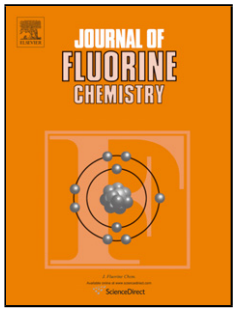


Title: Biotransformation of halogenated nucleosides by immobilized *Lactobacillus animalis* 2′-N-deoxyribosyltransferase

Author: Claudia N. Britos María José Lapponi Valeria A. Cappa Cintia W. Rivero Jorge A. Trelles

PII: S0022-1139(16)30100-2
DOI: http://dx.doi.org/doi:10.1016/j.jfluchem.2016.04.012
Reference: FLUOR 8768

To appear in: *FLUOR*

Received date: 23-2-2016
Revised date: 21-4-2016
Accepted date: 22-4-2016

Please cite this article as: Claudia N.Britos, María José Lapponi, Valeria A.Cappa, Cintia W.Rivero, Jorge A.Trelles, Biotransformation of halogenated nucleosides by immobilized Lactobacillus animalis 2′-N-deoxyribosyltransferase, Journal of Fluorine Chemistry http://dx.doi.org/10.1016/j.jfluchem.2016.04.012

Biotransformation of halogenated nucleosides by immobilized *Lactobacillus animalis* 2´-N-deoxyribosyltransferase

Claudia N. Britos, María José Lapponi, Valeria A. Cappa, Cintia W. Rivero, Jorge A. Trelles*

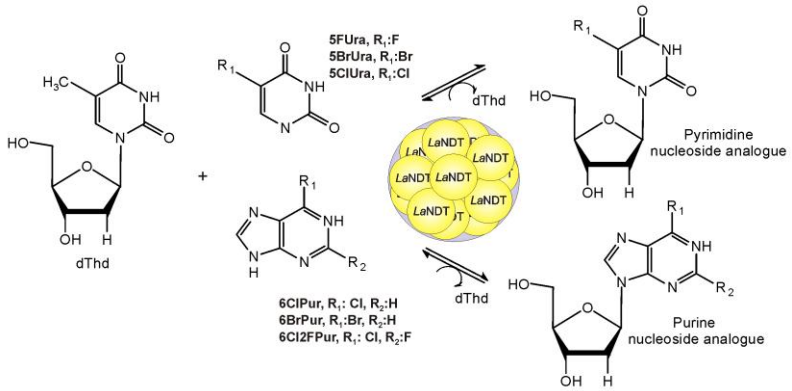
Laboratorio de Investigaciones en Biotecnología Sustentable (LIBioS), Universidad Nacional de Quilmes. Roque Sáenz Peña 352, Bernal (B1868BXD), Argentina.

*Corresponding author. Tel.: +054 1143657100 (ext. 5645); fax: +54 11 43657132.

E-mail address: jtrelles@unq.edu.ar (Jorge A. Trelles)

Graphical abstract

An immobilized biocatalyst with 2'-N-deoxyribosyltransferase activity (NDT) was developed from cell free extracts, resulting in a derivative with an activity of 2.6 U/g for the biosynthesis of the 5-fluorouracil-2´-deoxyriboside, an antimetabolite known as Floxuridine, used in gastrointestinal cancer treatment. Furthermore, immobilized NDT was satisfactorily used to obtain other halogenated pyrimidine and purine 2´-deoxynucleosides of pharmaceutical interest as antiviral or antitumor compounds at short reaction times. Besides, increasing the biocatalyst amount 8 times per volume unit allowed obtaining a 5-fold improvement in floxuridine biosynthesis. The developed biocatalyst proved to be effective for the biosynthesis of a wide spectrum of halogenated nucleosides by employing an economical, simple and environmentally friendly methodology.



Highlights

- This work describes a fast, simple and economical immobilized biocatalyst
- *Lactobacillus animalis* NDT was successfully stabilized in DEAE-Sepharose
- The developed immobilized biocatalyst was able to sinthetize floxuridine
- Halogenated nucleoside analogues of clinical interest were also obtained

Abstract

An immobilized biocatalyst with 2´-N-deoxyribosyltransferase (NDT) activity, *Lactobacillus animalis* NDT (*La*NDT), was developed from cell free extracts. *La*NDT was purified, characterized and then immobilized by ionic interaction. Different process parameters were optimized, resulting in an active derivative (2.6 U/g) able to obtain 1.75 mg/g of 5-fluorouracil-2´-deoxyriboside, an antimetabolite known as Floxuridine, used in gastrointestinal cancer treatment. Furthermore, immobilized *La*NDT was satisfactorily used to obtain at short reaction times other halogenated pyrimidine and purine 2´-deoxynucleosides such as 6-chloropurine-2´-deoxyriboside (4.9 U/g), 6-bromopurine-2´-deoxyriboside (4.3 U/g), 6-chloro-2-fluoropurine-2´-deoxyriboside (5.4 U/g), 5-bromo-2´-deoxyuridine (2.8 U/g) and 5-chloro-2´-deoxyuridine (1.8 U/g) compounds of pharmaceutical interest in antiviral or antitumor treatments. Besides, increasing the biocatalyst amount 8 times per volume unit allowed obtaining a 5-fold improvement in floxuridine biotransformation. The developed biocatalyst proved to be effective for the biosynthesis of a wide spectrum of nucleoside analogues by employing an economical, simple and environmentally friendly methodology.

