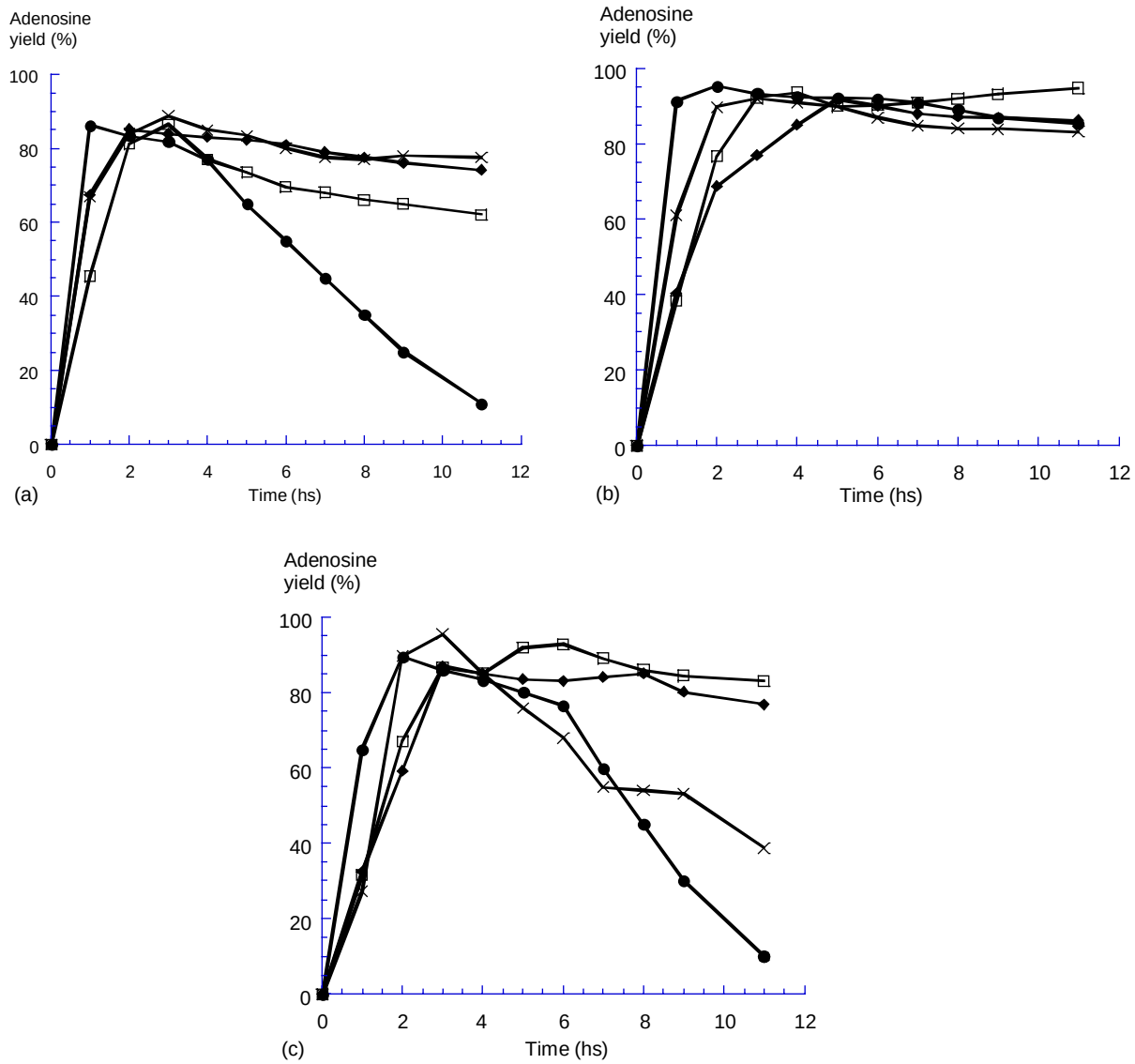


Fig. 1. Time course of adenosine synthesis biocatalysed by free or immobilised micro-organisms. (a) *Escherichia coli* ATCC 47092 (b) *Enterobacter gergoviae* CECT 857; and (c) *Citrobacter amalonaticus* CECT 863 (●) free cells (×) 2% agarose support (◆)2% agar support (□) 8% polyacrylamide support. Reaction conditions: see Section 2.

cells. These results confirm that the use of these immobilization techniques does not affect the biocatalytic activity of the intracellular enzyme. The only difference observed was the reaction time, greater for immobilized cells (3 h) than for free whole cells (1–2 h), that seems to be related to diffusion restrictions.

