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ORIGINAL ARTICLE

Aeromonas hydrophila strains as biocatalysts for transglycosylation

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Abstract

Microbial transglycosylation is useful as a green alternative in the preparation of purine nucleosides and analogues, especially for those that display pharmacological activities. In a search for new transglycosylation biocatalysts, two *Aeromonas hydrophila* strains were selected. The substrate specificity of both micro-organisms was studied and, as a result, several nucleoside analogues have been prepared. Among them, ribavirin, a broad spectrum antiviral, and the well-known anti HIV didanosine, were prepared, in 77 and 62% yield using *A. hydrophila* CECT 4226 and *A. hydrophila* CECT 4221, respectively. In order to scale-up the processes, the reaction conditions, product purification and biocatalyst preparation were analyzed and optimized.

Keywords: *Aeromonas hydrophila, microbial transglycosylation, whole cell biocatalysts, ribavirin, didanosine*

Introduction

Nucleoside analogues have proved to be useful in the treatment of viral infections and cancer (De Clercq 2005). Biocatalytic strategies that have previously been applied for the synthesis of these molecules include transglycosylation reactions carried out by microbial nucleoside phosphorylases (NPs), enzymes belonging to the transferase class (Lewkowicz & Iribarren 2006).

In one of the earliest papers, Imada and Igarasi (1967) reported the nucleoside phosphorylase activity of some bacterial cell-free extracts, almost all from *Enterobacteriaceae*. Nowadays, it is known that NPs are widespread in nature due to their essential role in purine and pyrimidine salvage pathways.

In a typical transglycosylation, NPs used sequentially catalyze ribosyl transfer from a pyrimidine nucleoside to a purine base. Several nucleoside analogues have been synthesized using this reaction (Utagawa 1999; Medici et al. 2006; Li et al. 2010) and new sources of NPs are continually being sought in order to further improve biocatalyst performance. In this context, we have previously reported the transglycosylation activity of whole cells of *Aeromonas hydrophila* CECT 4226 for synthesis of benzimidazole riboside (Bentancor et al. 2004).

A. hydrophila is an ubiquitous, oxidase positive, facultative anaerobic, glucose fermenting, gram-negative bacteria found in brackish, fresh, estuarine, marine, chlorinated and non-chlorinated water supplies worldwide (Agarwal et al. 2007). As far as we are aware, the only previous report dealing with the biocatalytic activity of this micro-organism used a crude enzyme derived from *A. hydrophila* to produce D-glucosamine and *N*-acetyl D-glucosamine from chitin (Sashiwa et al. 2002).

In this work we have studied the substrate specificities of NPs from *A. hydrophila* CECT 4221 and *A. hydrophila* CECT 4226. The synthesis of biologically active nucleosides like ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and didanosine (2’,3’-dideoxyinosine, DDI) was catalyzed by these bacteria (Scheme 1) and, therefore, the optimization of these reactions was also assessed.