Keywords: Green chemistry, floxuridine, DEAE-Sepharose, antiviral agents, antitumor compounds, enzyme stabilization.

Chemical compounds studied in this article

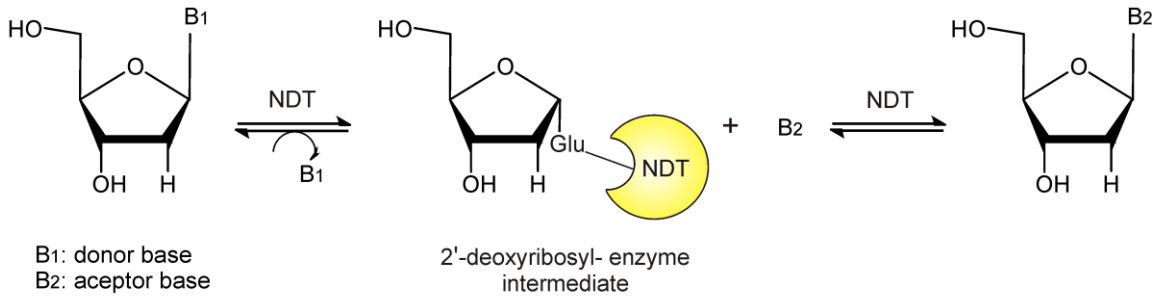
Floxuridine (PubChem CID: 5790)

1. Introduction

Nucleoside analogues (NAs) belong to a large family of chemically modified nucleosides with great structural diversity and broad spectrum of biological activity [1]. NAs represent one of the most important classes of antiviral and antitumor drugs extensively used in clinical applications [2].

These compounds are mainly synthesized by chemical methods that require the use of organic solvents, multiple reaction steps and the removal of protecting groups, causing unwanted accumulation of racemic mixtures that affect further purification [3]. In this sense, the use of biocatalysis for the synthesis of these NAs emerged as an alternative because reactions are regio- and stereoselective and take place in mild conditions. Two types of

enzymes have been mainly used to obtain NAs, such as nucleoside phosphorylases (NPs) and 2´-N-deoxyribosyltransferases (NDTs, EC 2.4.2.6) [1]. NDTs catalyze the cleavage of the N-glycosidic bond of a 2´-deoxyribonucleoside via a ping-pong bi- bi mechanism and a covalent deoxyribosyl-enzyme intermediate is generated, which in turn react with a purine or pirimidine as acceptor base (Scheme 1). These enzymes are classified into two classes according to their substrate specificity: NDT type I (PDT), specific for purine exchange, and NDT type II (NDT), which catalyzes the transfer between purines and/or pyrimidines. NDTs, in comparison with nucleoside NPs, have the advantage of catalyzing transglycosylation reactions between purine or pyrimidine bases and nucleosides in one step instead of two [4], as with NPs the synthesis of purine NAs using pyrimidine nucleosides as donors and purine bases as acceptors must be done with two types of NPs, one that is specific for purines (PNPs; EC 2.4.2.1) and another for pyrimidines (PyNPs; EC 2.4.2.2). NDTs have specificity for 2´-deoxyribonucleosides [5], although it has been demonstrated that NDTs can accept other related sugar donors as substrates [6]. Interestingly, it has been reported that some members of lactic acid bacteria (LAB) express NDTs as part of the nucleoside salvage pathway [7-9] .

