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Use of *Citrobacter koseri* whole cells for the production of arabinonucleosides: A larger scale approach

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ABSTRACT

Purine arabinosides are well known antiviral and antineoplastic drugs. Since their chemical synthesis is complex, time-consuming, and polluting, enzymatic synthesis provides an advantageous alternative. In this work, we describe the microbial whole cell synthesis of purine arabinosides through nucleoside phosphorylase-catalyzed transglycosylation starting from their pyrimidine precursors. By screening of our microbial collection, *Citrobacter koseri* (CECT 856) was selected as the best biocatalyst for the proposed biotransformation. In order to enlarge the scale of the transformations to 150 mL for future industrial applications, the biocatalyst immobilization by entrapment techniques and its behavior in different reactor configurations, considering both batch and continuous processes, were analyzed. *C. koseri* immobilized in agarose could be used up to 68 times and the storage stability was at least 9 months. By this approach, fludarabine (58% yield in 14 h), vidarabine (71% yield in 26 h) and 2,6-diaminopurine arabinoside (77% yield in 24 h), were prepared.

1. Introduction

The use of drugs such as AZT, DDI and other modified nucleosides, whose specificity is due to the selective inhibition of enzymes involved in viral nucleoside and nucleotide metabolism, are usual in the treatment of viral diseases [1].

Generally, these compounds are industrially obtained through chemical synthesis, using expensive and multiple step reactions [2]. The growing interest for the market of these products, as well as the increasing request for new industrial processes involving chemo-enzymatic methods, has driven the development of new methodologies for the preparation of nucleosides analogs employing biocatalysts [3,4].

It is known that microorganisms, particularly bacteria, have salvage pathways to synthesize purine nucleosides from pyrimidine ones. This process, called transglycosylation, is based on the action of nucleoside phosphorylases (NPs) that catalyze the phosphorolysis of the glycosidic bond (Scheme 1). Due to the small number of commercially available NPs and their characteristics, the use of whole cell bacteria as biocatalysts appears as a more convenient strategy [5].

Purine arabinosides, such as 9-β-D-arabinofuranosyladenine (vidarabine, AraA) that is currently used in the treatment of different viral infections [6], and 9-β-D-arabinofuranosyl 2-fluoroadenine (fludarabine), active in chronic lymphocytic leukemia therapies [7], have generated considerable attention as chemotherapeutic drugs [8]. Moreover, 2,6-diaminopurine arabinoside (DAPA) and 2-amino-6-methoxypurine arabinoside [9] are prodrugs of 9-β-D-arabinofuranosylguanine (AraG) since they are rapidly hydrolyzed *in vivo* by adenosine deaminase (ADA).

Regarding their chemoenzymatic preparation, arabinonucleosides have been previously synthesized by transglycosylation, using isolated uridine phosphorylase (UP) and purine nucleoside phosphorylase (PNP) from *Escherichia coli* and 1-β-D-arabinofuranosyluracil (AraU) as sugar donor [10]. Utagawa [11] reported the preparation of adenine- and other substituted purine arabinosides using whole cells of *Enterobacter aerogenes* (70–90% yield, 24 h). Medici et al. [12] showed that *Enterobacter gergoviae* afforded DAPA in 72% yield in 48 h. DAPA was also synthesized from *Enterobacter aerogenes* DGW-07 reaching above 90% conversion in 48 h [13].

N-deoxyribosyltransferases (NdRT), which transfer glycosyl residues to acceptor bases, have also been used in the synthesis of 2′-deoxynucleosides. Recently a novel NdRT from *Lactobacillus reuteri* has been cloned and over-expressed in *E. coli* and used in arabinoside synthesis [14].

Another strategy, based on the coupled action of two whole cells, to perform the simultaneous transglycosylation and deamination reactions, was applied to synthesize AraG [15]. The

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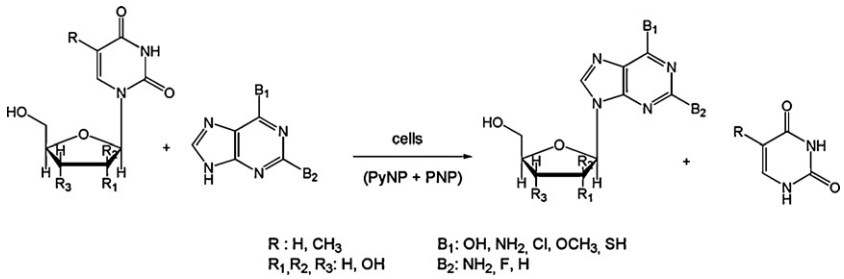

Scheme 1. Transglycosylation reaction biocatalysed by the combined action of a pyrimidine phosphorylase (PyNP) and a purine nucleoside phosphorylase (PNP) or by microbial cells that contain theses enzymes.

deamination step was performed by ADA from *Arthrobacter oxydans* [16] or adenylate deaminase produced by *Aspergillus oryzae* DAW-01 [13].