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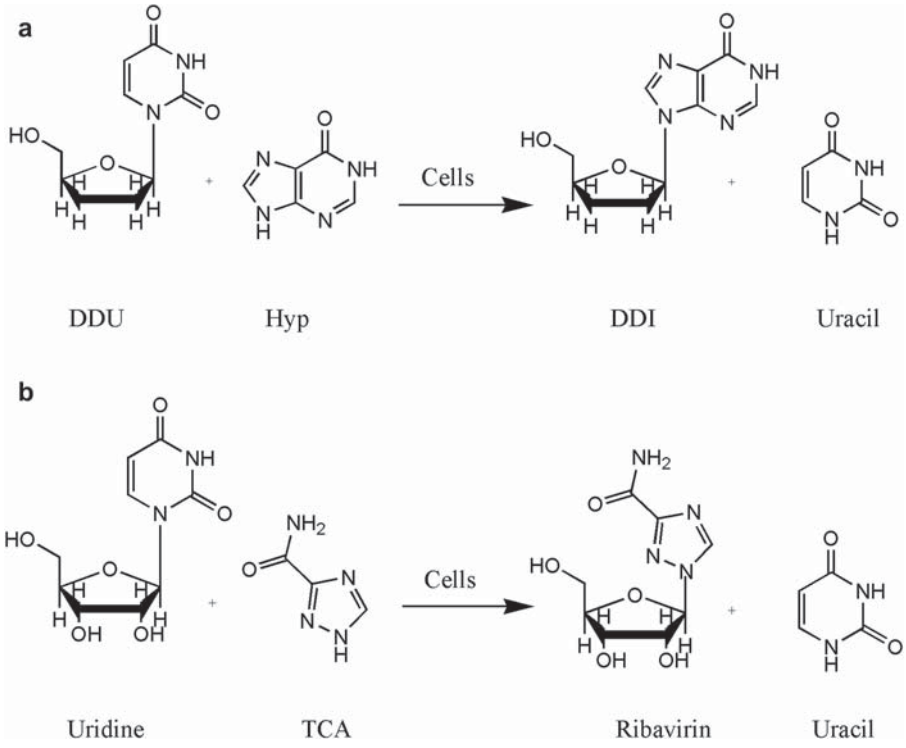

Scheme 1. Synthesis of (a) didanosine; (b) ribavirin, by transglycosylation. DDI: 2',3'-dideoxyinosine; DDU: 2',3'-dideoxyuridine; Hyp: hypoxanthine; TCA: 1,2,4-triazole-3-carboxamide.

Materials and methods

Chemicals and micro-organisms

Nucleosides and bases were purchased from Sigma-Aldrich (St Louis, MO) or ICN (Ohio). The culture media components were obtained from Merck (Darmstadt, Germany) and Difco (Sparks, MD). HPLC grade methanol and acetonitrile were from Carlo Erba (Rodano, Italy) or Sintorgan (Buenos Aires, Argentina). *Aeromonas hydrophila* strains were supplied by the *Colección Española de Cultivos Tipo* (CECT), Universidad de Valencia (Spain).

Growth conditions

Aeromonas hydrophila strains were cultured for 1 day at 30°C in Luria Broth medium (NaCl 10 g L^{-1}, tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}). Growth was measured by determining the optical density at 600 nm (equivalence: 0.4 DO units: 2×10^8 cells mL^{-1}). The resulting cultures were centrifuged at 12 000 g for 10 min and the resulting cell pellets used as biocatalysts.

Standard biotransformation

A standard reaction mixture, comprised of wet cell paste containing 1×10^{10} cells mL^{-1}, 10 mM purine base, 30 mM pyrimidine nucleoside and 30 mM potassium phosphate buffer pH 7 (final volume 0.5 mL), was shaken at 200 rpm and 60°C or 45°C. Aliquots taken at 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h were centrifuged at 10 000 g for 30 s and the supernatants analyzed by HPLC. The products were further characterized by MS.

Analytical methods

HPLC analyses were performed in a modular Gilson instrument (321Pump, 156 UV/VIS detector and 234 Autoinjector Series; Middleton, WI) with an Alltech Apollo RP18 column (150 × 4.6 mm, 5 μ) (Deerfield, IL), at room temperature. Products were eluted using water/acetonitrile or water/methanol mixtures at a flow rate of 1 mL min^{-1} using authentic materials as reference standards, when they were available.

MS analysis was carried out using a Thermo-Finnigan LCQ Advantage Max spectrometer (San Jose, CA) by direct injection, in positive mode, after solid phase extraction (SPE). The SPE was performed using a C18 silica gel cartridge (Phenomenex, Torrance, CA) and eluted with 1% methanol/formic acid (70:30, v/v). Satisfactory MS data were obtained for all compounds.