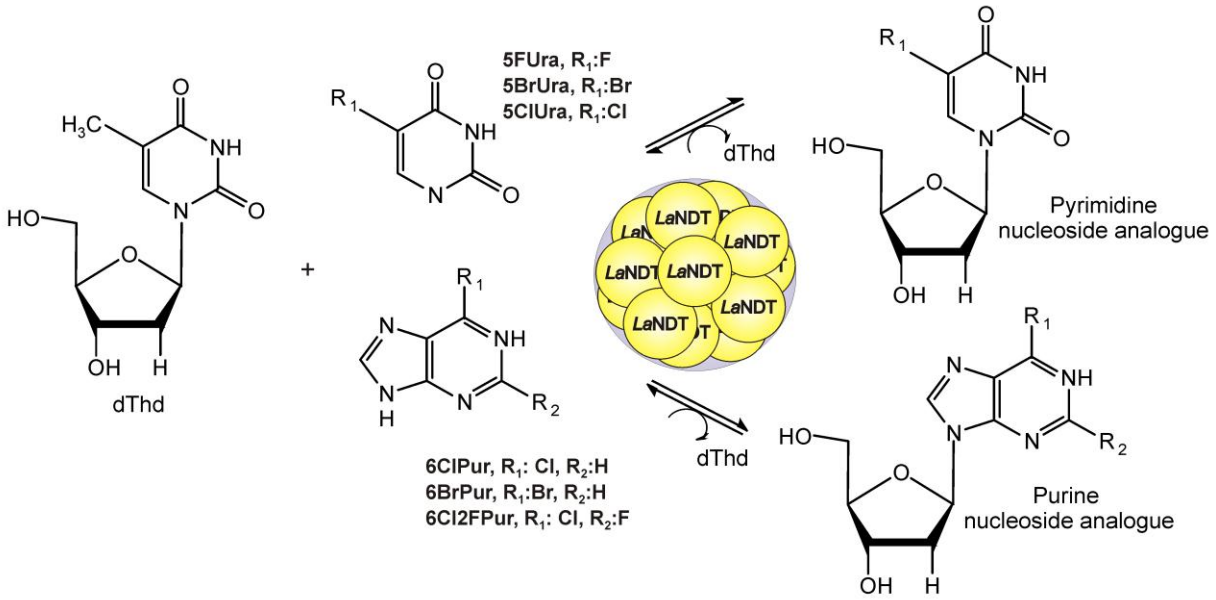


Scheme 1. Enzymatic synthesis of NAs and catalytic mechanism of NDTs. The nucleophile glutamic acid (Glu) present in the enzyme active site participates in the formation the covalent intermediate.

Nowadays, the use of enzymes for industrial applications is limited by several factors, such as the high cost of recombinant enzymes, instability, and availability in small amounts. Protein stabilization by immobilization has been exploited to enhance enzyme properties such as activity and specificity for their successful utilization in industrial processes [10]. Immobilization also promotes product recovery and improves biocatalyst reusability. The NA 5-fluorouracil-2´-deoxyriboside, better known as Floxuridine (FdUrd), is currently employed in the treatment of colorectal, pancreatic, kidney and stomach cancer, among others [2, 11]. The fluoro group present in biologically active molecules gives better characteristics to medicinal compounds. The presence of fluorine atom(s) into a bioactive nucleoside as an isosteric replacement of hydrogen or as an isopolar mimic of hydroxyl group, frequently leads to a dramatic change in biological activities and becomes an important strategy in the

design and discovery of novel drug candidates[12]. Besides, a related compound such as 5-chlorouracil-2´-deoxyuriboside proved to be useful in cancer treatment, and both of these halogenated analogues have been used as substrates (o building block) for the design of new prodrugs [13-15].

The aim of this study was to obtain diverse nucleoside analogues with potential antitumor and antiviral activity. In this work, we developed a novel immobilized biocatalyst (*La*NDT) obtained by an easy and economical procedure that could reduce the cost of eventual scale-up, and was able to biosynthesize nucleoside analogues useful for current therapeutics (Scheme 2).



Scheme 2. Biotransformation of pyrimidine and purine NAs performed by the developed immobilized *L. animalis* NDT (*La*NDT).

2. Results and Discussion

2. 1. Preparation of *La*NDT

The presence of NDT activity in *Lactobacillus animalis* ATCC 35046 was previously described by our group [9, 16]. In order to extract the protein, different lysis methods were employed. When both methods were assayed separately, poor protein yields were obtained. The use of a combined enzymatic lysis-sonication procedure improved protein extraction yield (1.3–1.6 mg/mL) by more than 4 times without activity loss.

Then, to enhance the activity of crude extracts, enrichment by ionic exchange and molecular exclusion chromatography was performed. NDT activity was detected in the elution fractions with the highest ionic strength (500 mM NaCl).

2.2. *La*NDT immobilization

NDTs are enzymes that act at mild reaction temperatures; previous results with *L.animalis* whole cells showed that the highest activity was obtained at 30 ºC [16], which was further confirmed with *L.animalis* protein extracts (data not shown), so this was the selected temperature for the rest of the assays [9]. These results were in agreement with related NDTs [17] and represent an even friendlier temperature than the reported 40 and 45°C for other biotransformations using NDTs [18, 19] . Also, different pH values were assayed to characterize *La*NDT and the enzyme showed stability between pH 5 and 8, and optimal activity was observed at neutral to slightly alkaline pH (data not shown).

Different supports were assayed for NDT immobilization. *La*NDT derivatives immobilized in IDA-Agarose, boronate-agarose and EC-EP Sepharose showed no activity (Table 1). The best immobilization yields were obtained using cationic supports such as DEAE-Sepharose and Q-Agarose. Then, both derivatives were analyzed for operational stability by performing subsequent FdUrd reactions, Q-Agarose maintained its activity for 24 h, while DEAE-Sepharose retained its initial activity for more than 64 h (Table 1). Therefore, this derivative was selected to optimize immobilization parameters.

