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Development of an immobilized biocatalyst based on *Bacillus psychrosaccharolyticus* NDT for the preparative synthesis of trifluridine and decytabine

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ABSTRACT

The immobilization of *Bacillus psychrosaccharolyticus* nucleoside 2′-deoxyribosyltransferase was deeply investigated and finally optimized. The best immobilization procedure resulted to be ionic adsorption on PEI 600 Da agarose followed by crosslinking with 70% oxidized dextran (20 kDa). The percentage of recovered activity was further improved (from 21% to 33%) by the addition of 20% glycerol to the immobilization mixture. The resulting biocatalyst showed a stability profile similar to that of the soluble enzyme and it was used for the preparative synthesis of trifluridine and decytabine obtaining conversions ranging from 50% to 76%.

1. Introduction

The use of glycosyl-transferring enzymes applied to the synthesis of modified nucleosides is an established procedure endowed with several advantages over the traditional multistep chemical methods [1–3]. These advantages arise from the inherent properties of enzymes such as stereo-, regio-, enantiospecificity and mild reaction conditions.

To this aim, the use of nucleoside phosphorylases (NPs) has been extensively described [4–7]. These enzymes are used to catalyze, in presence of inorganic phosphate, the transfer of the sugar moiety from a sugar donor nucleoside to a sugar acceptor base leading to the production of a second nucleoside through a two-step reaction. Moreover, several NPs from different sources have been also conveniently immobilized by following various approaches and obtaining, in most of cases, robust biocatalysts for preparative reactions [5,8–10]. However, the poor ability of NPs to recognize cytosine derivatives as substrates [11] and, often, the need to follow a two-enzyme one-pot scheme (PyNP-PNP or UP-PNP) to synthesize purine nucleosides, represent the main obstacle to their broad application. In particular, this last issue is a consequence of the fact that the thermodynamic equilibrium for PNP, but not for PyNP/UP is shifted towards the nucleoside synthesis [12].

More recently, a second class of enzymes that mediate the glycosyl transfer is gaining importance in the field of nucleoside synthesis [13–15]. N-Deoxyribosyltransferases (NDTs), in fact, catalyze the direct transfer of the 2′-deoxyribosyl moiety from a 2′-deoxynucleoside donor to a nucleobase acceptor through a one-step reaction [16,17]. NDTs have been divided into two classes depending on their substrate specificity. In particular, type I NDT (also known as PDT) exclusively catalyzes the purine–purine transfer, whereas type II NDT catalyzes the transfer between pyrimidines and/or purines and also accepts cytosine derivatives as substrates [12]. Taking into account the aforementioned properties, it clearly appears that NDTs are an important completion to NPs in the enzyme toolset aimed at the enzymatic synthesis of nucleoside analogues. In fact, the use of type II NDTs allows not only to obtain cytidine analogues but also to carry out the reaction in a one-enzyme one-pot mode.

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Immobilization often represents a key point to make a step forward in the view of industrial application, since it provides the biocatalyst with enhanced stability and recyclability. Differently from NPs, immobilization of NDTs is still little studied and only a few examples have been reported to date [15,18–20].

Recently, a new NDT from *Bacillus psychrosaccharolyticus* (*Bp*NDT) has been cloned, produced and characterized [19]. *Bp*NDT can be considered a valuable enzyme since it holds several beneficial properties, such as psychrophilic behavior coupled with good thermal and pH stability.

In order to obtain an improved biocatalyst for preparative purposes, a first attempt to immobilize this enzyme has been also reported in the same study. Immobilization of *Bp*NDT by direct covalent reaction with aldehyde activated carriers caused a complete loss of the enzyme activity. Results were slightly improved using the approach already described for other multimeric enzymes: adsorption on a strong anionic exchange carrier (agarose coated with PEI) followed by crosslinking with aldehyde-dextran [8–10].

This strategy also allows overcoming an important drawback associated to the immobilization of multimeric enzymes. In fact, multimeric enzymes can undergo subunit dissociation if they are not properly linked to the support, causing enzyme inactivation and product contamination. Conversely, the promotion not only of the attachment to the support but also inter-subunit bonds (with a crosslinking agent such as dextran) allows the covalent linkage of all the enzyme subunits onto the carrier [21].

Even in this case, *Bp*NDT activity was almost lost during the covalent crosslinking. On the other hand, when the crosslinking degree was reduced, the enzyme preserved a good activity, in spite of very poor stability, lower than that of the soluble enzyme [19].

The best derivative in terms of stability was achieved when the enzyme was immobilized on 600 Da PEI-agarose and crosslinked with dextran (20 kDa), oxidized at 70%. Nevertheless, the recovered activity at the end of the immobilization process was too low even when the immobilization time was reduced to one hour (about 20% of recovered activity). This made the biocatalyst poorly exploitable for preparative reactions. In fact, the synthesis of trifluridine was carried out at substrate concentration not higher than 20 mM [19].

In the present work, in order to achieve an immobilized derivative with suitable activity and stability to be used in preparative processes, immobilization of *Bp*NDT was optimized. In particular, the aim of this study was to modulate the process to ensure a high crosslinking degree (that assures a suitable stability of the enzyme preparation) but avoiding an important distortion of the 3D structure that reduces the recovered enzymatic activity after immobilization.

To this purpose, different factors that could influence the immobilization outcome were taken into account. First, we evaluated the effect of the reduction with $NaBH_4$, comparing the derivative obtained by crosslinking (before reduction of the imino bonds) with that previously obtained with the complete procedure (crosslinking and reduction).