Although free bacterial cells or extracted and purified NPs are appropriate for carrying out transglycosylation reactions at laboratory scale, for industrial applications the use of immobilized biocatalysts, which offer increased productivity and easy handling, is preferred. Properly designed immobilization on solid supports enables the reuse of the catalyst and may increase the stability of enzymes and cells under a broad range of experimental conditions [17–19]. *E. coli* UP and PNP have been co-immobilized by covalent linkage for the biocatalytic preparation of a variety of natural and modified purine nucleosides [20]. Hori et al. [21] immobilized PNP and PyNP from *B. stearothermophilus* by ionic binding and used these immobilized biocatalysts in a continuous reaction for the production of 5-methyluridine from inosine and thymine. To our best knowledge, immobilized whole cells have not been used as biocatalyst to synthesize purine arabinosides by transglycosylation.

Immobilization cell technologies have been widely developed since the 1980s, using both wholly artificial and naturally occurring methods [22]. In the first one, cells are artificially entrapped in or attached to various supports where they keep or not a viable state, depending on the degree of harmfulness of the immobilization procedure. Polysaccharide gel matrixes, more particularly Ca^{2+}-alginate hydrogels [23] are by far the most frequently used materials for cell entrapment. In contrast, the attachment of cells to an organic or inorganic matrix may be obtained by covalently binding cells to the support, using cross-linking agents such as glutaraldehyde or carbodiimide, although these immobilization procedures are usually incompatible with cell viability. At last, the spontaneous adsorption of microbial cells to different kind of carriers provides natural immobilized systems in which cells are attached to their support by weak, generally non-specific contacts such as electrostatic interactions. Regarding the application of immobilized whole cells to nucleoside synthesis, the use of immobilized *E. coli* [24,25], *Xanthomonas campestris* [26], *Enterobacter gergoviae* [27] and *Citrobacter amalonaticus* [28] by entrapment techniques on different supports (e.g. naturally occurring polysaccharides extracted from seaweed, like agar and agarose, and the synthetic matrix polyacrylamide) were reported.

The aim of the current study was to analyze the different operational parameters for the production of fludarabine and vidarabine using immobilized *Citrobacter koseri* whole cells in transglycosylation reactions, having the bacteria been selected from a screening from our cell collection. To pursue this objective, the performance and productivity of the immobilized cells were evaluated as function of a few critical parameters, such as biocatalyst load, immobilization matrixes, concentration of reagents, stability and reuse capacity of the biocatalyst. After optimization, different types of reactor configurations were investigated to enlarge the scale of the transformations from 3 to 150 mL in view to future scale up for industrial applications.

2. Materials and methods

2.1. Chemicals and microorganisms

Nucleosides and bases were purchased from Sigma–Aldrich (St Louis, MO, USA) or ICN (Ohio, USA). The culture media components were obtained from Merck (Darmstadt, Germany) and Difco (Sparks, MD, USA). HPLC grade methanol and acetonitrile were from Carlo Erba (Rodano, Italy) or Sintorgan (Buenos Aires, Argentina). Microorganisms were supplied by the *Colección Española de Cultivos Tipo* (CECT), Universidad de Valencia (Spain). All other chemical reagents were commercially available and of the best analytical grade.

2.2. Growth conditions

The strains were cultured in liquid media at the optimum temperature (T) and time (t), according to the American Type Culture Collection (ATCC). In particular, *C. koseri* strains were cultured for one day at 37 °C in liquid 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 saturated culture broths were centrifuged at 12000 × g for 10 min and the pellets were first washed with phosphate buffer, recentrifuged and used as biocatalysts.

2.3. Standard screening conditions

The standard reaction mixture comprising: 10 mM purine base, 30 mM pyrimidine nucleoside and 30 mM potassium phosphate buffer pH 7 (final volume 0.05 mL) and wet cell paste containing 1×10^{10} cells mL^{-1}, was stirred at 200 rpm and 60 °C. Aliquots taken at 4, 8, 12, 24 and 48 h were centrifuged at 10,000 × g for 30 s and the supernatants were analyzed by HPLC.