Synthesis of ribavirin

The reaction mixture comprised wet cell paste of *A. hydrophila* CECT4226 containing 5×10^{10} cells mL^{-1}, 30 mM 1,2,4-triazole-3-carboxamide (TCA, 13.4 mg), 30 mM uridine (29.3 mg) and 20 mM potassium phosphate buffer pH 7 (4 mL) which was stirred at 200 rpm and 60°C. After 26 h, the mixture was centrifuged and the ribavirin conversion (77%) in the supernatant was determined by HPLC using as eluent (1) 4 min water/acetonitrile (99.4:0.6, v/v), (2) 1 min gradient to water/acetonitrile (95:5, v/v) and (3) 4 min water/acetonitrile (95:5, v/v) and setting the detector at $\lambda = 235$ nm; R_t (min): uracil = 2.5; ribavirin = 3.5; uridine = 4; TCA = 7. Finally, ribavirin was obtained in 65% yield (14.2 mg, 99% purity by HPLC) after purification from the supernatant using first a variable volume column (10 × 200 mm, Kontes Flex-Column, Vineland, NJ), containing C18 silica gel (10 g, Phenomenex, Torrance, CA) eluting successively with five volumes of H_2O, acetonitrile 5% and 10%. The fractions containing TCA and ribavirin were further purified by normal flash chromatography with methanol:chloroform 20:80 v/v.

Synthesis of didanosine

The reaction mixture comprised wet cell paste of *A. hydrophila* CECT4221 containing 3×10^{10} cells mL^{-1}, 10 mM hypoxanthine (6.8 mg), 30 mM dideoxyuridine (31.8 mg) and 30 mM pH 7 potassium phosphate buffer (5 mL) which was stirred at 200 rpm and 45°C. After 16 h, the mixture was centrifuged and DDI conversion (62%) was determined by HPLC using water/acetonitrile (92.5:7.5 v/v) as eluent and setting the detector at $\lambda = 248$ nm; R_t(min): uracil = 1.8; dideoxyuridine = 2.2; hypoxanthine = 4.8; didanosine = 6.3. Finally, DDI was obtained in 58% yield (7.1 mg, 99% purity by HPLC) after purification from the supernatant using a variable volume column (10 × 200 mm, Kontes Flex-Column), containing C18 silica gel (10 g, Phenomenex) eluting successively with five volumes of H_2O, acetonitrile 2%, 10% and 50%.

A. hydrophila immobilization

The pellet, prepared as above described, was mixed with previously sterilized agarose (3 mL, 2% (w/v)). Then, the mixture was slowly added to stirred sunflower oil (10 mL) at 25°C for 5 min. The resulting gel beads (mean diameter 3.5 mm) were cooled, filtered, washed with hexane and then with physiological solution, to obtain solvent-free beads. They were used directly as the biocatalyst (catalyst load: 2.9×10^{10} cells g^{-1}).

Results

Substrate specificity of A. hydrophila strains

Several natural and unnatural pyrimidine nucleosides and purine bases were used as starting materials for transglycosylation biocatalyzed by two previously selected *A. hydrophila* strains (CECT 4226 and 4221) (Tables I–III). All biotransformations were performed using the standard methodology and the products were characterized by HPLC when commercial reference materials were available or by MS in the case of non-commercial products (2'-deoxyribavirin $M/Z^{+1} = 231.2$; 2-fluoradenine-2'-deoxyriboside $M/Z^{+1} = 270.1$; 2-amino-6-chloropurine-2'-deoxyriboside $M/Z^{+1} = 302.2$; 2-fluoradenine riboside $M/Z^{+1} = 286.1$; 2-amino-6-methoxypurine riboside $M/Z^{+1} = 298.5$; 6-mercaptopurine riboside $M/Z^{+1} = 285.2$; 8-azaguanosine $M/Z^{+1} = 258.5$; 6-iodopurine riboside $M/Z^{+1} = 369.4$; 6-methoxypurine riboside $M/Z^{+1} = 286.3$).