Table 1. Immobilization of *La*NDT in different supports

	DEAE-Sepharose	***Q-Agarose***	***IDA-Agarose***	***Boronate-Agarose***	***EC-EP Sepharose***
Immobilization yield[a] (%)	93	87	65	41	65
Specific activity[b] (U/g)	0.030	0.027	ND[c]	ND[c]	ND[c]
Product yield[b] (mM FdUrd)	0.27	0.26	ND[c]	ND[c]	ND[c]
Operational Stability (h)	64	24	-	-	-

[a] Immobilization conditions: See "Materials and Methods" for experimental details.
[b] Reaction conditions: 100 mg of immobilized *La*NDT in 400 μL reaction volume; donor 6 mM dThd, acceptor 2 mM 5FUra, 20 mM Tris-HCl buffer pH 7.0, 30 ºC, 8 h.
[c] ND, not detectable.

2.3. Optimization of *La*NDT immobilization

Numerous immobilization techniques have been developed to achieve enzyme stabilization. Among them, adsorption is one of the most efficient methods due to its capacity to retain catalytic activity and most importantly, because of the possibility to reuse the support after the enzyme has been inactivated [20]. It is worth mentioning that the ionic exchange of enzymes to charged supports represents a simple non-distorting immobilization method

that helps maintain the correct assembly of the oligomeric form and therefore, the quaternary structure is not altered. This kind of matrix also allows the reuse of the support after desorting the protein after its inactivation [21].

When the interaction between *La*NDT and DEAE-Sepharose was assessed at different pH values (4–8), it was found that acidic pH negatively affected immobilization yields and therefore, enzymatic activity (Fig. 1A), probably due to changes in the overall charge of the enzyme at acid pH values that affect its interaction with the cationic support [22].

Additionally, in order to optimize NDT loading, immobilization on DEAE-Sepharose was performed using increasing protein mass, and 4.8 times enzyme loading was obtained. This biocatalyst showed 2.6-fold more activity (0.71 mM of conversion) compared to the initial immobilization conditions (Fig. 1B).

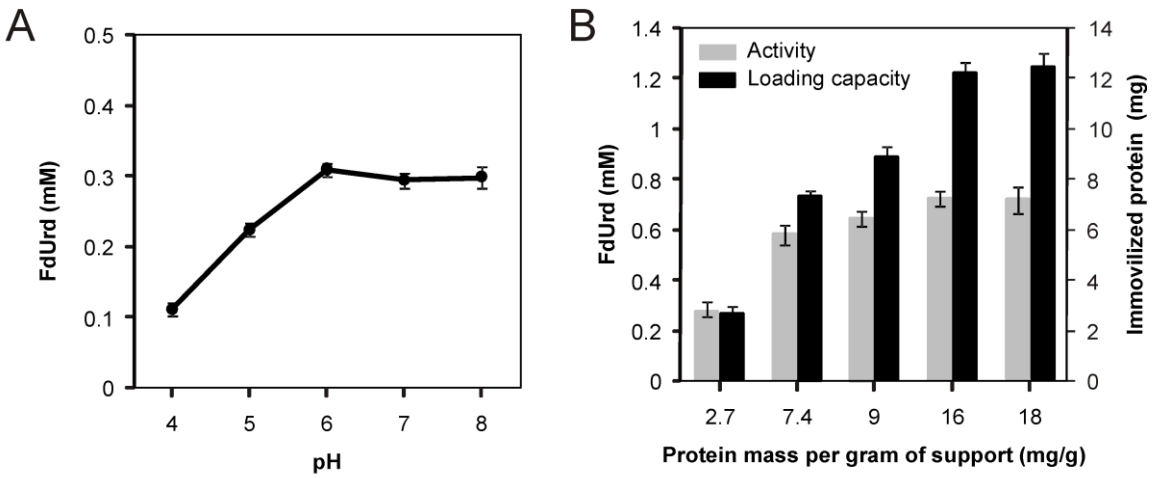


Fig. 1. Optimization of immobilized *La*NDT. A: DEAE-Sepharose was immobilized at different pH values, and FdUdr biotransformation was determined. B: Increasing enzyme amounts were added to 50 mg of support, and loading capacity of DEAE-Sepharose and FdUdr biotransformation were determined. Reaction conditions: 6 mM dThd and 2 mM 5FUra in Tris-HCl buffer (20 mM, pH 7.0*)* at 30ºC and 200 rpm shaking speed.

2.4. Biotransformation of nucleoside analogues

*La*NDT capacity to hydrolyze nucleosides with different sugar moieties was studied. 2'-deoxyribonucleosides (dUrd and dThd), uracil 1-β-D-arabinofuranoside (araUra) and 2´,3´-dideoxyuridine (ddUrd) and were partially hydrolyzed obtaining yields of 22, 19, 8 and 15 % at 1 h reaction, respectively.