Afterwards, we investigated the influence on the immobilization process exerted by different variables including the nature of the carrier, the composition of the reaction medium (*i.e.* addition of protective agents in order to reduce the denaturation of the enzyme) and the size of PEI and dextran.

Finally, the best enzyme derivative (in terms of both activity and stability), obtained through the optimized immobilization process, was used for the synthesis of 5-trifluorothymidine (trifluridine). Substrate concentrations used were two-fold higher than previously reported [19], in order to evaluate the performance of the selected biocatalyst in similar conditions to those required in preparative processes. In addition, the synthesis of 5-aza-2′-deoxycytidine (decytabine) was also performed.

Both trifluridine and decytabine are commercially important drugs with antiviral and antitumoral activities. 5-Trifluorothymidine, commercially known as trifluridine, is an antiviral agent employed in ophthalmic solutions for the treatment of herpes virus simple (HVS) [22]. This compound can also induce double-strand DNA breaks and inhibit the thymidylate synthase when it is transformed to its phosphorylated form, displaying antitumoral activity [23]. The combination of this compound with an inhibitor of the thymidine phosphorylase, to inhibit degradation of trifluridine, is currently undergoing a Phase III clinical trial with patients with refractory metastatic colorectal cancer (NCT01607957) [24].

Additionally, 5-aza-2′-deoxycytidine, decytabine, has been approved for use in the treatment of myelodysplastic syndromes. It can be incorporated into DNA when it is converted to aza-dCTP and produce the inhibition of DNA methylation, leading to enhanced gene expression and the consequently activation of repressed genes [25].

2. Materials and methods

2.1. Chemicals

2′-Deoxyuridine (dUrd) was a gift from Pro.Bio.Sint-Euticals (Varese, Italy) whereas adenine (Ade) was purchased from Sigma. 5-Trifluorothymine (5-tFThy), 5-trifluorothymidine (5-tFdThd), 5-azacytosine (5-azaCyt) and 5-aza-2′-deoxycytidine (5-azaCyd) were from Carbosynth Ltd. (Berkshire, UK). Crosslinked 6% agarose beads (Sepharose 6BCL) were from Amersham Biosciences AB (Uppsala, Sweden). Epoxy-activated Sepabeads® (EC-EP) were kindly donated by Resindion s.r.l. (Binasco, Milano, Italy). Branched polyethyleneimine (PEI) with 600 Da or 25,000 Da molecular mass, dextran with 20,000 Da or 100,000 Da molecular mass and PEG 600 Da were from Sigma-Aldrich (Milano, Italy). All other reagents and solvents (HPLC grade) were purchased from Sigma–Aldrich (Milano, Italy).

2.2. Enzyme preparation

NDT from *B. psychrosaccharolyticus* was prepared as previously reported [19]. Briefly, the *ndt* gene encoding *Bp*NDT was amplified by PCR using chromosomal DNA from *B. psychrosaccharolyticus* CECT 4074 as a template, cloned into the expression vector pET28a(+), and the recombinant plasmid was used to transform *Escherichia coli* BL21 (DE3) cells. After that, the overproduction of *Bp*NDT was carried out by the induction of the culture of *E. coli* BL21 (DE3) cells harboring pET28*Bpndt* (0.6 OD_{600nm}) with 0.5 mM IPTG for 2.5 h.

The recombinant *Bp*NDT was purified from the cell extract by three chromatographic steps consisting of an anionic-exchange chromatography, a molecular size exclusion chromatography, and an isofocusing chromatography. The protein fractions containing *Bp*NDT were detected by SDS-PAGE analysis [19].

2.3. Preparation of polyethyleneimine-functionalized supports and aldehyde-dextran

Functionalization of epoxy-activated Sepabeads was performed as previously described [8]. Briefly, the support (1 g) was suspended in 1 M NaCl solution pH 11 (12.6 mL) containing 10% (v/v) of PEI (MW 600 Da or 25,000 Da). The suspension was kept under mechanical stirring for 24 h at room temperature and then the support was filtered and washed with 1 M NaCl and deionized water.

PEI-functionalized agarose was obtained as formerly described [21]. Briefly, aldehyde-agarose (1 g), was suspended in 1 M NaCl solution pH 11 (12.6 mL) containing 10% (v/v) of PEI (MW 600). The

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reaction mixture was kept under mechanical stirring for 3 h at room temperature. $NaBH_4$ (57 mg) was then added and the reduction reaction was carried out for 2 h at room temperature. The activated support was washed with phosphate buffer pH 4 and deionized water.

Likewise, aldehyde-dextran was prepared as previously reported [9,26,27]. In particular, 1.67 g of dextran were suspended in 50 mL of deionized water and 3.04 g, 2.18 g, 0.87 g of $NaIO_4$ were added to obtain 70%, 50% and 20% oxidation degree, respectively. The reaction was carried out for 2 h at room temperature. The mixture was then dialyzed overnight against deionized water and stored at −20 °C.

2.4. Bp*NDT immobilization on PEI-coated Sepabeads and PEI-coated agarose*

In a total volume of 14 mL, the activated support (1 g) was suspended in 5 mM phosphate buffer at pH 7.5 containing the desired amount of enzyme and kept under mechanical stirring for 1 h. The immobilized preparation was filtered and washed with deionized water. The percentage of recovered activity was calculated as following: (recovered activity/loaded activity) × 100.