The biocatalyst obtained with calcium alginate is active (Table 1 entry 2 versus entry 1), but cannot be reused because it is destroyed by the phosphate buffer that leaches the Ca(II) from the gel [22,23]. The yields obtained with immobilized *E. gergoviae* were similar to those described by Yokozeki and Tsuji [29] using free whole cells of *E. aerogenes* AJ-11125. Nevertheless, they exceeded the yields described by Prasad et al [6] using other microorganisms. The optimum matrix for each strain is different. In fact, while *E. coli* gave better yields when it was immobilized in agarose or polyacrylamide, *E. gergoviae* gave the most active biocatalyst in polyacrylamide and the catalytic activity of *C. amalonaticus* was not affected by the nature of the matrix. This fact is well documented in the literature [19,20] and is related to differences in the cell wall and/or membrane composition.

3.2. Optimization of the immobilization parameters

Several operational parameters were investigated using *E. coli* immobilized on agarose as a model. The amount of entrapped cells per gram of biocatalyst, polymer concentration and immobilization conditions of the cells in the matrix (temperature and magnetic stirring speed of the oil

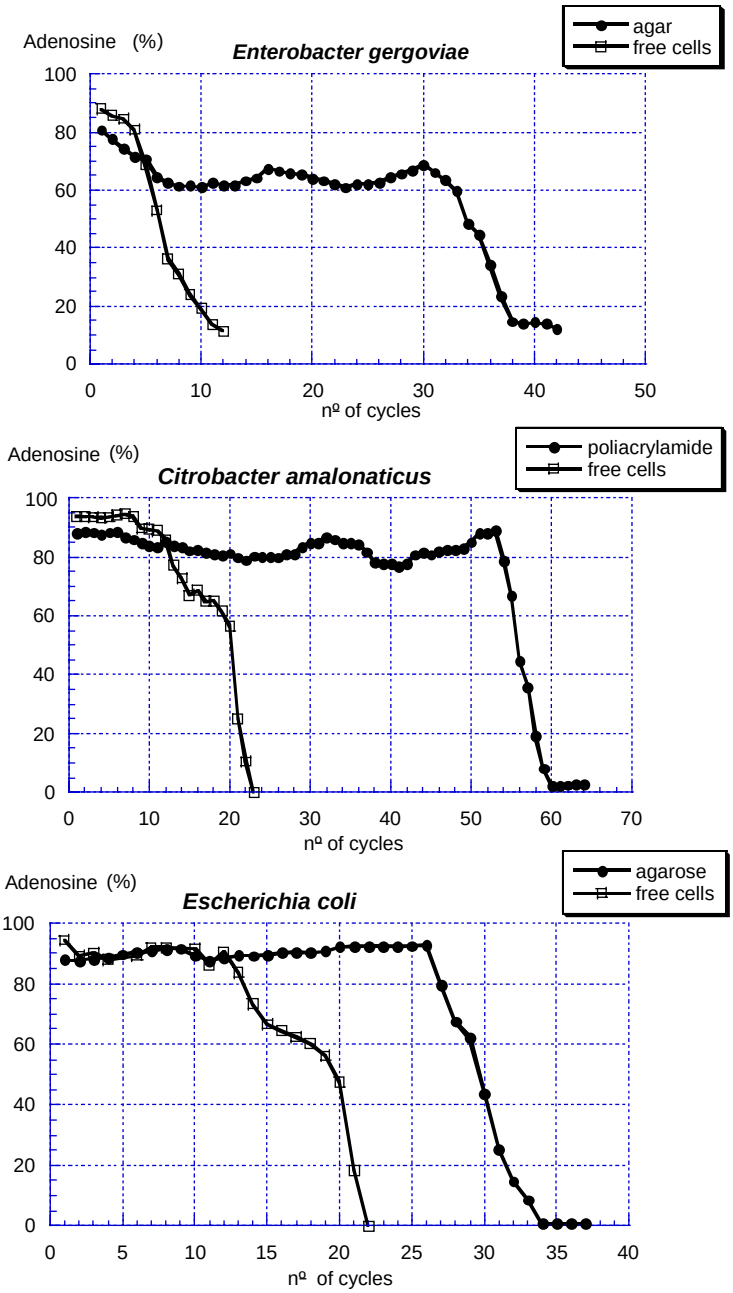


Fig. 2. Biocatalyst reuse (a) free and immobilised *Enterobacter gergoviae* CECT 857 (2% agar support) (b) free and immobilised *Citrobacter amalonaticus* CECT 863 (8% polyacrylamide support) (c) free and immobilised *Escherichia* coli ATCC 47092 (BL21) (2% agarose support). (□) Free cells (●) immobilised cells. Reaction conditions: see Section 2.