The base specificity in the synthesis of ribosides was studied initially with *A. hydrophila* CECT 4226 using uridine and different purine bases as substrates (Table I). The presence of substituents in the purine ring was necessary for biocatalyst activity since no transglycosylation was observed starting from purine alone (Table I, entry 14). Unexpectedly, among the 6-substituted purines (Table I, entries 1–6) adenine was not a substrate and the best result was obtained starting with 6-iodopurine. Incorporation of an amino group at the C-2 of the purine ring (Table I, entries 7–10) dramatically increased the yields, except when guanine was the substrate, although the latter may be attributed to the low solubility of this base (Medici et al. 2008). The presence of a 2-fluorine (Table I, entry 11) also improved the yield and the reaction time. Since benzimidazole, a 1,3-deazapurine ring (Bentancor et al. 2004) (Table I, entry 15) afforded good conversions in the transglycosylation reactions, other purine analogues with different nitrogen contents were assessed. Thus, TCA, a triazole derivative (Table I, entry 16) was also a good substrate whereas 7-deaza-adenine and 8-azaguanine (Table I, entries 12 and 13) were poorly accepted. The transglycosylation was not successful using bases containing indol, imidazole or pyridine rings (Table I, entries 17–19).

Table II shows the pyrimidine nucleoside acceptance by *A. hydrophila* CECT 4226. Nucleosides containing different sugar residues and uracil or thymine as bases were assessed for the synthesis of benzimidazole nucleosides. This base was chosen since it had already been demonstrated that benzimidazole displays good affinity for PNP but, in contrast, the corresponding nucleosides are poor

substrates for phosphorolysis due to the higher stability of their glycosidic bonds (Bentancor et al. 2004). The experiments were performed at both 45 and 60°C to assess the activity of the different enzymes involved. As expected, no reaction was observed with uridine at 45°C or with thymidine at 60°C (Table II, entries 1 and 6). 2'-Deoxyuridine was found to be the best substrate, giving benzimidazole 2'-deoxynucleoside in a high yield in a short reaction time at the two temperatures employed (Table II, entries 3 and 4). Less than 10% yield was obtained when DDU was used as substrate (Table II, entries 7 and 8) and neither 3'-azido-3'-deoxythymidine (AZT) nor arabinouridine were accepted (Table II, entries 9–12).

Taking into consideration these results, other 2'-deoxyribosides were prepared from 2'-deoxyuridine. Among them, 2'-deoxyribavirin and 2-fluor-2'-deoxyadenosine were produced in 1 h with 90 and 74% yield, respectively.

The behaviour of *A. hydrophila* CECT 4221 in the transglycosylation reaction was also studied (Table III). Adenine was accepted as the best substrate to prepare ribo- and deoxyribonucleosides from the corresponding uracil nucleosides (Table III, entries 1 and 8). In contrast, non-natural purine bases were poorly recognized, especially those with 2,6-disubstitutions or modified in the purine ring (Table III, entries 2–7 and 9–11). Regarding the sugar residue, DDU also afforded good yields in the preparation of dideoxynucleosides (Table III, entries 17–20), but only from natural bases. Thymidine and arabinouridine were not substrates for these bacterial phosphorylases.

Biotransformation optimization

Among the nucleosides obtained using *A. hydrophila* strains, ribavirine and DDI were of particular interest due to their use in anti-viral therapies. Different experimental conditions were studied in order to achieve the highest yields of these target nucleosides. The standard temperature (60°C) was used to prepare ribavirin, but 45°C was better for obtaining DDI since the enzymes involved in the phosphorolysis of pyrimidine substrates are different (Lewkowicz & Iribarren 2006). For HPLC analysis of the reaction mixtures, the wavelengths of the UV-detector were set at 235 nm and 248 nm for ribavirin and DDI, respectively. The best culture growth time for each *A. hydrophila* strain was evaluated by taking aliquots containing 1×10^{10} cells mL^{-1} at different growth stages and performing the biotransformations. The maximum activity of both biocatalysts was observed at 48 h, corresponding to later stationary phase.