2.5. *Crosslinking of immobilized* Bp*NDT with aldehyde-dextran*

An amount of 0.71 mL of 20 kDa or 100 kDa aldehyde-dextran (10%, v/v) was added to the aforementioned immobilization suspension (section 2.3). After stirring (1–4 h), pH was adjusted to 10 and $NaBH_4$ was added (1 mg/mL suspension). After 30 min of reduction, immobilized *Bp*NDT was washed twice with 50 mM potassium phosphate buffer pH 4.5 and deionized water. Finally, immobilized biocatalyst was stored at 4 °C prior to use.

2.6. *N-Deoxyribosyltransferase assay for soluble and immobilized* Bp*NDT*

Standard activity assay of immobilized enzyme was carried out as previously described [19]. The standard activity assay using soluble *Bp*NDT was started by the addition of 4 μg of pure enzyme to a 400 μL solution containing 10 mM 2′-deoxyuridine and 10 mM adenine in 50 mM HEPES buffer, pH 8.0. Reaction was performed as formerly reported [19].

In both cases samples were analyzed by HPLC (Agilent 1100 series) to quantitatively measure the production of nucleosides at 254 nm with a 5 μm C18-PFP column 250 mm × 46 mm (ACE, Advanced Chromatography Technologies) equilibrated with 100% trimethyl ammonium acetate at a flow rate of 1 mL/min. Elution was carried out by a discontinuous gradient: from 0 to 10 min, the mobile phase changed progressively from 100% trimethyl ammonium acetate and 0% of acetonitrile to 90% of trimethyl ammonium acetate and 10% of acetonitrile; and from 10 to 20 min, the mobile phase reverted to initial conditions, from 90% to 100% trimethyl ammonium acetate and from 10% to 0% acetonitrile. Retention times for the reference natural compounds were as follows: uracil (Ura): 5.41 min; 2′-deoxyuridine (dUrd): 9.16 min; adenine (Ade): 10.14 min; 2′-deoxyadenosine (dAdo): 15.50 min. All determinations were carried out by triplicate and the standard deviation was below 5%. One international activity unit (IU) was defined as the amount of enzyme producing 1 μmol/min of 2′-deoxyadenosine under assay conditions.

2.7. *Stability of* Bp*NDT at pH 10*

Three hundred mg of immobilized *Bp*NDT were incubated with 2 mL of 50 mM carbonate pH 10.0 for 24 h at 25 °C. 200 μL of suspension were withdrawn at different incubation times, and activity was measured by the standard assay.

On the other hand, 160 μg of soluble *Bp*NDT were added to 2 mL of 50 mM carbonate pH 10.0 and incubated for 24 h at 25 °C. At different incubation times, 0.40 μg of enzyme were withdrawn and activity was measured by the standard assay.

2.8. *Stability of* Bp*NDT in 20% of dimethylformamide*

Stability in presence of 20% of dimethylformamide was evaluated incubating 300 mg of immobilized enzyme in 2 mL of 50 mM HEPES pH 8.0 containing 20% DMF for 48 h at 37 °C. At different incubation times, 200 μL of suspension were withdrawn and activity was measured by the standard assay.

2.9. *Enzymatic synthesis of trifluridine and decytabine*

Nucleoside analogues synthesis was performed by adding 100 mg of immobilized biocatalyst (displaying 1.8 IU with the standard assay) to 5 mL of 10 mM potassium phosphate buffer pH 7.5 with different concentrations of trifluorothymine and 2′-deoxyuridine (from 10 to 40 mM), for 5-trifluorothymidine synthesis (trifluridine), or 5-azacytosine and 2′-deoxyuridine (from 10 to 20 mM), for 5-aza-2′-deoxycytidine synthesis (decytabine). Reactions were performed at 37 °C for 3 h and, at different reaction times, samples were withdrawn and filtered off using a pipette filter device. Afterwards, the supernatant was analyzed by HPLC to quantitatively measure the reaction products as aforementioned (section 2.6. of Materials and methods). Retention times were: uracil (Ura): 5.41 min; 2′-deoxyuridine (dUrd): 9.16 min; 5-trifluorothymine (5-tFThy): 8.0 min; 5-trifluorothymidine (5-tFThd): 13.5 min; 5-azacytosine (5-azaCyt): 4.5 min; 5-aza-2′-deoxycytidine (5-azadCyd): 8.9 min. The produced nucleoside was identified by comparison of its HPLC retention time with that of authentic samples [10].

3. Results and discussion

3.1. *Immobilization of recombinant* Bp*NDT on PEI-functionalized supports*

In order to immobilize recombinant *Bp*NDT maintaining its enzymatic activity, ionic adsorption of the enzyme to PEI-functionalized supports was carried out. In this sense, glyoxyl-agarose [28] and Sepabeads [29] were functionalized by two different PEI [30] (600 or 25,000 Da molecular weight). As shown in Table 1, when 600 Da PEI was used to coat both types of carriers, the recovered activity was not affected by the immobilization process. The immobilization yield was not even influenced by the quantity of enzyme loaded on the activated support (Table 1). When PEI 25,000 Da was employed to functionalize aldehyde-agarose, the percentage of recovered activity of immobilized *Bp*NDT diminished to 28%.

Functionalized supports with 600 Da PEI were then chosen for further stabilization since immobilized *Bp*NDT obtained by mere ionic adsorption to functionalized supports can suffer protein leakage, causing loss of activity and product contamination [31]. This drawback can be overcome by post-immobilization techniques, such as crosslinking with a bifunctional or polyfunctional polymer [32]. In this case, *Bp*NDT immobilized on PEI-agarose and PEI-Sepabeads were crosslinked with aldehyde-dextran, a polyaldehyde macromolecule obtained by oxidation with $NaIO_4$ [33]. Different oxidation degrees (meaning a different density of aldehyde groups) can be obtained depending on the amount of periodate used for oxidation [9,34].

