Journal of Molecular Catalysis B: Enzymatic 95 (2013) 16–22

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

A comparison between immobilized pyrimidine nucleoside phosphorylase from *Bacillus subtilis* and thymidine phosphorylase from *Escherichia coli* in the synthesis of 5-substituted pyrimidine 2′-deoxyribonucleosides

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ARTICLE INFO

Article history:
Received 14 January 2013
Received in revised form 27 April 2013
Accepted 15 May 2013
Available online xxx

Keywords:
Nucleoside phosphorylase
Transglycosylation
5-Halogenated pyrimidine
2′-deoxynucleosides
Floxuridine

ABSTRACT

Pyrimidine nucleoside phosphorylase from *Bacillus subtilis* (*Bs*PyNP, E.C. 2.4.2.3) and thymidine phosphorylase from *Escherichia coli* (*Ec*TP, E.C. 2.4.2.4) were used, as immobilized enzymes, in the synthesis of 5-halogenated pyrimidine 2′-deoxyribonucleosides (**14**–**18**) by transglycosylation in fully aqueous medium. From the comparative study of the two biocatalysts, no remarkable differences emerged about their substrate specificity, bioconversion yield, stability in organic cosolvents (DMF and MeCN). Moreover, both biocatalysts could be recycled for at least 5 times with no loss of the productivity.

Both enzymes do not accept arabinonucleosides and 2′,3′-dideoxynucleosides as substrates, whereas they catalyze bioconversions involving 5′-deoxyribonucleosides and 5-halogenated uracils. The synthesis of compounds **14**–**18** proceeded at a similar conversion (33–68% for *Bs*PyNP and 25–62% for *Ec*TP, respectively). Immobilization was found to exert, for both the biocatalysts, a dramatic enhancement of stability upon incubation in MeCN.

Optimization of 5-fluoro-2′-deoxyuridine (**14**) synthesis (pH 7.5, 10 mM phosphate buffer, nucleoside/nucleobase 3:1 molar ratio) and subsequent scale-up afforded the target compound in 73% (*Ec*TP) or 76% (*Bs*PyNP) conversion (about 9 g/L).

1. Introduction

By the late 1970s, when acyclovir came to the market, nucleoside analogues have been extensively investigated giving a new impetus to the search for more active and selective molecules. Such efforts have provided a large arsenal of antiviral and antitumor chemotherapeutic agents still used nowadays in clinical practice. 5-Substituted pyrimidine 2′-deoxynucleosides with a halogen (F, Br, I) or a halogenated group (CF_3, CH=CHBr) constitute a small class of drugs used for the topical treatment of local *Herpes simplex* virus and *Varicella zoster* virus infections (**15**, **17**, **18**), or of certain types of cancer (**14**, **16**) (see Scheme 2) [1–6].

5-Substituted pyrimidine nucleosides can be synthesized by C-5 halogenation of uracil-based nucleosides or their *O*-acetylated derivatives [7]. In spite of some advances [8], difficult handling of halogenating agents and their toxicity frequently discourage this approach which is also plagued by harsh experimental conditions, long reaction times and complex work-up. On the other hand, following a convergent approach, silylated uracil derivatives can be chemically coupled with protected 2-deoxyribose or ribose [9–11]. In the former case, a mixture of the α- and β-anomers is produced which must be chromatographically separated and finally deprotected. In the latter approach, the 2′-*O*-protecting group on the ribose unit controls the stereochemistry of the glycosidation to a single product. Notwithstanding, selective 2′-deoxygenation and final deprotection are eventually necessary to complete the synthetic sequence.

Enzyme-based syntheses have a tremendous potential over totally chemical methods. Nucleoside phosphorylases (NPs; E.C. 2.4.2), which participate by nature in the salvage pathway of nucleoside biosynthesis [12,13], can be exploited for the stereoselective synthesis of 2′-deoxynucleoside analogues [14–16]. NPs catalyze the reversible cleavage of the glycosidic bond of (deoxy)ribonucleosides in the presence of inorganic orthophosphate (P_i) as a second substrate to generate the nucleobase and α-D-(deoxy)ribose-1-phosphate (see Scheme 1) [12]. If a second

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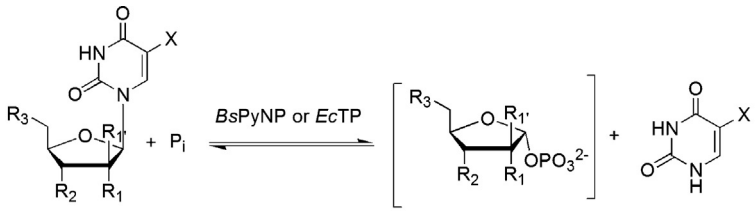
http://dx.doi.org/10.1016/j.molcatb.2013.05.007



	X	R_1	$R_{1'}$	R_2	R_3
1	H	H	H	OH	OH
2	CH_3	H	H	OH	OH
3	H	OH	H	OH	OH
4	H	OH	H	OH	H
5	H	H	OH	OH	OH
6	H	H	H	H	OH

Experimental conditions: 50 mM phosphate buffer, pH 7.5, room temperature, substrate=5 mM, volume=10 mL, *Bs*PyNP or *Ec*TP=1 IU.
Legend: **1**, 2'-deoxyuridine; **2**, thymidine; **3**, uridine; **4**, 5'-deoxyuridine; **5**, arabinosyluracil; **6**, 2',3'-dideoxyuridine; **7**, uracil (X=H) or **8**, thymine (X=CH_3), Pi, inorganic orthophosphate. *Bs*PyNP: pyrimidine nucleoside phosphorylase from *Bacillus subtilis*; *Ec*TP: thymidine phosphorylase from *Escherichia coli*.