2.4. C. koseri immobilization

2.4.1. Agarose or agar entrapment

The pellet of the saturated culture broths, prepared as above described was mixed with a previously sterilized and melted agarose solution (2.5 mL, 2.5% (w/v)) or agar solution (2.5 mL, 3% (w/v)). Then, the suspension was slowly added to stirred sunflower oil (10 mL) at 25 °C for 5 min. The resulting gel beads were cooled, filtered and washed successively with hexane and saline solution (NaCl 9 g L^{-1}), to obtain solvent-free beads. The obtained biocatalyst (catalyst load: 1.4×10^{11} or 2.9×10^{10} cells g^{-1} to prepare arabino or ribonucleosides respectively; beads diameter: 2.9 ± 0.2 mm) was stored in 30 mM potassium phosphate buffer pH 7 at 4 °C until use.

2.4.2. Polyacrylamide entrapment

For the preparation of polyacrylamide blocks, the bacterial pellet was first dissolved in 7.3 mL of 30 mM phosphate buffer pH 7 and added into 2.7 mL of an aqueous solution containing 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide. Then, 50 μL of ammonium persulphate (10%, w/v) and 14 μL of tetramethyl ethylene diamine (TEMED) were added. The contents were allowed to polymerize on a sterile plate and the resulting gel was cut into pieces (approximately 3 mm × 3 mm × 2 mm). The pieces were washed with saline solution and stored at 4 °C in 30 mM phosphate buffer pH 7 until use. Catalyst load: 1.4×10^{11} or 2.9×10^{10} cells g^{-1} to prepare arabino or ribonucleosides respectively.

2.4.3. Poly(vinyl alcohol) entrapment

PVA (average molecular weight of 89,000–98,000, degree of hydrolysis 99%) was mixed with deionized water and heated to obtain a 12.5% solution (w/v). The bacterial pellet was mixed thoroughly with 10 mL of PVA solution by gently stirring. The mixture was allowed to polymerize on a sterile plate and the resulting gel was cut into pieces (approximately 3 mm × 3 mm × 2 mm), then washed with saline solution and stored at 4 °C in 30 mM phosphate buffer pH 7 until use. Catalyst load: 1.4×10^{11} or 2.9×10^{10} cells g^{-1} to prepare arabino or ribonucleosides respectively.

2.5. Adenosine synthesis by immobilized C. koseri cells

The standard reaction mixture for adenosine synthesis containing: 30 mM adenine, 30 mM uridine and 30 mM potassium phosphate buffer pH 7 (final volume 3 mL) and 1 g biocatalyst, was stirred at 200 rpm and 60 °C. The reactions were allowed to proceed for 3 h (63% conversion). Aliquots taken at different reaction times were centrifuged at 10,000 × *g* for 30 s and the supernatants were analyzed by HPLC, using as operating conditions: 5 min water/methanol (90:10, v/v), 1.5 min gradient to water/methanol (80:20, v/v) and 5 min water/methanol (80:20, v/v), 1 mL min^{-1} flow rate and detector set at λ = 254 nm (R_t: uracil = 2.6; uridine = 3.1; adenine = 6.7; adenosine = 9.1).

2.6. Reuse capacity

To assess the reuse potential of the *C. koseri* entrapped cells, repeated batch transglycosylation reactions for the synthesis of adenosine were carried out under the experimental conditions described above. After each cycle, the beads were filtered from the reaction media, washed with phosphate buffer and transferred into a fresh reaction mixture.

2.7. Analytical methods

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

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

2.8. Synthesis of fludarabine

The reaction mixture comprising: *C. koseri* (wet cell paste or 1 g agarose beads) 5×10^{10} cells mL^{-1}, 3 mM 2-fluoradenine, 6 mM AraU and 30 mM potassium phosphate buffer pH 7 (3 mL), was stirred at 200 rpm and 60 °C. After 14 h, the mixture was centrifuged and 58% of fludarabine was determined in the supernatant by HPLC, using as eluent: (1) 3 min water/methanol (85:15, v/v), (2) 2 min gradient to water/methanol (70:30, v/v) and (3) 4 min water/methanol (70:30, v/v), 0.9 mL min^{-1} flow rate and setting the detector at λ = 260 nm (R_t: uracil = 2.4; AraU = 3.1; 2-fluoradenine = 6.6; fludarabine = 7.7). Finally, the fludarabine was purified from the supernatant using a variable volume column (10 mm × 200 mm, Kontes Flex-Column, Vineland, NJ, USA), containing C18 silica gel (Phenomenex, Torrance, CA, USA) eluting successively with 5 volumes of each: H_2O, 5% and 10% acetonitrile. Fludarabine was obtained in 48% yield (99% purity by HPLC).