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Table 1
Immobilization of *Bp*NDT on PEI-functionalized carriers.

Offered protein per gram of carrier (mg)	Offered activity per gram of carrier (IU)	Functionalized carrier	Molecular weight of PEI (Da)	Final activity (UI/g wet carrier)	Recovered activity (%)[a]
0.9	11.3 [19]	PEI agarose	600	11.3	100
0.5	6.1	PEI agarose	600	6.1	100
0.7	9.2	PEI agarose	25,000	2.6	28.3
1.0	12.0	PEI Sepabeads	600	11.8	98.0

[a] (Activity of immobilized *Bp*NDT/loaded activity) × 100.

The aldehyde-dextran can react with both free amino groups of the enzyme and PEI-coated support, resulting in a covalent multipoint attachment between the different protein subunits and the carrier. The number of bonds obtained during crosslinking depends on the dextran oxidation degree as well as on the reaction time. However, the formed imino bonds are not stable, but can be finally reduced with $NaBH_4$ to yield carbon-nitrogen irreversible covalent bonds [32,35].

The recovered activity of *Bp*NDT immobilized by ionic adsorption on 600 Da PEI-agarose and further crosslinked with 20% oxidized 20 kDa dextran was 38%, twice the recovered activity when 600 Da PEI-Sepabeads was used under the same crosslinking conditions (Table 2, entries 1 and 2). For this reason, 600 Da PEI-agarose was used for further optimization of the immobilization process. The effect of dextran oxidation degree and crosslinking time exerted on the immobilization process was investigated using 20 kDa dextran.

As the dextran oxidation level is increased, the number of aldehyde groups is incremented, therefore, an increased number of covalent bonds are produced during the crosslinking step. This confers rigidity to the protein 3D structure and allows less conformational changes resulting, thus, in high stability of the immobilized and crosslinked enzyme. Nevertheless, an increased rigidity of the enzyme structure could be also responsible for a reduced activity after immobilization.

In order to better understand the effect produced on the enzyme activity by the crosslinking process, two kinds of experiments were performed (Fig. 1):

- Preparation of different enzyme derivatives by adsorption on PEI carrier and incubation of the immobilized enzyme with dextran for diverse periods of time, avoiding the final reduction.
- Preparation of different derivatives by crosslinking of the PEI immobilized enzyme with dextran for diverse periods of time, followed by 30 min reduction.

As depicted in Fig. 1a, the crosslinking time appeared to be a crucial factor for determining the effect of such crosslinking on the enzyme activity. In fact, a progressive reduction of the recovered activity was observed when incubation time was increased, according to a time dependent increased number of covalent bonds [19]. However, this effect was only detected after the $NaBH_4$ reduction step, when the reversible imino bonds were transformed into covalent bonds (Fig. 1a) and it was much more evident when 70% oxidized dextran was used compared with 50% oxidized dextran (Fig. 1a). In fact, although the activity was completely retained up to 4 h of crosslinking, the derivative prepared with 50% oxidized dextran maintained about 40% of activity when the reduction was carried out after 2 h of crosslinking, whereas it lost almost completely its activity when the reduction was performed after 4 h of incubation.

Conversely, in similar conditions, the derivative prepared with 70% oxidized dextran lost almost completely its activity even when the reduction was performed after 2 h, as a consequence of the high number of covalent bonds produced.

These results can be confirmed following the residual activity of the suspension during the whole crosslinking process (Fig. 1b). In fact, the activity is completely maintained during the interaction with both the crosslinking agents (oxidized at 50% and 70%).

Similar results were obtained using 20% oxidized dextran, but the final residual activity (after reduction) was higher compared with the other derivatives (Fig. 1b).

The progressive loss of activity observed depending on the time of incubation and on the dextran oxidation degree confirms a distortion of the enzyme 3D structure, induced by the increased number of covalent bonds.

In order to minimize the effect of 3D structure distortion, protective agents such as 2′-deoxyuridine, glycerol and polyethylene glycol (PEG) were added to preserve the enzyme active site during the crosslinking process.

The use of substrates or inhibitors [36], as well as polyols [37], has been previously reported for preserving the 3D structure of several enzymes during immobilization, including multimeric nucleoside phosphorylases [5]. The interaction with these additives allows the enzyme to maintain a compact conformation and, thus, reduces the distortion of the 3D structure occurring during covalent immobilization or crosslinking.

In the case of *Bp*NDT, the addition of dUrd was not effective, but the addition of 20% glycerol to the immobilization medium increased the final activity (expressed as % of recovered activity

Table 2
Immobilization of *Bp*NDT on PEI-functionalized carriers and crosslinking with aldehyde-dextran (MM 20/100 kDa).