bath) were analyzed (Tables 2 and 3). Regarding this last point, the best beads (shape, diameter and strength) were obtained when the oil bath temperature was 25 °C, instead of 45 °C as previously reported [15]. The optimal stirring speed for the reaction with immobilized biocatalyst was 200 rpm (Table 3). The same optimum shaking speed was obtained for the biocatalysts immobilized in agar and polyacrylamide (data not shown).

The reactions were performed at 60 °C. At this temperature the intracellular PNP and PyNP of the microorganisms were active but the adenosine deaminase (that transforms adenosine in inosine) is practically deactivated [7]. Experiments carried out with the same biocatalyst but at different cell loading indicated that the highest activity was obtained with 3750×10^6 cells/g and 2 h of reaction (Table 2, entries 2,5, 8,10). Similar yields were also achieved using 1875×10^6 or 7500×10^6 cells/g (Table 2, entries 5 and 11). This behaviour shows that there is an optimal cell loading. At lower cell loading of 3750×10^6 cells/g, kinetic conditions prevail and at higher cell loading of 3750×10^6 cells/g the diffusion restrictions of the reagents to the cell were the rate-controlling step. Therefore, similar yields are

Table 3
Effect of agarose concentration and shaking speed in the adenosine synthesis from uridien by *E. coli* $T = 60\,^{\circ}C$; $t = 3\,h$; uridine = 0.15 mM; adenine = 0.05 mM

Entry	Biocatalyst amount (g)	Support concentration (%)[a]	Shaking speed (rpm)	Adenosine yield (%)[b]
1	Free cells[c]	None[d]	200	94
2	4.3	1	170	15
3	4.3	1	200	23
4	4.3	1	230	1
5	4.1	2	170	10
6	4.1	2	200	89
7	4.1	2	230	175
8	4	3	170	15
9	4	3	200	39
10	4	3	230	10

[a] Reaction time 3 h.
[b] % Adenosine = $[\text{adenosine}]_{\text{obtained}} \times 10^2/[\text{adenosine}]_{\text{theoretic}}$.
[c] 15,000 millions of cells.
[d] Reaction time 1 h.

achieved with 3.75 and 7.5×10^6 cells, at the same reaction times.

The influence on the reaction yield of the agarose percentage in the gel was also analyzed (Table 3). The optimum value was 2% in the case of agar and agarose. In the case of polyacrylamide the percentage was 8%. The shaking speed of the reaction flasks was from 170 to 230 rpm. In the case of adenosine the best yield was obtained when 2% of agarose and 200 rpm were employed (Table 3, entry 6). The reaction yields achieved under other experimental conditions were significantly lower than in this experiment. These results show that diffusion is the rate controlling step in the synthesis using the immobilized biocatalysts as described for other biocatalysts and reactions [22,23].

3.3. Reuse of the biocatalysts

The reaction profile with the selected microorganisms—free or immobilized is shown in Fig. 1. No cell growth was observed in the reaction conditions at 60 °C, using free cells [29]. Therefore, the reaction products and/or the substrates were not transformed into biomass under the reaction conditions. Nevertheless, the recovered cells can grow when they are put into culture medium under standard growth conditions. Therefore, we are working with "non-growing" cells under the reaction conditions. In addition, a low oxygen consumption (<5% in 29 min) was observed using the oxygen electrode technique, according to this physiological state. This finding is reported here for the first time. Traditionally, it was considered that dead cells are responsible for the synthesis, due to the abiotic reaction conditions (50–70 °C), necessary to deactivate the adenosine deaminase [12,13,30].

The reactions carried out with free cells produced 80–95% of adenosine in 1 h and then the percentage decreased as previously reported [11,12], with the exception of the *Enterobacter* strain (Fig. 1) [13]. The diminution of the adenosine percentage is probably due to the decomposition of adenosine by the adenosine deaminase plus a non specific purine nucleoside hydrolase, because hypoxanthine is obtained after long reaction times. Nevertheless, uridine is not re-synthesized because uridine nucleoside phosphorylases from *E. coli* and *C. amalonaticus* are not very active as observed in the screening process [30]. Only *E. gergoviae* free cells (Fig. 1b) do not decompose adenosine. This result agrees with previously reported data indicating that adenosine deaminase from *E. gergoviae* is deactivated at temperatures above 57 °C [7,11,12,30].