2.9. Synthesis of vidarabine

The reaction mixture comprising: *C. koseri* (wet cell paste or 1 g agarose beads) 5×10^{10} cells mL^{-1}, 10 mM adenine, 20 mM AraU and 20 mM potassium phosphate buffer pH 7 (3 mL), was stirred at 200 rpm and 60 °C. After 26 h, the mixture was centrifuged and 71% of vidarabine was determined in the supernatant by HPLC, using as eluent water/acetonitrile (95.2:4.8, v/v), 1 mL min^{-1} flow rate and setting the detector at λ = 254 nm (R_t: uracil = 2.1; AraU = 2.7; adenine = 3.6; vidarabine = 4.9). Finally, vidarabine was purified from the supernatant using a variable volume column (10 mm × 200 mm, Kontes Flex-Column, Vineland, NJ, USA), containing C18 silica gel (Phenomenex, Torrance, CA, USA) eluting successively with 5 volumes of each: H_2O, 5% and 10% acetonitrile. Vidarabine was obtained in 62% yield (99% purity by HPLC).

2.10. Synthesis of 2-6-diaminopurinarabinoside

The reaction mixture comprising: *C. koseri* (wet cell paste) 1.1×10^{10} cells mL^{-1}, 10 mM 2,6-diamopurine, 30 mM AraU and 30 mM potassium phosphate buffer pH 7 (3 mL), was stirred at 200 rpm and 60 °C. After 24 h, the mixture was centrifuged and 77% of DAPA was determined in the supernatant by HPLC, using as eluent water/methanol (90:10, v/v), 1 mL min^{-1} flow rate and setting the detector at λ = 254 nm (R_t: uracil = 2.4; AraU = 3.4; 2,6-diaminopurine = 4.7; DAPA = 6.3). Finally, DAPA was purified from the supernatant using first a variable volume column (10 mm × 200 mm, Kontes Flex-Column, Vineland, NJ, USA), containing C18 silica gel (Phenomenex, Torrance, CA, USA) eluting successively with 5 volumes of each: H_2O, 5% and 10% acetonitrile. DAPA was obtained in 66% yield (99% purity by HPLC).

2.11. Scaled reactions (25–200 mL)

The 25 mL scale syntheses of fludarabine and vidarabine were performed in glass reactors operated in batch mode (stirred-tank, packed-bed or bubble-column reactors). The reaction mixture comprised 0.35 g mL^{-1} agarose beads containing *C. koseri* whole cells (8 g of immobilized biocatalyst, 1.4×10^{11} cells g^{-1}), 3 mM 2-fluoroadenine and 6 mM AraU in case of fludarabine and 10 mM adenine and 20 mM of AraU for vidarabine, in 30 mM potassium phosphate buffer pH 7.

The stirred-tank reactor consisted of a spinner glass reactor (Celstir, Wheaton, Millville, USA) equipped with radial flow impellers. The stirring at 200 rpm was produced by the magnetic rotation of the impellers using a hot plate (Heidolph, UK).

The packed-bed reactor consisted of a cylindrical glass of 10 cm length and 1.5 cm width. The reaction mixture was introduced from the bottom using a peristaltic pump operating at 1.6 mL h^{-1}.

The bubble column reactor consisted of a 10 cm length and 2.6 cm width-glass column. To maintain the necessary fluidization of the bed, and external pump supplying an air flow of 0.15 L min^{-1} was coupled to the column. Evaporation of the reaction mixture was minimized (less than 1% in volume) attaching a condenser on top of the reactor. In all cases the temperature was maintained at 60 °C using a silicone flexible heating tape rolled around the reactor. Once the optimal reaction times were achieved, the mixtures were centrifuged and the supernatants analyzed by HPLC.

The synthesis of vidarabine in a final volume of 150 mL was performed using a 500 mL spinner glass reactor (Celstir, Wheaton, Millville,US) equipped with radial impellers. The reaction mixture comprised 0.35 g mL^{-1} agarose beads containing *C. koseri* whole cells (1.4×10^{11} cells g^{-1}), 10 mM adenine and 20 mM of AraU, in 30 mM potassium phosphate buffer pH 7. The reaction was kept at 60 °C and 200 rpm and was allowed to proceed during 26 h. After purification following the protocol previously detailed, 175.2 mg of vidarabine (62.5% yield) were recovered from the reaction mixture.

The synthesis of adenosine as model reaction was also carried out in continuous operating packed-bed reactors (100 and 200 mL volume glass columns; 0.25 cm × 20 cm and 40 cm, respectively) (Supplementary information Figs. S1 and S2). The reactors were pre-heated at 60 °C and this temperature was kept during the whole process. The synthesis of adenosine was performed using 72 g (100 mL reactor) or 141.5 g (200 mL reactor) of biocatalyst and the reaction mixture described above was circulated from a reservoir through a peristaltic pump. The determined optimal flow and residence time were 1.6 mL min^{-1}/124 min and 3.4 mL min^{-1}/110 min for 100 and 200 mL reactors respectively, affording a productive capacity of 0.45 and 0.96 mg/h of adenosine (61.7 ± 0.1% yield during 27 h for 100 mL reactor and 61.8 ± 0.1% yield during 24 h for 200 mL reactor).