Entry	Offered protein per gram of carrier (mg)	Offered activity (IU/g carrier)	Functionalized carrier	Dextran (kDa); oxidation degree (%)	Final (IU/g support); recovered activity (%)
1	1.0	12.0	PEI 600 Sepabeads	20; 20	2.2; 18.7
2 [19]	0.5	6.1	PEI 600 Agarose	20; 20	2.3; 37.7
3 [19]	0.5	6.1	PEI 600 Agarose	20; 50	2.2; 36.1
4 [19]	0.7	8.7	PEI 600 Agarose	20; 70	1.8; 20.7
5[a]	0.7	8.7	PEI 600 Agarose	20; 70	1.1; 13.3
6[b]	0.7	8.7	PEI 600 Agarose	20; 70	2.8; 32.8
7[c]	0.7	8.7	PEI 600 Agarose	20; 70	2.6; 30.0
8	0.7	8.7	PEI 600 Agarose	100; 20	4.6; 53.1
9	0.5	6.1	PEI 600 Agarose	100; 50	0.2; 2.9
10	0.5	6.1	PEI 600 Agarose	100; 70	0.25; 4.2
11[b]	2.9	34.8	PEI 600 Agarose	20; 70	19.5; 56

Crosslinking time: 1 h, followed by 30 min reduction; [a]dUrd (5 mM); [b]glycerol (20%); [c]PEG 600 Da (20%).

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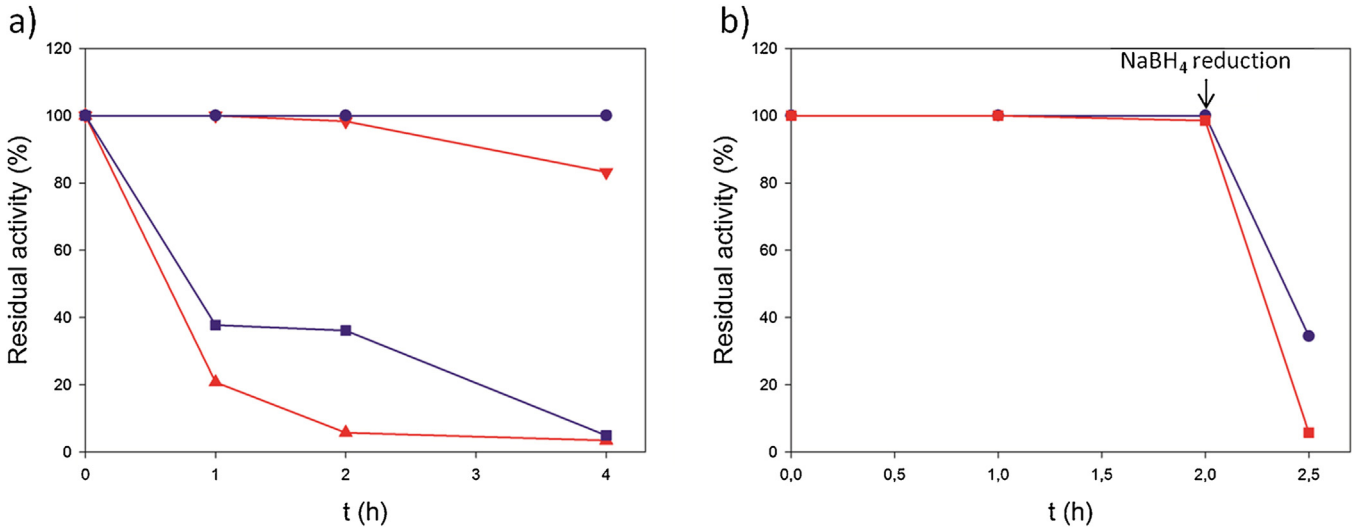

Fig. 1. Panel (a): Immobilized *Bp*NDT on 600 Da PEI agarose followed by crosslinking with 50% oxidized 20 kDa dextran: ● after crosslinking; ■ after crosslinking and 30 min reduction. Immobilized *Bp*NDT on 600 Da PEI agarose followed by crosslinking with 70% oxidized 20 kDa dextran: ▼ after crosslinking; ▲ after crosslinking and 30 min reduction. Panel (b): Crosslinking time and reduction dependence of immobilized *Bp*NDT. ● Immobilized *Bp*NDT on 600 Da PEI agarose followed by crosslinking with 50% oxidized 20 kDa dextran; ■ Immobilized *Bp*NDT on 600 Da PEI agarose followed by crosslinking with 70% oxidized 20 kDa dextran. $NaBH_4$ reduction was carried out in both cases after 2 h of crosslinking.

at the end of the immobilization process) when the immobilized enzyme was crosslinked with 70% oxidized 20 kDa dextran (Table 2, entries 5 and 6). Similar results have been obtained including 20% of PEG 600 in the immobilization mixture (Table 2, entry 7). Lastly, in order to obtain a highly active biocatalyst, the enzyme load was increased, obtaining a very good residual activity at the end of the complete immobilization process in presence of 20% of glycerol (56%) (Table 2, entry 11).

A similar behavior was described for the hexameric pyrimidine nucleoside phosphorylase from *Clostridium perfringens* (*Cp*UP), since uracil was added in order to stabilize this multimeric enzyme during covalent attachment to aldehyde-agarose, but no improvement in the percentage of recovered activity could be achieved [5,8]. On the other hand, the protective effect of glycerol during reduction was also observed in the immobilization of different PNP and PyNP on aldehyde agarose [5].

Alternatively, 100 kDa dextran was also tested: 20% oxidation allowed a residual activity after crosslinking which was much higher than that observed with 20 kDa dextran (about 50%), although the activity loss was also dramatic with a higher oxidation degree (Table 2, entries 8–10).

3.2. *Stability of immobilized* Bp*NDT*

Many unnatural nucleosides of clinical use are poorly soluble in physiological conditions. Consequently, the use of a high pH value [35] and/or the presence of a co-solvent [5,38] can be required to achieve suitable product concentrations for the development of preparative and industrial processes. However, enzyme stability may be affected by reaction conditions different from the physiological ones.