Phosphate buffer concentrations of 10, 20 and 30 mM were assessed as reaction media. When 10 mM buffer was employed, the final yields were reduced to almost 50%. Ribavirin yields were similar using either 20 or 30 mM buffers, so 20 mM was used thereafter. For DDI synthesis, the best yield was achieved using 30 mM buffer.

The amounts of biocatalyst and reagents were also analyzed. The results are shown in Table IV. Improvements were observed by moving from the standard 3:1 uridine:TCA ratio to carrying out the biotransformation with a 1:1 ratio of 30 mM substrate (Table IV, entries 1 and 2). As a consequence, the final amount of ribavirin was higher despite the yield being slightly

Table I. Purine ribosides and analogues synthesized by transglycosylation from uridine and catalyzed by *A. hydrophila* CECT 4226.

Entry	Base	Purine nucleoside	Yield[a] (%)	Time[b] (h)
1	6-Chloropurine	6-Chloropurine riboside	18	2
2	6-Iodopurine	6-Iodopurine riboside	72	8
3	6-Methoxypurine	6-Methoxypurine riboside	21	1
4	6-Mercaptopurine	6-Mercaptopurine riboside	11	4
5	Adenine	Adenosine	—	—
6	Hypoxanthine	Inosine	26	48
7	2-Amino-6-chloropurine	2-Amino-6-chloropurine riboside	69	2
8	2-Amino-6-methoxypurine	2-Amino-6-methoxypurine riboside	94	8
9	2-Amino-6-mercaptopurine	2-Amino-6-mercaptopurine riboside	80	1
10	Guanine	Guanosine	—	—
11	2-Fluoradenine	2-Fluoradenosine	82	0.5
12	7-Deazaadenine	7-Deazaadenosine	5	1
13	8-Azaguanine	8-Azaguanosine	13	1
14	Purine	Purine riboside	—	—
15	Benzimidazole	Benzimidazole riboside	88	24
16	1,2,4-triazole-3-carboxamide	Ribavirin	77	26
17	Aminoimidazolecarboxamide	Aminoimidazolecarboxamide riboside	—	—
18	Nicotinamide	Nicotinamide riboside	—	—
19	Tryptophan	Tryptophan riboside	—	—

[a]% yield = $[\text{nucleoside}]_{exp}.10^2/[\text{nucleoside}]_{the}$.
[b]see Materials and methods for conditions, Standard biotransformation section. All reactions were performed at 60°C.

Table II. Benzimidazole nucleosides synthesized by transglycosylation using *A. hydrophila* CECT 4226 as biocatalyst.

Entry	Pyrimidine nucleoside	Temperature[a] (°C)	Yield[b] (%)	Time (h)
1	Uridine	45	—	—
2	Uridine	60	88	24
3	Deoxyuridine	45	92	2
4	Deoxyuridine	60	92	2
5	Thymidine	45	61	24
6	Thymidine	60	—	—
7	DDU	45	6	1
8	DDU	60	5	1
9	AZT	45	—	—
10	AZT	60	—	—
11	Arabinouridine	45	—	—
12	Arabinouridine	60	—	—

DDU, 2',3'-Dideoxyuridine; AZT, 3'-azido-3'-deoxythymidine.
[a]see conditions in Materials and methods, Standard biotransformation section.
[b]% yield = $[\text{nucleoside}]_{exp}.10^2/[\text{nucleoside}]_{the}$.

lower. Additional advantages were lower uridine wastage and easier purification. Regarding the biocatalyst, increasing the number of cells mainly reduced the reaction time (Table IV, entries 3–5). Thus, with 5×10^{10} *A. hydrophila* CECT 4226 cells per millilitre, 77% conversion was obtained in 26 h.

In the case of DDI preparation, a 30:10 mM ratio between DDU and hypoxanthine afforded the best results (Table IV, entries 6–8) and increasing the biocatalyst amount also reduced the reaction time (Table IV, entries 6, 9 and 10). The best condition for DDI preparation was 3×10^{10} *A. hydrophila* CECT4221 cells per milliliter, allowing 62% of conversion in 14 h.