3. Results and discussion

3.1. Biocatalyst selection

Microorganisms supply a large diversity of biocatalysts with different substrate specificities. In order to make use of this enzymatic diversity for the synthesis of bioactive purine arabinosides, a screening process capable of identifying the strains with the proper transglycosylation activity has been carried out based on the methodology previously developed in the group [29]. Starting from AraU and the corresponding purine bases, almost 100 strains were screened to afford vidarabine, fludarabine and DAPA. Since it is known that AraU is substrate of UP [30], all the transglycosylations were carried out at 60 °C. This temperature was used to avoid deamination and to improve NP activities. The most representative results are shown in Table 1.

The only microorganism that efficiently catalyzed the synthesis of the three arabinonucleosides was *C. koseri* (CECT 856). Although *C. koseri* is frequently used as microbial sensor in bioremediation [31,32], its transglycosylation activity had not been previously reported.

3.2. 3 mL scale reaction

3.2.1. Biocatalyst load

The influence of the biocatalyst load on vidarabine transglycosylation reaction was investigated varying from 3.6×10^9 to 5×10^{10} cells mL^{-1}. The results (Table 2, entries 4–8) showed that 1×10^{10} *C. koseri* cells mL^{-1} was sufficient to reach a good transglycosylation yield but, as expected, shorter time was observed increasing the biomass amount. Then, 5×10^{10} cells mL^{-1} were used to prepare vidarabine and fludarabine in 26 and 14 h respectively. This reaction profile was not conserved in the case of

Table 1
Microbial transglycosylation for arabinosides synthesis. Primary screening.

CECT	Microorganisms	Vidarabine		DAPA		Fludarabine	
		%[a]	*t* (h)	%[a]	*t* (h)	%[a]	*t* (h)
333	*Achromobacter cycloclastes*	–	48	4	24	–	48
839	*Aeromonas hydrophila*	8	48	ND		3	48
4221	*Aeromonas hydrophila*	–	48	23	48	–	48
4226	*Aeromonas hydrophila*	–	48	52	8	7	24
4225	*Aeromonas hydrophila*	–	48	75	48	–	48
4223	*Aeromonas punctata*	–	48	ND		19	24
896	*Aeromonas salmonicida*	–	48	26	48	5	8
863	*Citrobacter amalonaticus*	33	24	14	24	4	24
401	*Citrobacter freundii*	–	48	14	24	–	48
856	*Citrobacter koseri*	58	24	83	48	54	48
	Chromobacterium violaceum	–	48	4	24	4	8
684	*Enterobacter aerogenes*	3	48	22	24	3	8
4214	*Enterobacter cloacae*	1	24	7	24	–	48
960	*Enterobacter cloacae*	–	48	9	48	–	48
194	*Enterobacter cloacae*	–	48	11	24	–	48
857	*Enterobacter gergoviae*	–	48	72	48	2	24
222	*Erwinia amylovora*	–	48	5	24	–	48
225	*Erwinia carotovora*	–	48	2	8	–	48
509	*Erwinia chrysanthemi*	–	48	11	48	ND	
877	*Escherichia coli*	14	24	22	24	7	8
433	*Escherichia coli*	18	8	ND		14	24
105	*Escherichia coli*	17	8	12	24	6	8
100	*Escherichia coli*	7	24	14	4	3	24
	Escherichia coli BL21	–	48	11	4	29	24
843	*Klebsiella planticola*	7	24	11	4	2	8
367	*Klebsiella sp*	9	24	7	8	–	48
4101	*Proteus mirabilis*	4	24	4	48	–	48
171	*Proteus rettgeri*	2	24	5	24	–	48
865	*Proteus rettgeri*	–	48	3	24	–	48
4557	*Proteus rettgeri*	–	48	2	24	–	48
174	*Proteus vulgaris*	–	48	7	48	–	48
4077	*Proteus vulgaris*	6	8	–	24	2	24
165	*Proteus vulgaris*	–	48	6	8	–	48
324	*Pseudomonas putida*	–	48	15	48	–	48
159	*Serratia marescens*	–	48	3	24	–	48
233	*Sthaphylococcus capitis*	–	48	7	24	–	48
4643	*Xanthomonas translucens*	–	48	5	24	–	48

ND: not determined.
[a] Conversion calculated by HPLC, based on the purine base.