The immobilization produces a diminution in the adenosine decomposition rate, especially in the case of *E. coli* (Fig. 1a). Only in the case of *C. amalonaticus* immobilized

Table 4
Synthesis of different adenosine nucleosides using immobilized whole cells

Microorganism	Matrix	Pyrimidine nucleoside	Reaction time (h)	Yield in adenosine nucleoside (%)
E.gergoviae	Agar (2%)	U	3	87
E.gergoviae	Agar (2%)	DU	1	42
E.gergoviae	Agar (2%)	DDU	–[a]	0
E.gergoviae	Agar (2%)	MU	3	35
E.gergoviae	Agar (2%)	AraU	–[a]	0
E. coli	Agarose (2%)	U	3	89
E. coli	Agarose (2%)	DU	1	35
E. coli	Agarose (2%)	DDU	24	40
E. coli	Agarose (2%)	MU	1	42
E. coli	Agarose (2%)	AraU	–[a]	0
C. amalonaticus	Polyacrylamide (8%)	U	3	91
C. amalonaticus	Polyacrylamide (8%)	DU	1	23
C. amalonaticus	Polyacrylamide (8%)	DDU	24	10
C. amalonaticus	Polyacrylamide (8%)	MU	1	17
C. amalonaticus	Polyacrylamide (8%)	AraU	24	55

Synthesis of some nucleosides using *E. gergoviae* immobilised in agar (2%). $T = 60\,^{\circ}C$; pyrimidine nucleoside = 0.15 mM; adenine = 0.05 mM.
[a] Contact time greater than 24 h.

on agarose (Fig. 1c) the decomposition profile of adenosine is similar to that observed with free cells. This is because this agarose biocatalyst is destroyed under the shaking conditions used for the reactions and cells are released to the reaction medium. In the case of *E. coli* (Fig. 1a) we can attribute the slow decrease in the adenosine yield to the slow degradation of the biocatalyst in all cases, as observed during this study.

Finally, the feasibility of reusing the immobilized biocatalyst was also analyzed. Fig. 2 shows the comparison between free cells and the most stable catalyst obtained for each microorganism. *E. coli* ATCC 47092, *E. gergoviae* CECT 857 and *C. amalonaticus* CECT 863 entrapped on 2% agar, 2% agarose and 8% polyacrylamide were reused in different cycles of 3 h.

In the case of reusing free cells, the solution was centrifuged at 10,000 × *g* to be sure that bacteria were not remaining in the liquid. The supernatant does not catalyze the reaction and free cells were not observed in the liquid under the microscope. After a few cycles, the yield achieved is dramatically reduced, probably because the viability of "non-growing" cells decreases. Indeed, after the reuse cycles, the concentration of viable cells decreases as proved by seeding solid medium plates with diluted solutions of cells harvested after the synthesis at 60 °C (10^{-1}, 10^{-2}, ..., 10^{-6}) in order to count viable cells.

As expected, immobilization reduces the reaction rate but increases the stability of the biocatalyst. The immobilized biocatalysts can be reused at least for several cycles of 3 h with a minimum reduction in activity (<10% yield). The recycling of the best biocatalysts is shown in Fig. 2. The stabilization is very important in the case of *C. amalonaticus*—immobilized in polyacrylamide, where 54 cycles (3 h) can be achieved without strong deactivation of the biocatalyst (Fig. 2b). In the other cases, 30 (Fig. 2a) or 25 (Fig. 2c) reuse cycles can be performed without appreciable activity loss. These results suggest that the biocatalysts here described fulfill the requirements for low cost production in an industrial application, it means: high activity, proper longevity, absence of by-products and short reaction times. Therefore, they can be useful in preparative biotransformations.

3.4. Synthesis of different adenine nucleosides

The immobilized whole cells were used in the synthesis of several adenosine nucleosides with different sugars (Table 4). The selected biocatalysts were those that gave the most active and stable biocatalysts (Fig. 2). We can observe that the activity profile of biocatalysts is different depending on the microorganism. Probably, this result indicates that the enzymes are different in each microorganism and a generalization is not possible, as a new screening in progress has also demonstrated.