Isolation and purification of reaction products

The isolation of transglycosylation reaction products is tricky due to the presence of phosphate buffer and the poor solubility of nucleosides and bases formed. A preliminary purification approach was carried out following traditional procedures that involve an initial step of adsorption chromatography employing Amberlite XAD 1180 in order to remove the phosphate (Murakami et al. 1991). Nucleosides and bases were eluted using acetonitrile–water mixtures and the eluate was applied to a silica column in normal or reverse phase depending on the expected nucleoside.

Subsequently, a one step purification procedure using reverse phase silica gel C18 flash chromatography was successfully applied. The purification of DDI was performed in a variable volume column in 95% purification yield. From 5 mL of reaction mixture 7.1 mg DDI was isolated. However, a second step using a normal phase silica gel column was necessary to separate ribavirin and uridine. From 4 mL of reaction mixture 14.2 mg of ribavirin was obtained (purification yield: 84%).

Biocatalyst immobilization

Whole cells of *A. hydrophila* strains were immobilized by entrapment in agarose, as previously optimized

Table III. Transglycosylations biocatalyzed by *A. hydrophila* CECT 4221.

Entry	Pyrimidine nucleoside	Base	Purine nucleoside	Yield[a] (%)	Time[b] (h)	Temperature (°C)
1	Uridine	Adenine	Adenosine	62	4	60
2	Uridine	6-Mercaptopurine	6-Mercaptopurine riboside	11	4	60
3	Uridine	6-Methoxypurine	6-Methoxypurine riboside	21	1	60
4	Uridine	6-Chloropurine	6-Chloropurine riboside	10	1	60
5	Uridina	2-Amino-6-chloropurine	2-Amino-6-chloropurine riboside	—	—	60
6	Uridine	Benzimidazole	Benzimidazole riboside	6	3	60
7	Uridine	TCA[a]	Ribavirin	—	—	60
8	Deoxyuridine	Adenine	Deoxyadenosine	89	4	60
9	Deoxyuridine	Hypoxanthine	Deoxyinosine	4	1	60
10	Deoxyuridine	6-Chloropurine	6-Chloropurine deoxyriboside	—	—	60
11	Deoxyuridine	2-Amino-6-chloropurine	2-Amino-6-chloropurine deoxyriboside	—	—	60
12	Thymidine	Adenine	Deoxyadenosine	—	—	45
13	Thymidine	Hypoxanthine	Deoxyinosine	4	1	45
14	Thymidine	6-Chloropurine	6-Chloropurine deoxyriboside	—	—	45
15	Thymidine	2-Amino-6-chloropurine	2-Amino-6-chloropurine deoxyriboside	—	—	45
16	Arabinouridine	Adenine	Arabinoadenosine	—	—	60
17	DDU	Adenine	DDA[c]	59	48	45
18	DDU	Hypoxanthine	DDI[d]	62	14	45
19	DDU	6-Chloropurine	6-Chloropurine dideoxyriboside	—	—	45
20	DDU	2-Amino-6-chloropurine	2-Amino-6-chloropurine dideoxyriboside	—	—	45

TCA, 1,2,4-triazole-3-carboxamide; DDU, 2',3'-dideoxyuridine; DDA, 2',3'-dideoxyadenosine; DDI, 2',3'-dideoxyinosine.
[a]% yield = $[\text{nucleoside}]_{exp}.10^2/[\text{nucleoside}]_{the}$.
[b]see conditions in Materials and methods, Standard biotransformation section.

Table IV. Experimental conditions optimization.