DAPA production and the best yield was observed in 24 h using 1×10^{10} cells mL^{-1}.

3.3. Phosphate concentration

Typically, microbial transglycosylation screenings have been carried out in 30 mM phosphate buffer [33]. As previously mentioned, this biotransformation is a two-step biocatalyzed reaction where the first one involves the phosphorolysis of the pyrimidine nucleoside to afford α-pentofuranoside-1-phosphate which is consumed in the second step to produce the target purine nucleoside [5]. In this mechanism, phosphate plays an important role since it influences the rate of both reversible reactions but in opposite directions. Moreover, high phosphate concentration makes

Table 2
Effect of experimental variables in the transglycosylation reaction of vidarabine, fludarabine and DAPA performed by *C. koseri* free cells.

Entry	Base	Buffer (mM)	AraU (mM)	Base (mM)	*C. koseri* biomass (10^{10} cell mL^{-1})	Conversion (%)[a]	Time (h)
1	Adenine	30	30	10	1	74	72
2	Adenine	20	30	10	1	72	72
3	Adenine	10	30	10	1	10	96
4	Adenine	20	20	10	1	69	72
5	Adenine	20	10	10	1	42	72
6	Adenine	20	10	10	0.36	26	72
7	Adenine	20	20	10	3	74	72
8	Adenine	20	20	10	5	71	26
9	2-Fluoradenine	30	6	3	1	69	72
10	2-Fluoradenine	20	6	3	1	45	72
11	2-Fluoradenine	10	6	3	1	2	96
12	2-Fluoradenine	30	9	3	1	64	72
13	2-Fluoradenine	30	3	3	1	12	72
14	2-Fluoradenine	30	6	3	3	60	26
15	2-Fluoradenine	30	6	3	5	58	14
16	2,6-Diamino purine	30	10	10	1	45	24
17	2,6-Diamino purine	30	30	10	1	77	24

[a] Determined by triplicate by HPLC based on the purine base.

purification more difficult. Then, there is an optimal phosphate concentration, usually for each substrate–enzyme combination.

As observed in Table 2, 10 mM phosphate (entries 3 and 11) was low enough to decrease the rate of UP catalyzed phosphorolysis of AraU affording small quantities of arabinose 1-phosphate. When phosphate concentration increased to 20 mM, the reaction performed with the natural base adenine achieved optimal yields (Table 2, entry 2). In contrast, 30 mM was necessary to promote the reaction with non-natural bases (Table 2, entries 9 and 17) in order to get α-pentofuranoside-1-phosphate concentration above the enzyme K_M [34].

3.4. Substrates: concentration and molecular ratio

To obtain the highest yields, the employed substrates concentration is usually a relevant point to be investigated since the low solubility of the purine bases in the reaction media is a well-known limiting factor. In addition, different AraU: purine base ratios were tested (Table 2 entries 2, 4, 5, 9, 12, 13, 16 and 17). The best conversion to vidarabine was observed when the ratio was 3:1. Ratio 2:1 leads to a small decrease in conversion, however, to contain the cost of the process and considering the difficulties in the isolation of the products, this ratio was selected for further experiments for vidarabine and fludarabine. In contrast, ratio 3:1 was employed for DAPA synthesis.

3.5. C. koseri immobilization

The use of immobilized biocatalysts in industrial applications is due to several reasons that include: the biocatalyst can be easily removed from the reaction mixture, rinsed and reused thus reducing operational costs; it can be utilized in continuous operation and, in general, the immobilized biocatalyst shows greater stability under both storage and operational conditions [35]. In particular, we carried out the selection of immobilization matrices considering their physical properties and structural characteristics (like mechanical strength, integrity of the particles and cell release into the medium), the toxicity of the immobilization process or the matrix itself, the easy of preparation and the transglycosylation activity. To perform these studies, the synthesis of adenosine from uridine and adenine was adopted as reaction model (for detailed experimental conditions, see Section 2.5), which affords a 63% yield in 1.5 h using *C. koseri* free cells.

Different types of supports were evaluated: ionic hydrogels (alginate and chitosane) with divalent ions (Ca^{2+}) or polycations (e.g. polyethylenimine); thermogels (agar and agarose) and synthetic polymers (polyvinylalcohol (PVA) and polyacrylamide).

In this way, the immobilization matrices selected at this stage were agar, agarose, polyacrylamide and PVA. Later, the feasibility of repeated uses of the four immobilized biocatalysts and free-cells was analyzed in reaction cycles of 3 h. The obtained results are shown in Fig. 1.