The reaction conditions were the same as described in Table 1. We can observe that the reaction time increases in order to achieve the maximum yield with 2′-deoxyuridine and 2′3′-dideoxyuridine compared to the synthesis using ribose nucleosides such as uridine. This result could be due to the low affinity of uridine-nucleoside phosphorylase for the dehydroxylated sugars [8,9]. The problems for the phosphorolysis of these deoxy and dideoxy-nucleosides by a PyNP are related to the fact that phosphate anion binds through H-bonding to the hydroxyl groups in C2′ and in C3′ independently if the enzyme is included in the NP-type-I or in NP-type-II enzymes [8]. It has been shown that the location of the phosphate ion in adequate position to attack in α-position of sugar, in C1′, is very important. In the case of DDU, phosphate ion should only be bound to the amino acids of the active site described for many nucleoside phosphorylases [8,30]. This situation would reduce the activity of the uridine nucleoside phosphorylase in the case of these deoxynucleosides.

Table 5
Synthesis of some nucleosides using *E. gergoviae* immobilised in agarose (2%)

Pyrimidine nucleoside	Base	Yield in nucleoside (%)
U	6-Mercaptopurine	56
DU	6-Mercaptopurine	18
U	3-Carboxamide1,2,4-triazol	45
DU	6-Methoxypurine	27 (24 h)
U	Purine	80
DU	Purine	40 (5 h)
DDU	Purine	38 (21 h)

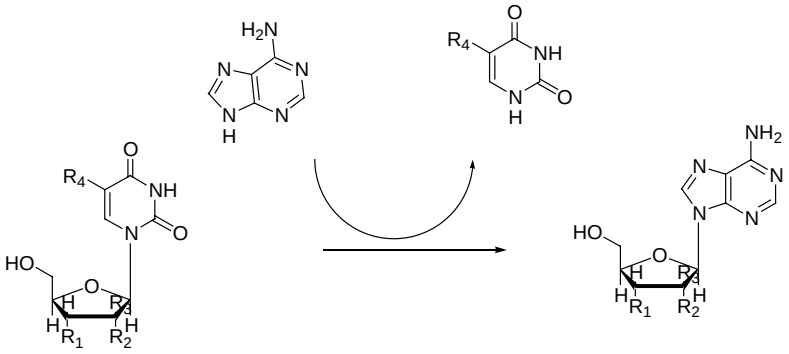
$T = 60$ °C; $t = 3$ h; pyrimidine nucleoside = 0.15 mM; base = 0.05 mM.

The nature of the sugar is more important than that the structure of the base as we can deduce by comparing the yields obtained with U and MU versus DU, DDU and araU. Generally, the pyrimidine nucleosides with ribose (U and MU) are better substrates than the other nucleosides (DU, DDU and araU). Only *C. amalonaticus* immobilized in polyacrylamide gave a moderated yield of ara-nucleosides, after 24 h. *E. coli* is the only microorganism that gave an acceptable yield with DDU in accordance with a previous report of Rogert et al. [12] regarding the catalytic activity of this strain. Therefore, the catalytic activity and selectivity is strongly related to the nature of the microorganism.

3.5. Synthesis of other nucleosides

In Table 5, we show some results obtained in the synthesis of other nucleosides (Scheme 2), using the biocatalyst prepared from *E. gergoviae* whole cells immobilized in agar (2%).

We can observe in Table 5 that uridine (U) is a better reagent than 2′-deoxyuridine or 2′,3′-dideoxyuridine, using the same base. Different structures of bases can be used to obtain unnatural nucleosides as in the case of *E. gergoviae* immobilized in agar. The yields obtained are similar to those described by Prasad et al. [6] with whole cells.



R_1= R_2= OH , R_3 =H, R_4 = H ; uridine (U)
R_1=OH, R_2=R_3=R_4 = H ; 2'-deoxyuridine (DU)
R_1= R_2 = R_3 = R_4 = H ; 2´,3´-dideoxyuridine (DDU)
R_1 = R_3 = OH; R_3 =H, R_4 = H; ara-uridine (AU)
R_1 = R_2 = OH ; R_3 = H ; R_4 = CH_3 ; 5-methyluridine (MU)

Scheme 2.

The structure of the second base seems to be less important than that of the sugar. We could also consider that a reduction in the steric hindrance favors the process comparing the yields obtained at 3 h. with 6-mercaptopurine and purine using U as nucleoside. Finally the virazole yields (HPLC) (45%, in Table 5) is similar to the 44% in isolated virazole described by Hennen and Wong [31]. Nevertheless, in our case 25 cycles of this synthesis were performed.

Acknowledgements

This work was supported by the Project BQU 2001-1301 from Ministerio de Ciencia y Tecnología of Spain and the Project PR158/01-1065 from Agencia Española de Cooperación Internacional (AECI).

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