Entry	Pyrimidine nucleoside	Base	Pyrimidine nucleoside (mM)	Base (mM)	*Aeromonas hydrophila* strain	Biocatalyst amount (cells mL^{-1})	Buffer (mM)	Yield[a] (%)	Time (h)
1	Uridine	TCA	30	10	CECT4226	3.6×10^9	20	88	72
2	Uridine	TCA	30	30	CECT4226	3.6×10^9	20	83	72
3	Uridine	TCA	30	30	CECT4226	1×10^{10}	20	71	72
4	Uridine	TCA	30	30	CECT4226	3×10^{10}	20	75	48
5	Uridine	TCA	30	30	CECT4226	5×10^{10}	20	77	26
6	DDU	Hyp	30	10	CECT4221	1×10^{10}	30	45	24
7	DDU	Hyp	10	10	CECT4221	1×10^{10}	30	24	24
8	DDU	Hyp	30	30	CECT4221	1×10^{10}	30	28	24
9	DDU	Hyp	30	10	CECT4221	3×10^{10}	30	62	14
10	DDU	Hyp	30	10	CECT4221	5×10^{10}	30	45	14

DDU, 2',3'-Dideoxyuridine; TCA, 1,2,4-triazole-3-carboxamide; Hyp, hypoxanthine.
[a]% yield = $[\text{nucleoside}]_{exp}.10_2/[\text{nucleoside}]_{the}$.

(Trelles et al. 2004). Agarose beads containing corresponding amounts of each biocatalyst were added to 3 mL of the reaction mixtures. Similar kinetic behaviour of free and immobilized *A. hydrophila* strains for the syntheses of DDI and ribavirin was observed (Figure 1). Additionally, the immobilized biocatalysts were re-used three times without loss of activity.

Discussion

In the transglycosylation reaction, two sequential steps are necessary to biotransform a pyrimidine nucleoside into a purine one. First, pyrimidine nucleoside is converted to α-ribose-1-phosphate (R1P) by the action of a pyrimidine nucleoside phosphorylase (PyNP). In the second step, PNP catalyzes the transfer reaction between R1P and a purine base to afford the corresponding purine nucleoside. Uridine phosphorylase (UP) is typically active up to 80°C. while thymidine phosphorylase (TP), the enzyme that catalyzes the phosphorolysis of pyrimidine 2'-deoxynucleosides, is only active below 50°C. Therefore, it is necessary to set the temperature of the reaction depending on the pyrimidine nucleoside substrate (Lewkowicz & Iribarren 2006).

A previous study had shown that *A. hydrophila* CECT 4226 was an efficient biocatalyst in the preparation of benzimidazole, TCA and 2-amino-6-substituted purine ribosides, but did not accept adenine as a substrate, suggesting that its PNP resembles the mammalian one (Lewkowicz & Iribarren 2006). The PyNPs also show a similar behaviour to the corresponding mammalian enzymes since only natural occurring pyrimidine nucleosides were well recognized. In the case of *A. hydrophila* CECT 4221, adenine and hypoxanthine were accepted as substrates while non-natural bases afforded poor yields. In contrast, this strain was the best producer of dideoxynucleosides.

Ribavirin and DDI produced in this study are pharmacologically important. Ribavirin is a guanosine analogue first synthesized by Witkowski et al. (1972). It was reported to have broad spectrum activity against a variety of DNA and RNA viruses (Wu et al. 2005). The mechanism of action includes inhibition of inosine monophosphate dehydrogenase, which is the key step in *de novo* guanosine synthesis, a requirement for viral replication (Gish 2006). Shirae and Yokozeki (1991a; b) reported ribavirin production from orotidine and 1,2,4-triazole-3-carboxamide (TCA) using *Erwinia caratovora* as a biocatalyst as well as from guanosine or inosine