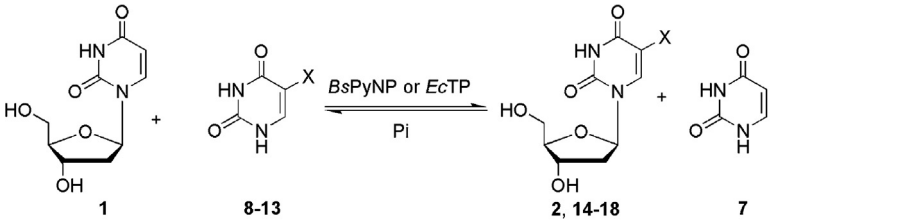
Scheme 1. Phosphorolysis of sugar donor nucleosides (**1**–**6**) catalyzed by immobilized *Bs*PyNP or *Ec*TP.

nucleobase is added to the reaction medium, the formation of a new nucleoside can result (transglycosylation, Scheme 2). Thus, following this pattern, the stereocontrolled synthesis of 2′-deoxynucleosides can be achieved without the need for protection/deprotection, in a "two-step, one-pot" reaction.

We have recently described the immobilization and a few synthetic applications of purified uridine phosphorylase from *Bacillus subtilis* (E.C. 2.4.2.3) [17–19], herein *Bs*PyNP, and thymidine phosphorylase from *Escherichia coli* (*Ec*TP, E.C. 2.4.2.4) [20]. According to the classification proposed by Pugmire and Ealick [12], both these enzymes belong to the NPII family, being characterized by specificity for pyrimidine nucleosides.

Immobilization of *Bs*PyNP and *Ec*TP [18,20] provided more stable biocatalysts than the soluble counterparts in non-physiological conditions (pH 10 and 45 °C or 37 °C), fundamental for biocatalytic applications, *i.e.* to increase reagent solubility (and concentration, thereof).

Immobilized *Bs*PyNP, coupled to immobilized purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1) from *B. subtilis*, catalyzed the synthesis of 2′-deoxyguanosine and 2′-deoxyinosine [19]. Immobilized *Bs*PyNP also catalyzed the synthesis of doxifluridine (5′-deoxy-5-fluorouridine), a prodrug of 5-fluorouracil [17,21]. Immobilized *Ec*TP was tested in the synthesis of floxuridine by using 2′-deoxyuridine or thymidine and 5-fluorouracil as reagents [20]. Whereas the synthesis of purine 2′-deoxynucleosides catalyzed by immobilized *Bs*PyNP and *Bs*PNP was deeply investigated by assaying the effect of pH, temperature, reagent concentration till the achievement of 85–92% yield, "one-enzyme"



		*Bs*PyNP (v_s)	*Ec*TP (v_s)
8, X = CH_3	**2**, X = CH_3	67% (1.3)	67% (3.3)
9, X = F	**14**, X = F	68% (1.7)	62% (3.3)
10, X = CF_3	**15**, X = CF_3	57% (0.4)	51% (0.4)
11, X = Br	**16**, X = Br	57% (1.0)	56% (1.5)
12, X = I	**17**, X = I	57% (0.5)	56% (0.8)
13, X = CH=CHBr	**18**, X = CH=CHBr	33% (0.07)	25% (0.2)

Percentage of produced nucleosides (**2**, **14**-**18**) and rate of synthesis (vs, μmol $min^{-1}g^{-1}$) are reported.
Experimental conditions: 10 mM phosphate buffer, pH 7.5, room temperature, sugar donor (**1**)=20 mM, nucleobase (**8**-**13**)=10 mM, volume=10 mL, *Bs*PyNP=10 IU, *Ec*TP=10 IU.
For **13**, pH was set to 10 by using potassium carbonate (see Experimental for details).
Legend: **1**, 2'-deoxyuridine; **8**-**13**, 5-substituted pyrimidine; **2**, thymidine; **14**, 5-fluoro-2'-deoxyuridine (floxuridine); **15**, 5-trifluoromethyl-2'-deoxyuridine (trifluridine); **16**, 5-bromo-2'-deoxyuridine (broxuridine); **17**, 5-iodo-2'-deoxyuridine (idoxuridine); **18**, (E)-5-(2-bromovinyl)-2'-deoxyuridine (brivudin); Pi, inorganic orthophosphate. *Bs*PyNP: pyrimidine nucleoside phosphorylase from *Bacillus subtilis*; *Ec*TP: thymidine phosphorylase from *Escherichia coli*.
Maximum conversions were reached at 10 h and 3 h for *Bs*PyNP and *Ec*TP, respectively, with the only exception of compound **18** (48 h) and **2** (6 h for *Bs*PyNP). All experiments were performed as duplicate.