Thus, the election of an optimized enzyme biocatalyst for the production of nucleoside analogues requires the evaluation of enzyme stability in non-physiological conditions, such as alkaline pH or the presence of water-miscible organic co-solvents. In that way, a range of experimental conditions in which the immobilized enzyme can be used without relevant loss of activity can be established.

Surprinsingly, soluble *Bp*NDT retained 60% of its activity after 24 h at pH 10 (Fig. 2), whereas the activity of immobilized enzyme on PEI agarose was completely abolished, in accordance to the results previously reported for some multimeric PyNP and PNP [8,9]. Likewise, the immobilized biocatalyst obtained after crosslinking with 100 kDa dextran (20% oxidation) was also rapidly void of activity. Although highest residual activity was achieved when 20% oxidized dextran was employed in crosslinking, the obtained immobilized biocatalyst was unstable at pH 10 (Fig. 2).

The stability of immobilized *Bp*NDT was progressively improved when crosslinking was performed with an increased dextran oxidation degree (Fig. 2), and was fairly similar to soluble enzyme when *Bp*NDT was immobilized on agarose coated with PEI (600 Da) and crosslinked using 70% oxidized 20 kDa dextran (crosslinking performed in presence of 20% glycerol to preserve the activity of the absorbed enzyme). This enzyme derivative maintained 50% of activity after 24 h of incubation (Fig. 2).

High concentrations of a large number of modified nucleosides (g of product per liter of reaction medium) may not be soluble even at alkaline pH, therefore, the addition of an organic solvent is sometimes necessary. Since dimethylformamide (DMF) has been successfully used as co-solvent in preparative synthesis of nucleosides [10], the effect of this co-solvent on the stability of soluble and immobilized *Bp*NDT was studied.

Once again, free *Bp*NDT showed the highest stability in presence of 20% DMF compared with all the immobilized derivatives (Fig. 3), maintaining 100% of activity for at least 48 h. Furthermore, similarly to pH studies, the immobilized enzyme by mere ionic adsorption was completely deactivated (Fig. 3), as well as the one obtained after crosslinking with 100 kDa dextran (20% oxidized). Likewise, immobilized *Bp*NDT on 600 Da PEI agarose crosslinked with 70% oxidized dextran in presence of 20% glycerol was the most stable immobilized biocatalyst, retaining 70% of activity after 48 h in presence of 20% DMF (Fig. 3).

The very low stability of enzymes adsorbed on ionic carriers is surprising because it was never observed before in other enzymes, but confirms the results obtained in the immobilization study, where the crosslinking of the enzyme adsorbed on PEI-coated carriers with dextran caused a complete loss of activity. Probably, the strong ionic microenvironment surrounding the immobilized enzyme highly promotes, in certain conditions, the distortion of

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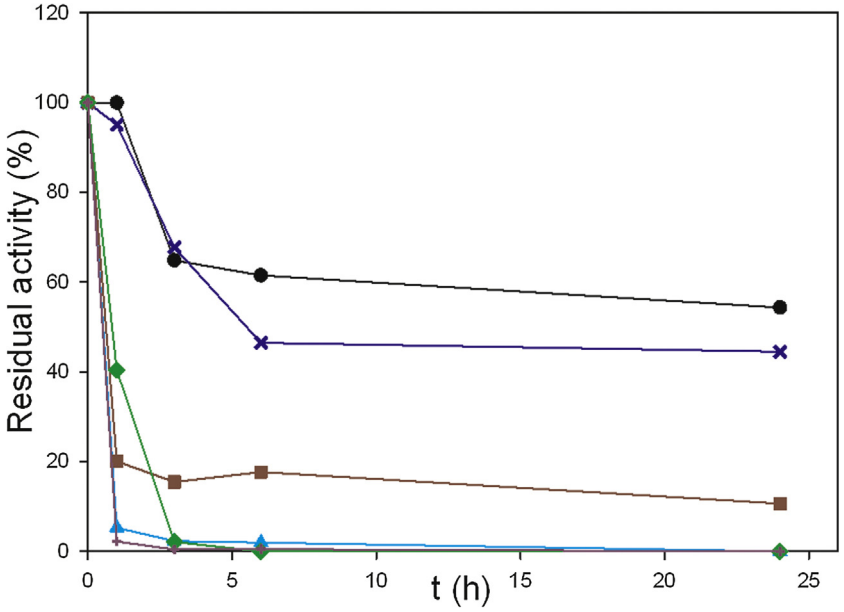

Fig. 2. Stability of *Bp*NDT at pH 10. ● Free *Bp*NDT; × Immobilized *Bp*NDT on 600 Da PEI agarose followed by crosslinking with 70% oxidized 20 kDa dextran (crosslinking performed in presence of 20% of glycerol); ■ Immobilized *Bp*NDT on 600 Da PEI agarose followed by crosslinking with 50% oxidized 20 kDa dextran; ▲ Immobilized *Bp*NDT on 600 Da PEI agarose followed by crosslinking with 20% oxidized 20 kDa dextran; ♦Immobilized *Bp*NDT on 600 Da PEI followed by crosslinking with 20% oxidized 100 kDa dextran; + *Bp*NDT adsorbed on 600 Da PEI agarose.

the 3D structure (by crosslinking or by using extreme pH conditions). However, further studies would be needed to confirm the detrimental effect of PEI on the stability of *Bp*NDT.