Immobilized *La*NDT was used to biosynthesize different nucleoside analogues of pharmaceutical interest. *La*NDT is a NDT II-type, since it can catalyze the synthetic reaction of transglycosylation between pyrimidine and purine bases (Table 2). 5-Halogenated 2'-deoxyribonucleosides are commonly used as anticancer agents [14]. In this sense, FdUrd exerts its effects by inhibiting thymidylate synthase [12]. Likewise, BrdUrd and CldUrd have also

been used with similar therapeutic effects, [23] and are also used for *in vivo* studies of cancer cell proliferation [24]. In this work, FdUrd, CldUrd and BrdUrd were obtained at short reaction times, with specific activities around 3 U/g, higher than reported for other NDTs [18].

Table 2. Biotransformation of 2'-deoxyribonucleosides by immobilized *La*NDT[a]

Donor	***Acceptor***	***Product***	***Activity (U/g)***	***Yield (%)***	***Product conversion (mg/g)***
dThd	5FUra	**FdUrd**	2.6	35	1.75
	5BrUra	**BrdUrd**	2.8	25	1.54
	5ClUra	**CldUrd**	1.8	6	0.84
dThd	6BrPur	**6BrPdR**	4.3	61	3.84
	6ClPur	**6ClPdR**	4.9	69	3.76
	6Cl2FPur	**6Cl2FPdR**	5.4	51	2.77

[a] Reaction conditions: 10 mg of immobilized *La*NDT in 100 μL reaction volume: donor 6 mM, acceptor 2 mM, 20 mM Tris-HCl buffer pH 7.0, 30 ºC.

Purine bases modified at the 6-position and their derivatives have received considerable attention, due to their structural similarity to DNA damage products arising from the modification of N-6 of 2′-deoxyadenosine or O-6 of 2′-deoxyguanosine [25]. These compounds possess a wide range of biological properties. It was shown that 2ChPur or 2FPur nucleosides have anticancer activity [26]. 6-modified purine 2´-deoxyribosides (6ClPdR, 6BrPdR and 6Cl2FPdR) have been obtained with high values of specific activity and yields between 50 and 70 % using immobilized *La*NDT (Table 2). Also, the bioconversion of 6ClPdR and CldUrd nucleosides was achieved with *La*NDT and not with immobilized whole cells as previously reported [9]. This nucleoside can act as building block to synthesis guanosine derivatives [6] or may be used for the synthesis of modified antisense oligonucleotides [27].

Many 2´,3´-dideoxynucleosides have been used because of their anti-HIV activity [28]. Immobilized *La*NDT could synthesize 5-fluoro-2´,3´-dideoxyuridine (FddUrd) and 5-fluorouracil-arabinonucleoside (ara5FUra) after 24 and 8 h, respectively (data not shown). Additionally, in view of a potential bioprocess scale-up, increasing the biocatalyst amount 8 times per volume unit resulted in a 5-fold increase in FdUrd activity (Fig. 2), making *La*NDT a promising biocatalyst for future application in the development of compounds with high added value and pharmacological interest.

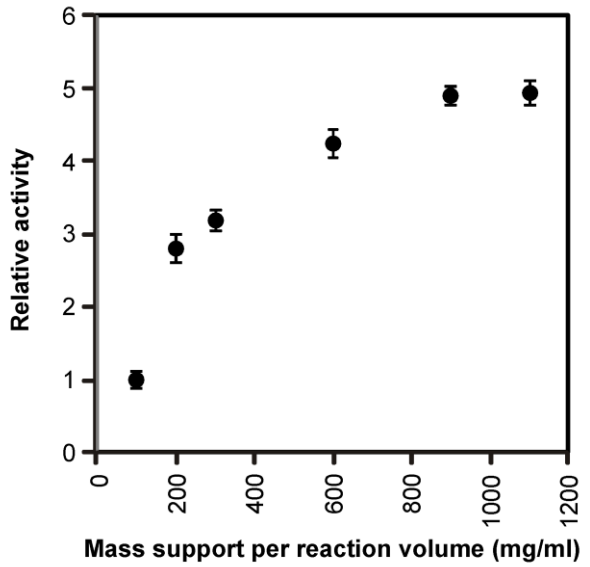


Fig. 2. Different derivative masses per volume were added, relative activity was determined. Reaction conditions: 6 mM dThd and 2 mM 5FUra in Tris-HCl buffer (20 mM, pH 7.0) at 30 ºC and 200 rpm shaking speed.

3. Conclusions

In this work a novel biocatalyst from *Lactobacillus animalis* (*La*NDT) was developed, prepared and purified from cell free extracts. When *La*NDT was immobilized in DEAE-Sepharose, it remained associated at high ionic strength, which allowed purification and immobilization in a single step, simplifying the process and reducing the costs of derivative preparation. Besides, the immobilized biocatalyst can be recovered after deactivation by subjecting the support to a new enzyme loading. Optimization of immobilization parameters allowed reaching 1.75 mg/g of FdUrd. Besides, immobilized *La*NDT was used to biosynthesize other purine and pyrimidine 2'-deoxyribonucleosides with yields above 50 % at short reaction times.