Scheme 2. Synthesis of 5-substituted nucleosides **2** and **14**–**18** *via* transglycosylation catalyzed by immobilized *Bs*PyNP or *Ec*TP.

transglycosylations for the synthesis of pyrimidine 2′-deoxyribonucleosides (*i.e.* floxuridine) [20] and congeners (*i.e.* doxifluridine) [17] do lack such a systematic study.

In this paper we report on a comparison between immobilized *Bs*PyNP and *Ec*TP in the synthesis of 5-substituted pyrimidine nucleosides **14**–**18** (Scheme 2). Floxuridine (**14**) was selected as the target compound to optimize the experimental conditions. Apart from its higher clinical relevance, this choice was addressed by practical reasons: we aimed at developing a scalable synthetic process in fully aqueous medium and floxuridine is the most water soluble among the considered nucleosides. Bioconversion of 5-fluorouracil (**9**, X = F, see Scheme 2 and Table 3) was carried out till 50 mM. Halogenated nucleosides and nucleobases other than 5-fluoro derivatives suffer from low solubility in aqueous solvents and this property can be a shortcoming in scaling-up enzyme-catalyzed transformations in fully aqueous medium. For this reason, we also assayed the stability of immobilized *Bs*PyNP and *Ec*TP in the presence of acetonitrile (MeCN) and *N*,*N*-dimethylformamide (DMF) as cosolvents in the view of preparative applications.

An example of floxuridine synthesis by transglycosylation has been recently reported [22]. However, a cell-based bioconversion was studied whilst our attention was here focused on purified and immobilized enzymes as biocatalysts which are more viable when running pharmaceutical syntheses.

2. Experimental

2.1. General

Nucleosides (**1**–**6** and **14**–**18**), nucleobases (**7**–**13**), dextran (MW 20 kDa) and PEI (polyethylenimine, branched, MW 25 kDa) were purchased from Sigma–Aldrich and/or VWR International (Milano, Italy). 2′-Deoxyuridine (**1**) was supplied by Pro.Bio.Sint. (Varese, Italy). 5′-Deoxyuridine (**4**) was prepared as previously reported [17]. Sepabeads EC-EP® were kindly provided by Resindion Mitsubishi Chemical Co. (Binasco, Milano, Italy). All solvents were of HPLC grade.

Enzymatic reactions were monitored by using a HPLC Merck Hitachi L-7100 equipped with a UV detector L-7400 and a column oven L-7300 (Darmstadt, Germany). The column was a Lichrocart RP18 (5 μm, 250 mm × 4.6 mm, Merck) kept at 35 °C; flow rate was 1 mL/min and analyses were detected at λ = 260 nm.

Upon 15 L fermentation, cells were disrupted using a GEA Niro Soavi Homogenizer (Parma, Italy) and cell debris was precipitated by centrifugation using an Avant J25 Beckman Coulter (Cassina De' Pecchi, Milan, Italy). The column for protein purification was from Tosoh Bioscience (Rivoli, Turin, Italy). Enzyme activity [18,20] and protein concentration [23] were evaluated on a Shimadzu spectrophotometer UV 1601 (Milano, Italy).

2.2. Enzymes

Thymidine phosphorylase from *E. coli* (E.C. 2.4.2.4) was purchased from Sigma-Aldrich (Milano, Italy). Protein concentration was 33 mg/mL with a specific activity towards 2′-deoxyuridine (**1**) of 46 IU/mg (spectrophotometrically determined).

Expression and purification of *Bs*PyNP (E.C. 2.4.2.2) were carried out at 15 L fermentation volume following the procedure previously reported [19], with some modifications to maximize the protein expression.

Thus, a three-phase process (pre-seed, seed and fermentation) was carried out. The pre-seed cells were prepared in 50 mL LB medium and incubated at 37 °C. After 6 h, 500 mL of pre-fermentative medium (yeast extract 1 g/L, $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 15 g/L, $MgSO_4$ 5 g/L, dextrose 10 g/L) were inoculated with the pre-seed culture to a final concentration of 0.5% (v/v). The seed culture was incubated at 30 °C for 16 h and was subsequently diluted to 5% into a stirred bioreactor containing 15 L of fermentative medium ($(NH_4)_2SO_4$ 2 g/L, K_2HPO_4 15 g/L, KH_2PO_4 7.5 g/L, $MgSO_4$ 2 g/L, dextrose 50 g/L). The pH was controlled by automatic addition of diluted NH_4OH (25%, v/v), the stirring rate was adjusted to 500 rpm and the pO_2 was maintained automatically at 50% with aeration and agitation. The strain was grown to mid-exponential phase at 30 °C until OD_{600} reached 50, the temperature was then lowered to 22 °C and the protein expression was induced by adding 0.5 mM isopropylthio-β-D-galactoside (IPTG). After a 24 h expression phase, the final OD_{600} of the culture was 80.

Cells were disrupted by two cycles of homogenization at 1000 bar and the suspension was clarified by adding 0.5% quaternary ammonium salt followed by centrifugation. The enzyme was purified by loading the crude extract onto a Toyopearl Giga-Cap Q-650 M column previously equilibrated with Tris–HCl buffer pH 7.5. The adsorbed enzyme was then eluted with a linear gradient (0–0.5 M) of NaCl in 20 mM Tris–HCl pH 7.5 and 1 mM $MgCl_2$. Fractions containing the active enzyme were collected and dialyzed against 10 mM Tris–HCl pH 7.5 containing 1 mM $MgCl_2$ and 250 mM NaCl and stored at −20 °C.