Except for the derivative immobilized in PVA that showed a similar reuse profile to *C. koseri* free cells, those obtained from the other supports retained more than 50% of the initial activity by at least 50 cycles. Among them, *C. koseri* immobilized in agarose showed the highest reuse number (68 cycles) together with a high initial performance, and therefore, was the chosen biocatalyst.

Additionally, the particle size was analyzed to know the role that it plays on mass transfer. The Weisz module [36] was calculated for 1–5 mm diameter spherical beads obtaining values lower than 0.3 indicating that diffusion does not represent a significant impact. Therefore, and taking also into account the ease of handling and the filterability for the efficient recovery of the biocatalyst, particles of 3 mm were chosen.

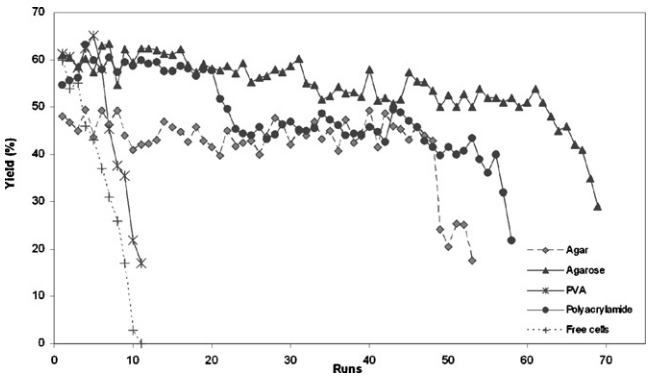

Fig. 1. Dependence on adenosine production upon repeated uses of *C. koseri* immobilized in different supports. All biotransformations were carried out using the same reaction conditions.

Furthermore, the stability of the immobilized cells upon storage at 4 °C was also analyzed. The agarose-immobilized cells were kept in buffer and no significant loss of activity after 9 months was detected.

Finally, the immobilized biocatalyst was employed for the synthesis of vidarabine and fludarabine under the best reaction conditions showed in Table 2 for free cells. Neither the yields nor the reaction times were seriously affected by using immobilized cells (58% fludarabine and 71% vidarabine in 14 and 26 h, respectively).

3.6. 25 and 150 mL scale reactions

In view to further pilot processes, the reactions were scaled to 25 mL in a first approach to study the influence of the reactor configuration, and finally to 150 mL.

3.7. Reactor configuration – effects of parameters and optimization

Three types of bioreactors, stirred tank reactor (STR), packed-bed reactor (PBR) and bubble column reactor (BCR) were used in order to select the best configuration for scaling-up studies in batch processes. Initial studies were performed in 25 mL for fludarabine and vidarabine synthesis. As it is shown in Fig. 2, no significant variations regarding reaction times, maximum yields and type of bioreactor were observed in the 25 mL-scale studies, affording the biocatalytic reactions a similar profile than those at 3 mL-scale.

Finally, a 150 mL STR reactor was chosen to perform a further scaled transglycosylation. For economical reasons, only vidarabine synthesis was tested and again comparable results were obtained with those reached earlier. In this case, after purification, excess reagents and 175 mg of vidarabine were recovered (71.2% yield, 26 h, data not shown).

In addition, preliminary data using continuous processes for adenosine synthesis in 100 and 200 mL packed-bed reactors suggest that this system could also be suitable for arabinosides production (Supporting Information Figs. S1 and S2).

3.8. Substrate specificity

In view to widen the applications of *C. koseri* cells as transglycosylation biocatalyst, the preparation of a variety of purine nucleoside analogs was carried out (Table 3). While in mesophilic microorganisms UP keeps its activity up to 75 °C, the activity of thymidine phosphorylase (TP), the specific enzyme for pyrimidine deoxynucleosides phosphorolysis, drops at 50 °C [5]. Consequently, temperatures of 45 or 60 °C were used, depending on the thermal stability of the pyrimidine phosphorylase involved in the

Table 3
Other nucleosides obtained by transglycosylation reaction catalyzed by *C. koseri*.