3.3. *Synthesis of trifluridine and decytabine catalyzed by immobilized* Bp*NDT*

Preparative synthesis of 5-trifluorothymidine (trifluridine, **3a**) and 5-aza-2′-deoxycytidine (decytabine, **3b**) were performed by *Bp*NDT adsorbed on 600 Da PEI agarose and crosslinked with 70% oxidized 20 kDa dextran in presence of 20% glycerol, since it was the best immobilized biocatalyst in terms of activity and stability (Scheme 1).

The enzymatic synthesis of trifluridine was thus carried out at different concentrations of deoxyuridine (dUrd) as sugar donor and 5-trifluorothymine (5tFThy, **2a**) as base acceptor. As for any equilibrium controlled synthesis, the increase of the molar excess of one of the substrates induces an improvement in the final conversion.

Nevertheless, we decided to study the synthesis in presence of an equal molar amount of the sugar donor dUrd and substrate

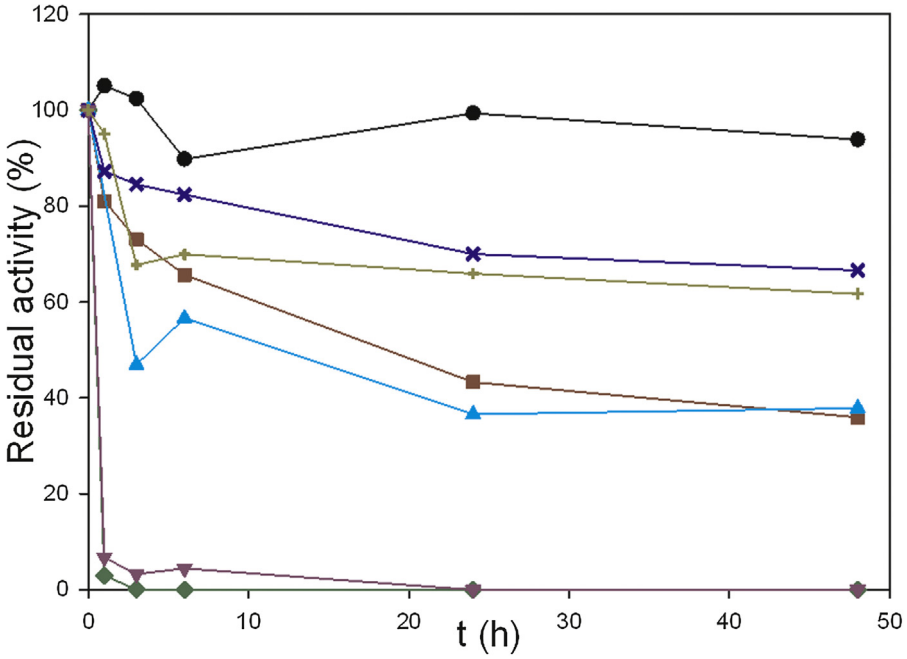

Fig. 3. Stability of *Bp*NDT in presence of 20% DMF. ● Free *Bp*NDT; × Immobilized *Bp*NDT on PEI 600 Da agarose followed by crosslinking with 70% oxidized 20 kDa dextran (crosslinking performed in presence of 20% of glycerol); + Immobilized *Bp*NDT on PEI 600 Da agarose followed by crosslinking with 70% oxidized 20 kDa dextran; ■ Immobilized *Bp*NDT on PEI 600 Da agarose followed by crosslinking with 50% oxidized 20 kDa dextran; ▲ Immobilized *Bp*NDT on PEI 600 Da agarose followed by crosslinking with 20% oxidized 20 kDa dextran; ♦Immobilized *Bp*NDT on PEI 600 Da followed by crosslinking with 20% oxidized 100 kDa dextran; ▼ Immobilized *Bp*NDT on PEI 600 Da agarose.

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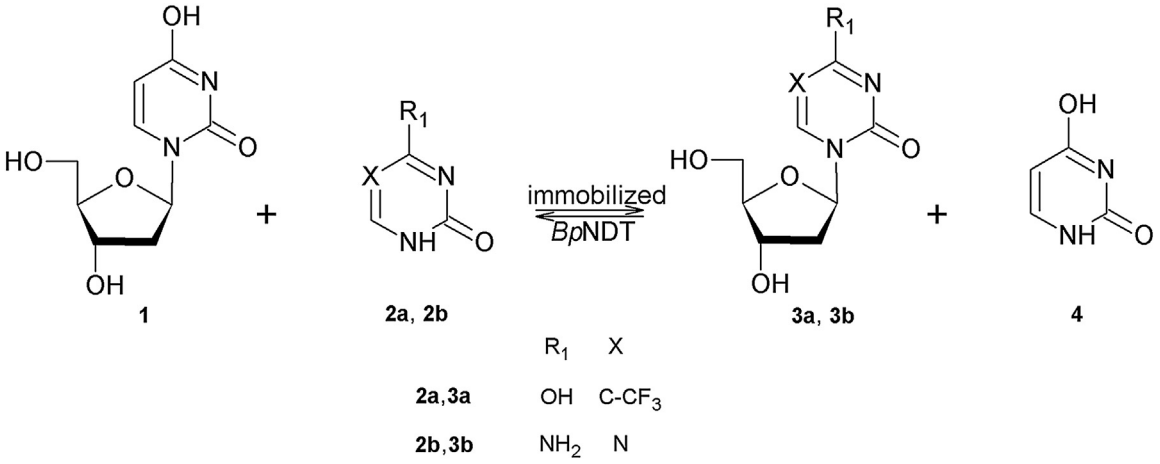

Scheme 1. Synthesis of trifluridine (5tFThd, **3a**) and decytabine (5azadCyd, **3b**) catalyzed by immobilized *Bp*NDT (600 Da PEI agarose followed by crosslinking with 70% oxidized 20 kDa dextran).