After purification, 60 mL of protein extract were obtained; protein concentration was 5 mg/mL with a specific activity towards 2′-deoxyuridine (**1**) of 15 IU/mg (spectrophotometrically determined). The purity of the protein (>90%) was assessed by SDS-PAGE electrophoresis (4–12%).

2.3. Spectrophotometric enzyme activity assay

Enzyme activity assay was performed as previously reported [19]. Briefly, the activity of both *Bs*PyNP and *Ec*TP was determined by measuring the absorbance at 297 nm of uracil produced from 2′-deoxyuridine (**1**) at 37 °C in a cuvette of 1 cm light path ($\varepsilon = 1912\ M^{-1}\ cm^{-1}$). One IU corresponds with an amount of enzyme that liberates 1 μmol of uracil (**7**) from 2′-deoxyuridine (**1**) per minute.

2.4. Enzyme immobilization

Immobilization of both *Bs*PyNP and *Ec*TP was performed according to the procedure previously described [20]. Briefly, the enzyme (*Ec*TP, load: 1.3 mg/g; loaded activity: 60 IU/g; *Bs*PyNP, load: 3 mg/g; loaded activity: 45 IU/g) was added to a suspension of the matrix (Sepabeads EC-EP® derivatized with PEI) [18] in 5 mM phosphate buffer pH 7.5 and gently stirred for 20 min at room temperature. A freshly prepared solution of aldehyde dextran was added to the immobilization suspension. After stirring for 1 h, pH was adjusted to 10.05 and $NaBH_4$ was added keeping the reaction under mechanical stirring for 30 min. The immobilized preparation was then filtered and washed with 10 mM potassium phosphate buffer pH 5 and deionized water.

2.5. Enzyme stability assay

Immobilized enzyme preparation (200 mg) was added to a solution (1.5 mL) containing 50 mM phosphate buffer and 20% of MeCN or DMF (v/v) at pH 7.5. The mixture was stirred at room temperature for 24 h. At fixed times, 40 μl of suspension was withdrawn and activity was tested by standard spectrophotometric activity assay.

2.6. Phosphorolysis: general protocol

To a solution of the nucleoside (**1**–**6**) (10–12 mg, 5 mM) in 50 mM phosphate buffer pH 7.5 (10 mL), immobilized *Ec*TP or *Bs*PyNP

(1 IU) was added and the mixture was stirred at room temperature. Aliquots (0.2 mL) of the reaction mixture were withdrawn at fixed time intervals (5, 10, and 15 min), filtered through a pipette filter device to remove the biocatalyst and analyzed by HPLC (see below for chromatographic conditions and R_t).

2′-Deoxyuridine (**1**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (97:3). Uracil (**7**, X = H), R_t = 4.92 min, 2′-deoxyuridine (**1**), R_t = 12.19 min.

Thymidine (**2**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (93:7). Thymine (**8**, X = CH_3), R_t = 4.36 min, thymidine (**2**), R_t = 12.27 min.

Uridine (**3**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (97:3). Uracil (**7**, X = H), R_t = 4.92 min, uridine (**3**), R_t = 5.86 min.

5′-Deoxyuridine (**4**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (85:15). Uracil (**7**, X = H), R_t = 2.10 min, 5′-deoxyuridine (**4**), R_t = 10.23 min.

Arabinosyluracil (**5**) and 2′,3′-dideoxyuridine (**6**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (90:10). Uracil (**7**, X = H), R_t = 2.60 min, arabinosyluracil (**5**), R_t = 5.25 min, 2′,3′-dideoxyuridine (**6**), R_t = 8.67 min.

2.7. Transglycosylation: general protocol

To a solution of phosphate buffer (10 mM) at pH 7.5 (10 mL) containing 2′-deoxyuridine (**1**, 45.6 mg, 20 mM) and the 5-substituted base (**8–13**, 13–22 mg, 10 mM), 10 IU of immobilized enzyme (*Ec*TP or *Bs*PyNP) were added and the suspension was kept under mechanical stirring at room temperature. In the case of 5-bromovinyluracil (**13**), pH was set to 10 by using a 10 mM phosphate buffer containing 13.8 mg of potassium carbonate (final concentration 10 mM). Aliquots (0.2 mL) were periodically withdrawn, filtered through a pipette filter device to remove the biocatalyst and analyzed by HPLC (see below for chromatographic conditions and R_t). When the highest conversion was achieved, the reaction was stopped by filtration of the immobilized biocatalyst on a Büchner funnel with a sintered glass disc under reduced pressure. The produced nucleosides (**2** and **14–18**) were identified by comparison of their HPLC R_t with that of authentic samples.

Thymidine (**2**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (90:10). Uracil (**7**), R_t = 3.30 min, 2′-deoxyuridine (**1**), R_t = 4.84 min, thymine (**8**, X = CH_3), R_t = 5.54 min, thymidine (**2**), R_t = 9.74 min.