Entry	Base	Pyrimidine nucleoside	Product	*T* (°C)	Yield (%)[a]	Time (h)
1	Adenine	Uridine	Adenosine	60	62	1.5
2	Benzimidazole	Uridine	Benzimidazole riboside	60	6	3
3	2,6-Diaminopurine	Uridine	2,6-Diaminopurine riboside	60	71	4
4	Benzimidazole	Thymidine	Benzimidazole-2′-deoxyriboside	45	78	24
5	2,6-Diaminopurine	Thymidine	2,6-Diaminopurine-2′-deoxyriboside	45	85	4
6	Adenine	2′-Deoxyuridine	2′-Deoxyadenosine	60	67	1
7	Hypoxanthine	2′,3′-Dideoxyuridine	Didanosine	45	7	48
8	2,6-Diaminopurine	2′,3′-Dideoxyuridine	2,6-Diaminopurine-2′,3′-dideoxyriboside	45	5	48
9	Guanosine[b]	Arau	Arabinoguanosine	60	27	12

[a] Determined by triplicate by HPLC based on the purine base. See standard screening procedure in materials and methods section for experimental conditions.
[b] Guanosine was used instead guanine as donor and was added in pulses every 4 h.

biotransformation. *C. koseri* NPs tolerated changes at carbon 2′ of the pentose ring, but presence of OH 3′ is significant for activity since dideoxynucleosides were obtained in very low yields. Different 6 and 2,6-substituted purines as well as benzimidazole, a ring analog, were tested and almost all of them were accepted as substrates. As an additional benefit, *C koseri* ADA and adenine deaminase showed low activity which enables the use of 6-amino nucleosides without strict control of temperature and time.

For the synthesis of AraG, guanosine was used as guanine donor in order to solve the low solubility of this base at pH 7 [37] but the desired product was not obtained. After a kinetical study of the reaction, it was verified that, after 1 h, the presence of guanosine in the reaction medium was not detected while the concentration of AraU remained almost unchanged. Probably, guanosine and guanine were used in other metabolic paths. To ensure the availability of guanine at longer reaction times, guanosine pulses were added every 4 h. With the fourth pulse of guanosine (12 h), AraG reaches its final concentration and remains constant up to 48 h (Table 3 entry 9).

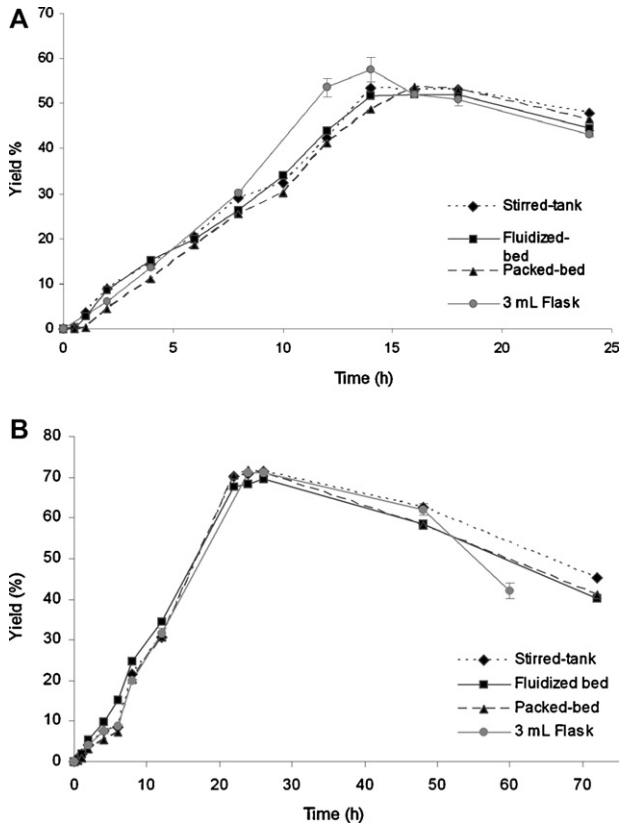

Fig. 2. 25 mL-scale time course transglycosylation reaction performed in different reactor configurations. (A) Fludarabine and (B) vidarabine synthesis. The reaction carried out at 3 mL was inserted as reference.

4. Conclusion

The influence of variables such as culture time, buffer concentration, biocatalyst and substrates amount, immobilization support and reactor configuration on the production of arabinonucleosides from AraU and the corresponding bases by transglycosylation reaction biocatalyzed by *C. koseri* whole cells have been analyzed. Under the optimal operating conditions, three pharmacologically active nucleosides, fludarabine (58% yield in 14 h), vidarabine (71% yield in 26 h) and 2,6-diaminopurine arabinoside (77% yield in 24 h), were prepared. The scale-up from 0.05 mL (screening conditions) to 150 mL (stirred tank reactor batch operating conditions) did not lead to significant changes in the profile of the reactions. These facts together with the high catalyst productivity provided by the immobilized biocatalyst (600 g adenosine/kg biocatalyst), suggest that this methodology can potentially be an economically viable way for the production of arabinonucleosides.

Author contributions

M.L.N., R.M., E.S.L. and A.M.I. designed the research; M.L.N. and R.M. performed the experiments; E.S.L., M.T. and A.M.I. supervised the project; R.M. and E.S.L. wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2012.08.011.

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