On the other hand, the developed biocatalyst showed capacity to accept 2´,3´-dideoxyribose and arabinose moieties as substrates, obtaining FddUrd and ara5FUra in one step reaction. These results indicate that NDT from *Lactobacillus animalis* ATCC 35046 could be used to produce a wide range of nucleoside analogues of pharmaceutical interest employing an environmentally friendly methodology.

4. Experimental

4.1. Materials

Nucleosides and bases were purchased from Sigma Chem. Co. (Brazil). Culture media compounds were obtained from Britania S.A. (Argentina). Chemicals were purchased from Sigma Chem. Co. (Brazil). HPLC solvents used were supplied by Sintorgan S.A. (Argentina). Supports (DEAE-Sepharose, IDA-Agarose, Q-Agarose, Boronate,

EC-EP Sepharose, Sephadex G-75) were purchased from Sigma Aldrich (Argentina). Ultrafiltration devices were acquired from Sartorius (Argentina).

4.2. Growth conditions

Lactobacillus animalis ATCC 35046 was grown according to the required conditions of temperature and culture medium until saturation, harvested by centrifugation for 10 min at 11000 x *g*, washed twice with Tris-HCl buffer (20 mM, pH 7) and stored at -20 ºC until use.

4.3. Enzyme preparation from *L. animalis* crude extracts

Cell free protein extracts of *L. animalis* were obtained by subsequent rupture methods: enzymatic lysis and sonication. First, bacterial suspension was incubated in Tris-HCl buffer (10 mM, pH 8) with 1 mg/mL lysozyme at 37 ºC and 200 rpm for 2 h. Crude extracts were recovered by centrifugation at 5500x *g* for 30 min. Then, sonication was carried out using 5 cycles of 5 min pulses at 8–9 W (Sonic Dismembrator F60, USA), and protein extract was obtained by centrifugation at 5500x *g* for 30 min at 4 ºC. Then, *La*NDT was recovered and enriched by ion exchange and then, by gel filtration chromatography. A DEAE-Sepharose column was equilibrated with Tris-HCl buffer (10 mM, pH 7), and stepwise elution was performed with increasing concentrations of NaCl (0–500 mM). The fraction with NDT activity was further purified by gel filtration chromatography through a Sephadex^{TM} G-75 column equilibrated with Tris-HCl buffer (10 mM, pH 7). Elution fractions were quantified by UV absorption at 280 nm and assayed for NDT activity. The protein fractions containing *La*NDT were pooled and concentrated by ultrafiltration (Vivaspin^{TM} devices, 10,000 MWCO, Sartorius). The protein extracts were quantified by the coomassie blue method, and analyzed by polyacrylamide gel electrophoresis (PAGE) and NDT activity as described below.

4.4. Standard assay for *La*NDT

Biotransformation of FdUrd from thymidine (dThd) and 5-fluorouracil (5FUra) was selected as standard reaction to evaluate enzymatic activity. Free or immobilized *La*NDT was added to a solution containing 6 mM dThd and 2mM 5FUra in Tris-HCl buffer (20 mM, pH 7.0) at 30ºC and 200 rpm shaking speed. At different times, 20 µL aliquots were taken, centrifuged at 10,000 *g* and the supernatant was analyzed by HPLC. In such conditions, one unit of enzyme (U) was defined as the amount of enzyme that catalyzed the formation of 1 µmol of FdUrd in 1 min.

*4.5. La*NDT immobilization

4.5.1. Immobilization by adsorption

One hundred mg of support (Q-Agarose, DEAE-Sepharose, Boronate-Agarose or IDA-Agarose) was incubated with 0.27 mg of *La*NDT. The immobilization mixture was gently stirred for 4 h at 4 ºC, washed with 20 mM Tris-HCl buffer (pH 7), dried by vacuum filtration and stored at 4 ºC until use.

4.5.2. Immobilization by covalent bonding

One hundred mg of EC-EP Sepharose was incubated with 0.27 mg of *La*NDT in 20 mM Tris-HCl buffer (pH 7) with 1 M NaCl. The mixture was gently stirred for 8 h at 4 ºC, and the support was separated from the solution by filtration. The obtained derivative was incubated for 8 h at 4 ºC in 3 M glycine (pH 9) and stored at 4 ºC until use. In both cases, immobilization yields were determined as the difference in protein content of the mixture before and after incubation with the supports. NDT activity of the obtained derivatives was evaluated by FdUrd standard assay.

4.6. Operational stability of immobilized *La*NDT

FdUrd biotransformation with immobilized *La*NDT on DEAE-Sepharose and Q-Agarose was carried out several times until inactivation.

4.7. Optimization of *La*NDT immobilization

*La*NDT was immobilized on DEAE-Sepharose at different pH values (4, 5, 6, 7 and 8) using 2.7 mg of total protein per gram of support. Once the optimal value was selected, loading capacity was evaluated using 50 mg of support and increasing amounts of *La*NDT in the immobilization mixture. Immobilization yields were determined by quantification of total protein content, and the activity of the derivatives was evaluated by FdUrd standard assay.