5-Fluoro-2′-deoxyuridine (**14**) and 5-trifluoromethyl-2′-deoxyuridine (**15**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (97:3). Uracil (**7**), R_t = 4.92 min, 2′-deoxyuridine (**1**), R_t = 12.19 min, 5-fluorouracil (**9**, X = F), R_t = 5.65 min, 5-trifluoromethyluracil (**10**, X = CF_3), R_t = 10.89 min, 5-fluoro-2′-deoxyuridine (**14**), R_t = 15.89 min, 5-trifluoromethyl-2′-deoxyuridine (**15**), R_t = 16.91 min.

5-Bromo-2′-deoxyuridine (**16**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (95:5). Uracil (**7**), R_t = 4.07 min, 2′-deoxyuridine (**1**), R_t = 6.7 min, 5-bromouracil (**11**, X = Br), R_t = 8.79 min, 5-bromo-2′-deoxyuridine (**16**), R_t = 21.19 min.

5-Iodo-2′-deoxyuridine (**17**). Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (90:10). Uracil (**7**), R_t = 2.60 min, 2′-deoxyuridine (**1**), R_t = 5.41 min, 5-iodouracil (**12**, X = I), R_t = 9.35 min, 5-iodo-2′-deoxyuridine (**17**), R_t = 15.29 min.

5-Bromovinyl-2′-deoxyuridine (**18**) Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/acetonitrile, gradient elution, 0 min-5 min (5% acetonitrile), 5.1 min-22 min (15% acetonitrile). Uracil (**7**), R_t = 4.07 min, 2′-deoxyuridine (**1**), R_t = 6.1 min, 5-bromovinyluracil (**13**, X = CHCHBr), R_t = 18.59 min, 5-bromovinyl-2′-deoxyuridine (**18**), R_t = 19.67 min.

2.8. Recycling of the biocatalysts

Recycling of immobilized *Ec*TP or *Bs*PyNP was performed by evaluating the synthesis of thymidine (**2**) by transglycosylation, in the same conditions described in Section 2.7. When the highest conversion was achieved, the reaction mixture was filtered under reduced pressure and the immobilized biocatalyst was re-used for the next reaction.

3. Results and discussion

The aim of this work was the synthesis of some 5-halogenated pyrimidine nucleosides of medicinal interest (**14–18**) through a "one-enzyme, one-pot" transglycosylation in fully aqueous medium. We selected immobilized *Bs*PyNP from *B. subtilis* and *Ec*TP from *E. coli* as the biocatalysts, which have been shown to display promising properties in terms of activity and stability [17,19,20] when assayed in nucleoside biotransformation.

3.1. Screening of sugar donor (*1*–*6*) (phosphorolysis)

Target compounds of transglycosylation reactions are all 2′-deoxyribonucleosides (**14–18**, see Scheme 2). Therefore, the first issue was to select a pyrimidine 2′-deoxynucleoside as the sugar donor. 2′-Deoxyuridine (**1**) and thymidine (**2**) were thus assayed in phosphorolysis (Scheme 1) since they are the natural substrates of *Bs*PyNP and *Ec*TP, respectively. Besides, both these compounds can be easily synthesized at a moderate price resulting in affordable starting material for synthetic applications.

As expected, *Bs*PyNP and *Ec*TP are able to convert both 2′-deoxyuridine (**1**) and thymidine (**2**) into the parent nucleobases. However, it is worth noting that for both substrates the rate of the phosphorolysis (v_p) of *Ec*TP is 2.5-fold higher than that of *Bs*PyNP: being the equilibrium-controlled conversion comparable (**1**) or the same (**2**), the higher bioconversion rate of *Ec*TP can be advantageous in terms of reaction time and enzyme stability.

Unnatural sugar-modified nucleosides (**4–6**) were also tested as substrates in the phosphorolysis reaction. 5′-Deoxyuridine (**4**), arabinosyluracil (**5**) and 2′,3′-dideoxyuridine (**6**) are valuable sugar donor to synthesize nucleoside analogues used as antiviral or antitumour drugs, either through a "one-enzyme" transglycosylation, or following a "two-enzyme" synthetic sequence. However, both *Bs*PyNP and *Ec*TP displayed a narrow substrate specificity since their ability to cleave the glycosidic bond was restricted to 2′-deoxyribonucleosides (**1** and **2**), uridine (**3**) and 5′-deoxyuridine (**4**) (Table 1). Inversion of the rate of phosphorolysis (v_p) of *Bs*PyNP and *Ec*TP for substrates **3** and **4** is consistent with the evidence that,

Table 1
Phosphorolysis of sugar donor nucleosides (**1–6**) catalyzed by immobilized *Bs*PyNP or *Ec*TP.

Sugar donor	*Bs*PyNP (v_p)	*Ec*TP (v_p)
1	70 (3.6)	60 (9.0)
2	62 (2.6)	60 (7.1)
3	66 (3.7)	51 (0.5)
4	50 (3.4)	50 (0.7)
5	0 (–)	0 (–)
6	0 (–)	0 (–)

Percentage of produced nucleobases after 24 h and reaction rate (v_p, $\mu mol\ min^{-1}\ g^{-1}$) are reported. Experimental conditions: 50 mM phosphate buffer, room temperature, substrate (**1–6**) = 5 mM, volume = 10 mL, *Bs*PyNP or *Ec*TP = 1 IU.