Table 3
Enzymatic synthesis of 5-trifluorothymidine (trifluridine, **3a**) and 5-aza-2′-deoxycytidine (decytabine, **3b**) catalyzed by immobilized *Bp*NDT at different substrate concentrations.

dUrd (1) (mM)	Base acceptor (mM)	Conversion (%)	Product (mM)	Productivity (mM/h)
10	**2a** (10)	58	**3a** (5.8)	2.92
20	**2a** (10)	64	**3a** (6.5)	3.22
20	**2a** (20)	56	**3a** (11.1)	5.56
30	**2a** (30)	53	**3a** (16.0)	5.34
40	**2a** (40)	50	**3a** (20.1)	6.71
10	**2b** (10)	76	**3b** (7.6)	2.53
20	**2b** (20)	51	**3b** (10.1)	3.36

Experimental conditions: 100 mg of biocatalyst were added to 5 mL of **1** and **2a**/**2b** in 10 mM potassium phosphate pH 7.5 and incubated at 37 °C for 2–3 h.

2a, since this condition is more attractive in the view of a large scale preparative process. Different experiments have been thus performed increasing the concentration of both substrates up to 40 mM, corresponding to 7.2 g/L of modified base (Table 3).

As expected, the highest conversion (64%) was achieved at 20 mM dUrd and 10 mM 5tFThy (2:1 molar ratio), and it was diminished when using equimolar amounts of both substrates (Table 3). Nevertheless, productivity was enhanced by increasing the concentration of both substrates reaching the highest rate (6.71 mM/h) when 40 mM of dUrd (**1**) and 5-tFThy (**2a**) were employed. In these conditions, 5.9 g/L of trifluridine (**3a**) were obtained.

The performance of the immobilized *Bp*NDT was exactly the same compared with the soluble enzyme, providing about 64% of conversion of **2a** into product **3a**, using 2:1 of molar excess of dUrd (**1**) (data not shown).

Syntheses of **3a** by immobilized *Bp*NDT lead to higher productivities than those transglycosylations performed by immobilized UP from *Bacillus subtilis* (*Bs*UP) or TP from *Escherichia coli* (*Ec*TP) in the same reaction conditions [9,10]. In fact, immobilized *Bp*NDT was able to achieve 58% conversion yield after 2 h in presence of 1:1 substrate molar ratio (productivity: 1.6 mM/h/IU), whereas immobilized *Bs*UP and *Ec*TP afforded approximately the same conversion (57% and 51%, respectively) to compound **3a** using a 2:1 substrate molar ratio, in 10 h and 3 h respectively (productivity: 0.2 mM/h/IU) [9,10].

In addition, immobilized *Bp*NDT was also able to catalyze the synthesis of decytabine (**3b**) with similar conversion yields (Table 3). In this case, it is worth mentioning that *Ec*TP or *Bs*UP are not able to catalyze the synthesis of **3b** using 5-azacytosine (**2b**) as substrate (results not shown). This fact can be explained considering that UP and TP are inactive toward cytidine, according to previous literature data [11].

4. Conclusions

Optimization of the immobilization process of NDT from *B. psychrosaccharolyticus* (*Bp*NDT) has been accomplished by studying the effect of oxidation grade and molecular mass of dextran in crosslinking of the absorbed enzyme on PEI functionalized supports. It has been observed that the activity loss was likely due to the distortion of the 3D structure of the enzyme caused by the reduction of imino bonds towards irreversible C-N covalent bonds. This detrimental effect was higher as larger was the number of covalent bonds obtained during the crosslinking (by increasing the reaction time and/or the oxidation degree of the crosslinking agent). This evidence allowed the optimization of the immobilization process in order to obtain a highly active *Bp*NDT derivative that maintained the good stability induced by the formation of several covalent bonds during the immobilization.

A stabilized and active biocatalyst was obtained on 600 Da PEI-agarose followed by crosslinking with 70% oxidized dextran (20 kDa) in presence of 20% glycerol or PEG. These additives behave as protective agents during the final reduction step of the immobilization process, reducing the distortion of the 3D structure of the protein. In particular, by using 20% glycerol, a biocatalyst with a recovered activity (>50%) much higher than that previously reported (about 20%) was obtained [19], also maintaining good stability in presence of 20% DMF as well as at pH 10.

The optimized biocatalyst was successfully used to carry out the synthesis of two interesting therapeutic nucleoside analogues: trifluridine and decytabine. These syntheses were performed in presence of high substrate concentration and, in both cases, higher conversion yields and productivities were achieved in comparison to immobilized nucleoside phosphorylases (*Bs*UP and *Ec*TP), demonstrating the biotechnological interest of this NDT.

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The results now achieved in the immobilization of this enzyme complete the set of immobilized enzymes that can be used for the synthesis of antiviral and antitumoral nucleosides, allowing the selection of the most adequate biocatalyst depending on the desired product. In particular, the immobilized *Bp*NDT could be the first choice for the *one-pot*, *one-enzyme* synthesis of modified pyrimidine or purine nucleosides including decytabine and other cytosine derivatives.

Acknowledgments

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