4. 8. Biotransformation of nucleoside analogues

Thymidine (dThd) and 2´-deoxyuridine (dUrd) were assayed as sugar donors. Different purine and pyrimidine bases were tested: 5-fluorouracil (5FUra), 5-bromouracil (5BrUra), 5-chlorouracil (5ClUra), 6-chloropurine (6ClPur), 6-bromopurine (6BrPur) and 6-chloro-2-fluoropurine (6Cl2FPur). Reactions were performed using 100

mg/mL of immobilized *La*NDT, 6 mM nucleoside and 2 mM base, 30 ºC and 200 rpm. At different times (5-8 h), 20 μL aliquots were taken and centrifuged at 10,000x *g*, and the supernatant was analyzed by HPLC to evaluate yield expressed as percentage and product conversion expressed as mg of product per gram of support.

4.9. Amount of biocatalyst

In order to maximize the performance of the biocatalyst obtained, FdUrd biotransformation was performed using different amounts of immobilized *La*NDT at constant reaction volume.

4.10. Analytical Methods

Nucleoside analogues were quantitatively measured by HPLC (Gilson) with a Nucleodure™ 100 C-18 column (5 μm, 125 mm × 5 mm) at 254 nm using water/methanol as mobile phase. The ratios of mobile phase and retention time for the reaction products were : 95:5 (v/v) for 5-fluoruracil-2´-deoxyriboside (FdUrd, 5.8 min); 90:10 (v/v) for 5-bromouracil-2´-deoxyriboside (BrdUrd, 11 min), 6-chloropurine-2´-deoxyriboside (6ClPdR, 2.9 min), 6-bromopurine-2´-deoxyriboside (6BrPdR, 2.9 min) and 6-chloro-2-fluoropurine-2´-deoxyriboside (6Cl2FPdR, 6.2 min), 99:1 (v/v) for 5-chlorouracil-2´-deoxyriboside (CldUrd, 8.7 min) and 98:2 (v/v) for 5-fluorouracil-2´,3´-dideoxyriboside (FddUrd, 7.1 min) and 5-fluorouracil-1-β-D-arabinofuranoside (Ara5FUra, 3.9 min).
Product identification was performed by *MS-HPLC* LCQ-DECAXP4 Thermo Spectrometer with the electron spray ionization (ESI) method. Phenomenex C18 column (5 μm, 100 mm × 2 mm) and Xcalibur 1.3 software (Thermo-Finnigan, USA) were used. Mobile phase and flow were: (i) 95/5 (v/v) water/methanol + 0.1% acetic acid (F: 200 μL/min) for FdUrd (M^+: 246.8), CldUrd (M^+: 266.7) and BrdUrd (M^+: 307.2); and (ii) 15/85 (v/v) water/methanol + 0.1% acetic acid (F: 200 μL/min) for 6ClPdR (M^+: 271.8), 6Cl2FPdR (M^+: 289.9), 6BrPdR (M^+: 315.8), 5FddUrd (M^+: 231.1) and Ara5FUra (M^+: 262.9).

Acknowledgments

This research was supported by Agencia Nacional de Promoción Científica y Tecnológica (PICT 2013-2658), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2014-KA5-00805) and Universidad Nacional de Quilmes (PUNQ 1409/15). CWR and JAT are research members at CONICET. MJL and VAC are CONICET research fellows.