1, 2′-deoxyuridine; **2**, thymidine; **3**, uridine; **4**, 5′-deoxyuridine; **5**, arabinosyluracil; **6**, 2′,3′-dideoxyuridine; *Bs*PyNP, pyrimidine nucleoside phosphorylase from *Bacillus subtilis*; *Ec*TP, thymidine phosphorylase from *Escherichia coli*; v_p, rate of phosphorolysis.
All experiments were performed as duplicate.

differently from TPs, PyNPs do not discriminate at the 2-position of the ribose [12].

3.2. Synthesis of 5-halogenated pyrimidine nucleosides **14**–**18** (transglycosylation)

Substrate specificity of *Bs*PyNP and *Ec*TP against 5-halogenated pyrimidines (**9**–**13**, see Scheme 2) was evaluated through the transglycosylation reaction using 2′-deoxyuridine (**1**) as the sugar donor. According to the results of Table 1, regardless the used biocatalyst, phosphorolysis of **1** was higher or similarly yielding than the bioconversion of thymidine (**2**) and proceeded at a higher rate.

The reactions were performed by using a 2:1 molar ratio of 2′-deoxyuridine (**1**) in order to generate a slight excess of 2-deoxyribose-1-phosphate for the following coupling with the nucleobase (**9**–**13**). Whereas in the phosphorolysis the phosphate concentration was 10-fold higher than the nucleoside concentration (50 mM vs 5 mM), transglycosylations were performed by using a equimolar ratio of phosphate and the nucleobase (10 mM). In fact, in this case, phosphate acts as a substrate in the first step of the bioconversion (phosphorolysis) but it then becomes a reaction product being regenerated once the coupling between the nucleobase and the intermediate sugar-1-phosphate has occurred [24].

All the tested nucleobases (**9**–**13**) were converted into the corresponding 2′-deoxyribonucleosides (**14**–**18**) (Scheme 2). No striking differences between *Bs*PyNP and *Ec*TP were observed about the percentage of conversion of (**16**) and (**17**), but for the fluorinated compounds (**14** and **15**) and brivudin (**18**). *Bs*PyNP-Catalyzed synthesis of floxuridine (**14**) and trifluridine (**15**) afforded slightly higher conversion (68% and 57%, respectively) than *Ec*TP (62% and 51%). The overall low conversion of **13**, associated to both the biocatalysts, might be ascribed to the steric hinderance of the bulky 5-substituent, in agreement with previous reports [22].

The rate of synthesis (v_s, Scheme 2) emerged as the main difference between the two biocatalysts. Bioconversions were generally faster when the biocatalyst was *Ec*TP.

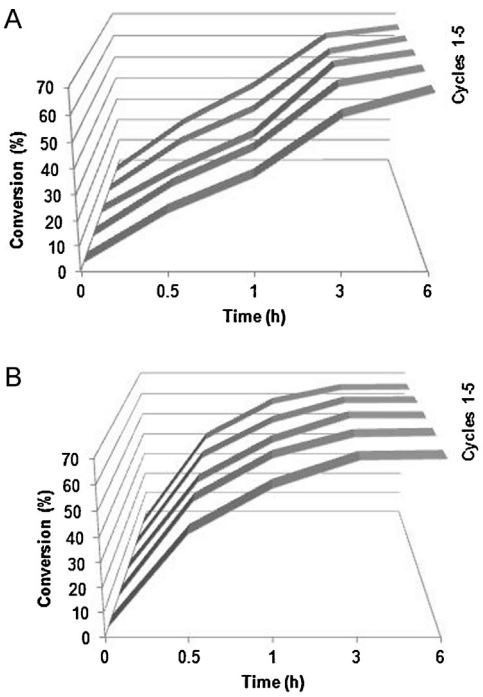
3.3. Recycling of the biocatalysts

Immobilized enzymes offer both technical and economical advantages over their soluble counterparts [25], one of them being the possibility to develop a continuous process. Immobilized *Bs*PyNP and *Ec*TP have been here tested in the *in continuum* synthesis of thymidine (**2**) by transglycosylation. To this aim, each biocatalyst was incubated in the reaction mixture containing 2′-deoxyuridine (**1**) and thymine (**8**) in the standard conditions used for the transglycosylation studies (2:1 molar ratio, 10 mM phosphate buffer pH 7.5, r.t., see Section 2). When the highest conversion was achieved, the mixture was separated from the biocatalyst *via* a simple filtration and the immobilized enzyme was then re-used for a new bioconversion. As depicted in Fig. 1, both biocatalysts retained all of their activity after 5 reactions showing optimal stability and recyclability.

No significant loss of activity was observed both for *Bs*PyNP and *Ec*TP after 5 cycles thus indicating that these biocatalysts could be re-used for additional reactions.