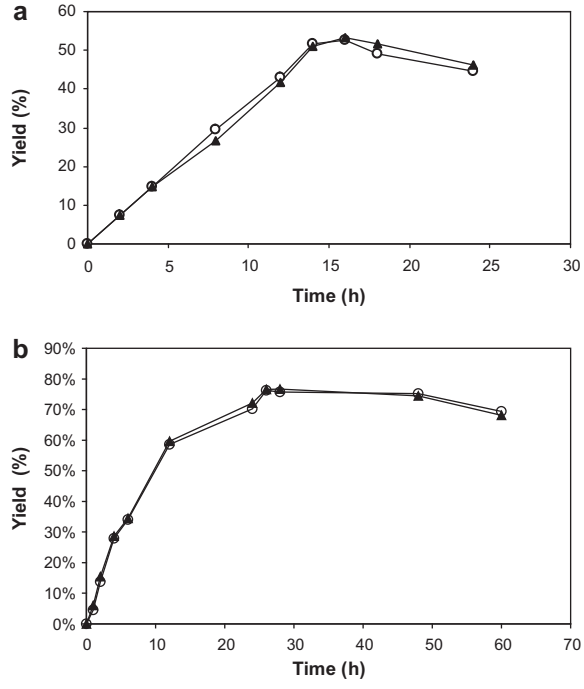

Figure 1. Time course of (a) DDI and (b) ribavirin synthesis catalyzed by (▲) free and (o) immobilized cells of *Aeromonas hydrophila* CECT 4221 and *Aeromonas hydrophila* CECT 4226, respectively.

employing *Brevibacterium acetylicum* whole cells. In all cases ~ 70% yield was obtained after 48 h. Konstantinova et al. (2004) provided a technological method, carrying out the transglycosylation reaction in recombinant *E. coli* BL21 immobilized on aminopropylated silochrome (68% yield). Previously, Iaskovich and Iakovleva (1999) had used *Xanthomonas campestris* immobilized by adsorption to prepare ribavirin with 50% yield from adenosine.

Didanosine is an effective HBV and HIV inhibitor (Kitos & Tyrrell 1995) acting, after phosphorylation, as a chain terminator during reverse transcription (De Clercq 2002). It was obtained from 2',3'-dideoxyuridine (DDU), by transglycosylation, using isolated deoxyribosyltransferases from *Lactobacillus helveticus* or *leichmanii* (Prasad et al. 1999) as well as whole cells of *E. coli* (Shiragami et al. 2001) (25 and 45% yield, respectively).

Based on these previous results, some experimental variables were optimized in order to improve the production of these compounds by *A. hydrophila* strains. The optimal temperatures for the transglycosylations were 60°C for ribavirin and 45°C in the case of DDI. This is in accordance with the stability of the respective enzymes (UP for uridine and TP for dideoxyuridine). The highest transglycosylation activity was observed when the micro-organisms were harvested in late stationary phase. This accords with the fact that the salvage paths, where the NPs play their natural action, display maximum activity at this stage in order to facilitate nucleic acid turnover (Yorgey & Kolter 1993).

The use of 20 mM buffer phosphate was preferred to 30 mM to synthesize ribavirin, since similar yields were obtained using both concentrations and a 30:30 nucleoside:base concentration ratio was selected to facilitate purification procedures. In contrast, a 30:10 ratio was preferred for DDI preparation since a high concentration of nucleoside substrates seems to be necessary for *A. hydrophila* CECT 4221 PNP.

Increasing the cells number increased the reaction rate. Ribavirin synthesis reached the highest yields when 5×10^{10} cells mL^{-1} were used, while 3×10^{10} cells mL^{-1} was selected in the case of DDI.

A rapid isolation of purine nucleosides was developed, using reverse phase flash chromatography. The produced pyrimidine and residual purine bases remained adsorbed in the support, while nucleosides were selectively eluted using methanol:water or acetonitrile:water mixtures affording high purification yields. This methodology could be suitable for scale-up processes since the stationary phase can be reused.

A. hydrophila was efficiently immobilized on agarose beads and surprisingly both free and immobilized forms showed similar kinetic behaviour. This result, together with the fact that the immobilized biocatalyst maintains its activity after at least three reuses, makes *A. hydrophila* appealing for industrial biocatalysis.

The performance of *A. hydrophila* species reported in this work suggests that a careful screening must be always carried out in the search of appropriate biocatalysts, since different strains of the same genus and species may afford diverse results.

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