References

[1] I.A. Mikhailopulo, A.I. Miroshnikov, New trends in nucleoside biotechnology, Acta naturae, 2 (2010) 36-59.
[2] D.D. Jordheim L. P., Zoulim F.,Dumontet C., Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases, Nat Rev Drug Discov, 12 (2013) 447-464.
[3] E. Ichikawa, K. Kato, Sugar-modified nucleosides in past 10 years, a review, Curr Med Chem, 8 (2001) 385-423.
[4] A. Fresco-Taboada, I. de la Mata, M. Arroyo, J. Fernandez-Lucas, New insights on nucleoside 2'-deoxyribosyltransferases: a versatile biocatalyst for one-pot one-step synthesis of nucleoside analogs, Appl Microbiol Biotechnol, 97 (2013) 3773-3785.
[5] R. Anand, P.A. Kaminski, S.E. Ealick, Structures of purine 2'-deoxyribosyltransferase, substrate complexes, and the ribosylated enzyme intermediate at 2.0 A resolution, Biochemistry, 43 (2004) 2384-2393.
[6] K. Okuyama, S. Shibuya, T. Hamamoto, T. Noguchi, Enzymatic synthesis of 2'-deoxyguanosine with nucleoside deoxyribosyltransferase-II, Bioscience, biotechnology, and biochemistry, 67 (2003) 989-995.
[7] P.A. Kaminski, Functional cloning, heterologous expression, and purification of two different N-deoxyribosyltransferases from Lactobacillus helveticus, J Biol Chem, 277 (2002) 14400-14407.
[8] J. Fernandez-Lucas, C. Acebal, J.V. Sinisterra, M. Arroyo, I. de la Mata, Lactobacillus reuteri 2'-deoxyribosyltransferase, a novel biocatalyst for tailoring of nucleosides, Appl Environ Microbiol, 76 (2010) 1462-1470.
[9] C.N. Britos, V.A. Cappa, C.W. Rivero, J.E. Sambeth, M.E. Lozano, J.A. Trelles, Biotransformation of halogenated 2′-deoxyribosides by immobilized lactic acid bacteria, Journal of Molecular Catalysis B: Enzymatic, 79 (2012) 49-53.
[10] R.K. Singh, M.K. Tiwari, R. Singh, J.K. Lee, From protein engineering to immobilization: promising strategies for the upgrade of industrial enzymes, Int J Mol Sci, 14 (2013) 1232-1277.
[11] P.S. Liu, A.; Chu, C. K., Fluorinated Nucleosides: Synthesis and Biological Implication, J Fluor Chem, 129 (2008) 743-766.
[12] P. Liu, A. Sharon, C.K. Chu, Fluorinated nucleosides: Synthesis and biological implication, Journal of fluorine chemistry, 129 (2008) 743-766.
[13] S.M. Park, H. Yang, S.K. Park, H.M. Kim, B.H. Kim, Design, synthesis, and anticancer activities of novel perfluoroalkyltriazole-appended 2'-deoxyuridines, Bioorg Med Chem Lett, 20 (2010) 5831-5834.
[14] C.W. Rivero, C.N. Britos, M.E. Lozano, J.V. Sinisterra, J.A. Trelles, Green biosynthesis of floxuridine by immobilized microorganisms, FEMS Microbiol Lett, 331 (2012) 31-36.
[15] D. Vivian, J.E. Polli, Synthesis and in vitro evaluation of bile acid prodrugs of floxuridine to target the liver, Int J Pharm, 475 (2014) 597-604.
[16] V.A. Cappa, C.W. Rivero, C.N. Britos, L.M. Martinez, M.E. Lozano, J.A. Trelles, An efficient biocatalytic system for floxuridine biosynthesis based onLactobacillus animalis ATCC 35046 immobilized in Sr-alginate, Process Biochemistry, 49 (2014) 1169–1175.
[17] Y. Miyamoto, T. Masaki, S. Chohnan, Characterization of N-deoxyribosyltransferase from Lactococcus lactis subsp. lactis, Biochim Biophys Acta, 1774 (2007) 1323-1330.
[18] J. Fernandez-Lucas, A. Fresco-Taboada, C. Acebal, I. de la Mata, M. Arroyo, Enzymatic synthesis of nucleoside analogues using immobilized 2'-deoxyribosyltransferase from Lactobacillus reuteri, Appl Microbiol Biotechnol, 91 (2011) 317-327.
[19] H.D.W. Hicks N., Synthesis of Nucleoside Analogues Using Immobilised N-Deoxyribosyltransferases, Biocatalysis, 11 (1994) 1-7.
[20] U. Hanefeld, L. Gardossi, E. Magner, Understanding enzyme immobilisation, Chemical Society reviews, 38 (2009) 453-468.
[21] A. Fresco-Taboada, I. Serra, J. Fernandez-Lucas, C. Acebal, M. Arroyo, M. Terreni, I. De la Mata, Nucleoside 2'-deoxyribosyltransferase from psychrophilic bacterium Bacillus psychrosaccharolyticus--preparation of an immobilized biocatalyst for the enzymatic synthesis of therapeutic nucleosides, Molecules, 19 (2014) 11231-11249.
[22] W. John, The Protein Protocols Handbook, 3º ed., Humana Press2009.
[23] M.L. Brandon, L. Mi, W. Chaung, G. Teebor, R.J. Boorstein, 5-chloro-2'-deoxyuridine cytotoxicity results from base excision repair of uracil subsequent to thymidylate synthase inhibition, Mutation research, 459 (2000) 161-169.
[24] T. Fujimaki, M. Matsutani, O. Nakamura, A. Asai, N. Funada, M. Koike, H. Segawa, K. Aritake, T. Fukushima, S. Houjo, et al., Correlation between bromodeoxyuridine-labeling indices and patient prognosis in cerebral astrocytic tumors of adults, Cancer, 67 (1991) 1629-1634.
[25] E.A. Véliz, P.A. Beal, 6-Bromopurine nucleosides as reagents for nucleoside analogue synthesis, The Journal of organic chemistry, 66 (2001) 8592-8598.

[26] P.L. Bonate, L. Arthaud, W.R. Cantrell, Jr., K. Stephenson, J.A. Secrist, 3rd, S. Weitman, Discovery and development of clofarabine: a nucleoside analogue for treating cancer, Nat Rev Drug Discov, 5 (2006) 855-863.
[27] P. Herdewijn, Heterocyclic Modifications of Oligonucleotides and Antisense Technology, Antisense and Nucleic Acid Drug Development, 10 (2000) 297-310.
[28] C. Mathé, G. Gosselin, l-Nucleoside enantiomers as antivirals drugs: A mini-review, Antiviral Research, 71 (2006) 276-281.