3.4. Optimization and scale-up of floxuridine (**14**)

The synthesis of floxuridine (**14**) was further investigated with the aim to highlight the influence of reaction parameters such as pH and substrate concentration on the percentage of bioconversion. We opted for *Bs*PyNP as the biocatalyst because the conversion of **9** into **14** was slightly higher compared to *Ec*TP: percentage of bioconversion is reasonably privileged over reaction rate in the optimization of a synthetic process. Moreover, *B. subtilis* was



Experimental conditions: 10 mM phosphate buffer, 20 mM 2'-deoxyuridine (1), 10 mM thymine (8), pH 7.5, room temperature, volume= 10 mL, *Bs*PyNP and *Ec*TP= 10 IU.

Fig. 1. Cycles of transglycosylation reaction catalyzed by immobilized *Bs*PyNP and *Ec*TP (panel A and B, respectively).

fermented till up 15 L and *Bs*PyNP could be therefore readily available in large amount. The study of fermentation and purification conditions allowed the production of *Bs*PyNP on a pre-industrial scale with low production cost and good results for that concerning yield and quality of the purified enzyme.

As reported in Table 2, the highest conversions were achieved in the pH range 7.5–10, whereas a weakly acid pH was detrimental for the reaction outcome; even if the use of strongly basic pH appeared to be eligible, the optimal compromise between percentage of conversion, reaction rate and long term stability of the biocatalyst was pH 7.5 which was therefore set in the next study on the effect of substrate concentration (phosphate, nucleobase and nucleoside).

According to data reported in Table 3, a decrease of phosphate concentration from 10 to 1 mM (entries 1–3) did not exert any effect on the final conversion but exclusively on the initial rate of the reaction. Neither the conversion, nor the rate of synthesis were affected when substrate concentration was increased from 20 and 10 mM to 80 and 40 mM for the sugar donor and the nucleobase, respectively: as mentioned above (Section 3.2) the *in situ* phosphate regeneration following the glycosylation step concurs to flatten the influence of the buffer concentration (5 mM or 10 mM, entries 4–5).

Table 2
Effect of pH on the synthesis of floxuridine (**14**) catalyzed by immobilized *Bs*PyNP (see Scheme 2).

pH	v_s (μmol min^{-1} g^{-1})	Conversion (%)
6	0.07	40
7	1.6	63
7.5	1.7	68
9	1.5	70
10[a]	1.3	70

Experimental conditions: 10 mM phosphate buffer, room temperature, substrate (**1**) = 20 mM, 5-fluorouracil (**9**) = 10 mM, volume = 10 mL, *Bs*PyNP = 1 IU. Conversions were recorded after 24 h. All experiments were performed as duplicate.

[a] Phosphate buffer was additioned with 10 mM K_2CO_3.

Table 3
Effect of substrate concentration on the synthesis of floxuridine (**14**) catalyzed by immobilized *Bs*PyNP or *Ec*TP (see Scheme 2).

Entry	Enzyme	**1** (mM)	**9** (mM)	Phosphate buffer (mM)	v_s (μmol min^{-1} g^{-1})	Conversion (%)
1	*Bs*PyNP	20	10	1	1.0	68
2	*Bs*PyNP	20	10	5	1.6	68
3	*Bs*PyNP	20	10	10	1.7	68
4	*Bs*PyNP	80	40	5	1.5	70
5	*Bs*PyNP	80	40	10	1.6	69
6	*Bs*PyNP	150	50	10	2.2	76
7	*Ec*TP	80	40	10	2.1	65
8	*Ec*TP	150	50	10	5.7	73

Experimental conditions: phosphate buffer pH 7.5, room temperature, volume = 10 mL, *Bs*PyNP or *Ec*TP = 10 IU. The reaction was monitored till 10 h. All experiments were performed as duplicate.

Mostly interesting for a preparative application is the effect of a higher substrate concentration (150 mM) and molar ratio (3:1) of the nucleoside donor (entries 6 and 8): as expected in a controlled-equilibrium biotransformation, both the yield and the rate of synthesis resulted positively affected. Immobilized *Bs*PyNP and *Ec*TP, compared in these conditions, catalyzed the synthesis of **14** with a similar conversion (76% and 73%, respectively) but different rate of synthesis, being *Ec*TP, once again, 2.5-fold faster than *Bs*PyNP. Starting from 50 mM of **9** (6.5 g/L), the theoretical volumetric yield of **14** is about 9 g/L, produced in about 3 h in fully aqueous medium and mild conditions (room temperature and pH 7.5).

3.5. Stability of immobilized BsPyNP and EcTP in the presence of cosolvents

The optimization of a synthetic process, mainly aimed at achieving the highest conversion and volumetric yield, requires that a biocatalyst is sufficiently stable to tolerate a wide range of experimental conditions. Immobilized *Bs*PyNP and *Ec*TP were shown to be very stable at pH 10 and 45 °C or 37 °C, respectively [19,20]. These conditions enhance the solubility of 5-halogenated bases (**9**–**13**) and their 2′-deoxyribonucleoside counterparts (**14**–**18**) in aqueous buffer but are frequently not sufficient to achieve suitable concentrations (g of product per litre of reaction) for in batch product downstream. 5-Halogenated nucleobases and nucleosides **15**–**18**, other than floxuridine (**14**), do possess this limitation. A moderate content of organic solvent can assist in the reaction and work-up thereof. Therefore, stability of immobilized *Bs*PyNP and *Ec*TP was assayed in acetonitrile (MeCN) and *N*,*N*-dimethylformamide (DMF) which are some of the most commonly used solvents for nucleoside reactions [8]. As depicted in Fig. 2, both immobilized enzymes maintained more than 65% of their activity after 24 h incubation regardless the solvent used.

Surprisingly, the residual activity in DMF of both the soluble enzymes was slightly higher than that of the parent immobilized biocatalyst. The sensitively improved stability of the immobilized preparations with respect to the native enzymes emerged upon incubation in MeCN: differently from DMF, MeCN exerted a detrimental effect on the soluble proteins, particularly in the case of *Ec*TP.

From an applicative viewpoint, however, both solvents seem to be useful to perform a transglycosylation scale-up catalyzed by immobilized *Bs*PyNP or *Ec*TP. In the case of DMF, being stability of soluble and immobilized enzymes similar, the use of the immobilized preparation is however to be preferred.

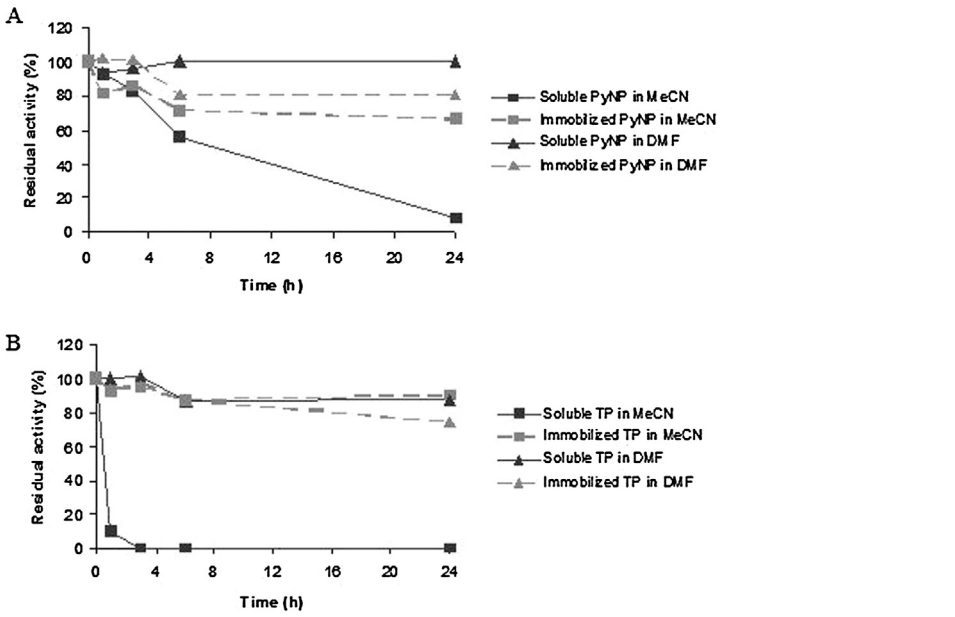


Fig. 2. Stability of immobilized *Bs*PyNP (panel A) and *Ec*TP (panel B) in presence of organic cosolvents.

4. Conclusions

On account of the overall simplicity, nucleoside synthesis through transglycosylation appears superior to chemical methods. Particularly, immobilized *Bs*PyNP and *Ec*TP were shown to catalyze the synthesis of 5-halogenated-2′-deoxynucleosides (**14**–**18**) in fully aqueous medium in moderate to quite high conversion. From the comparative investigation of *Bs*PyNP and *Ec*TP, a significative difference did not emerge, neither considering the substrate specificity in phosphorolysis, nor the performance in the studied transglycosylations.

Both enzymes catalyze the phosphorolysis of uracil-based 2′-deoxy-, 5′-deoxy- and ribonucleosides but are not able to cleave the C1′—N bond of arabino- or 2′,3′-dideoxyribosides. However, *Bs*PyNP and *Ec*TP complement one another if phosphorolysis rate is considered: the higher is the reaction rate of *Ec*TP against natural 2′-deoxyribonucleosides (**1** and **2**), whilst the faster is the *Bs*PyNP-catalyzed bioconversion of uridine (**3**) and 5′-deoxyuridine (**4**).

Transglycosylations proceed at a higher rate when *Ec*TP is used as biocatalyst. The same behaviour was registered when the synthesis of floxuridine was studied and scaled-up. Both biocatalysts catalyzed the synthesis of this nucleoside in more than 70% conversion when a 3:1 molar ratio of nucleoside donor was used, at pH 7.5 and in fully aqueous medium.

Upon standard conditions used for transglycosylation (10 mM phosphate buffer, pH 7.5, r.t., nucleoside *donor*/base *acceptor* ratio 2:1), both biocatalysts remained highly productive after 5 cycles. The optimal stability shown by both immobilized enzymes also in DMF and MeCN as cosolvents will assist in further scaling-up the synthesis of 5-halogenated pyrimidine 2′-deoxyribonucleosides.

Acknowledgments

We thank Dr. Milena Schiraldi and Lucia Di Gennaro for their help in the experimental, Dr. Giorgio Marrubini for helpful discussion and Dr. Caterina Temporini for English revision. This work was funded by Regione Lombardia (Metadistretti Call 2007, ID 4165).

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