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Short communication

Development of a high efficient biocatalyst by oriented covalent immobilization of a novel recombinant 2′-*N*-deoxyribosyltransferase from *Lactobacillus animalis*

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ABSTRACT

The 2′-*N*-deoxyribosyltransferases [NDT; **EC 2.4.2.6**] are a group of enzymes widely used as biocatalysts for nucleoside biosynthesis. In this work, the molecular cloning, expression and purification of a novel NDT from *Lactobacillus animalis* (*La*NDT) have been reported. On the other hand, biocatalyst stability has been significantly enhanced by multipoint covalent immobilization using a hetero-functional support activated with nickel-chelates and glyoxyl groups. The immobilized enzyme could be reused for more than 300 h and stored during almost 3 months without activity loss. Besides, the obtained derivative (Ni^{2+}-Gx-*La*NDT) was able to biosynthesize 88 mg floxuridine/g biocatalyst after 1 h of reaction. In this work, a green bioprocess by employing an environmentally friendly methodology was developed, which allowed the obtaining of a compound with proven anti-tumor activity. Therefore, the obtained enzymatic biocatalyst meets the requirements of high activity, stability, and short reaction times needed for low-cost production in a future preparative application.

Nucleoside analogues have a key role as antimetabolites due to their widely demonstrated antiviral and anticancer activity. Pyrimidine analogues such as floxuridine have activity in patients with colorectal, pancreatic, breast, head and neck cancers (Adema et al., 2002).

These kinds of compounds are mainly synthesized by chemical methods that often require time-consuming multistep processes, the use of organic solvents and the removal of protecting groups. Furthermore, chemical process may drive to accumulate undesired racemic mixtures that affect further purification (Ichikawa and Kato, 2001). The use of glycosyl-transferring enzymes for the biosynthesis of nucleoside analogues is an established procedure with several advantages over the traditional multistep chemical methods that arise from the inherent properties of enzymes such as high efficiency, regio-, stereo- and enantioselectivity, and mild reaction conditions (Li et al., 2010; Mikhailopulo, 2007). The 2′-*N*-deoxyribosyltransferases [NDT; **EC 2.4.2.6**] are a group of enzymes that have been used for nucleoside biosynthesis by mediating the transfer of glycosyl residues to acceptor bases (Fresco-Taboada et al., 2013). 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 (Lapponi et al., 2016).

Nevertheless, the industrial application of enzymes is often hindered by their low operational stability and difficult recovery (Sheldon, 2008; Sheldon and Van Pelt, 2013). These drawbacks can generally be overcome by different immobilization methods. Hetero-functional supports displaying several functionalities at their surface allow interactions with the enzyme groups under different immobilization conditions (Barbosa et al., 2013). Particularly, the combination of methal-chelates and the glyoxyl groups allows a first and rapid interaction of the enzyme with the support through a coordination bond between His-tagged proteins and the metals of the support. Subsequently, the unprotonated ε-NH_2 of the nearby lysines to the His-tag, can establish a covalent attachment with the aldehyde groups of support surface, forming Schiff's bases that must be mildly reduced to turn them into irreversible secondary amines as the final protein-surface bonds (Bolivar et al., 2006; Fernández-Lorente et al., 2015). This immobilization chemistry has revealed to be extremely efficient to stabilize many industrial enzymes and enable their continuous use and

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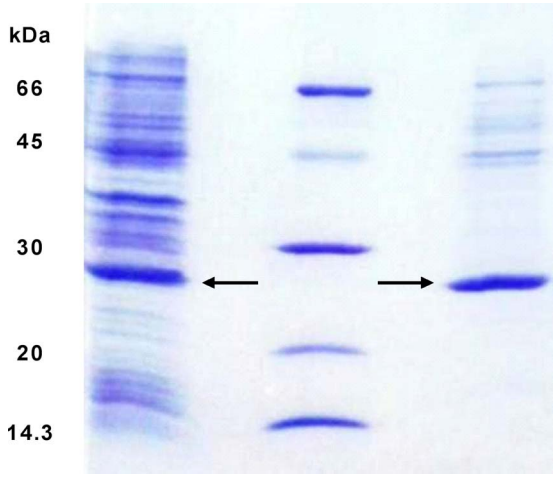

Fig. 1. SDS-PAGE of *La*NDT expression and purification. Lane 1, *La*NDT in soluble fraction after induction of 22 h at 19 °C in 2YT medium using 1 mM IPTG. Lane 3, protein standard marker. Lane 5, *La*NDT after purification by IMAC-Ni using as immobilization and washing buffer (Tris-HCl buffer 20 mM, supplemented with 200 mM sodium chloride and 10 mM imidazole). Attached protein was eluted by adding Tris-HCl buffer 20 mM supplemented with 200 mM sodium chloride and 25 mM imidazole. Arrows point to the recombinant *La*NDT.

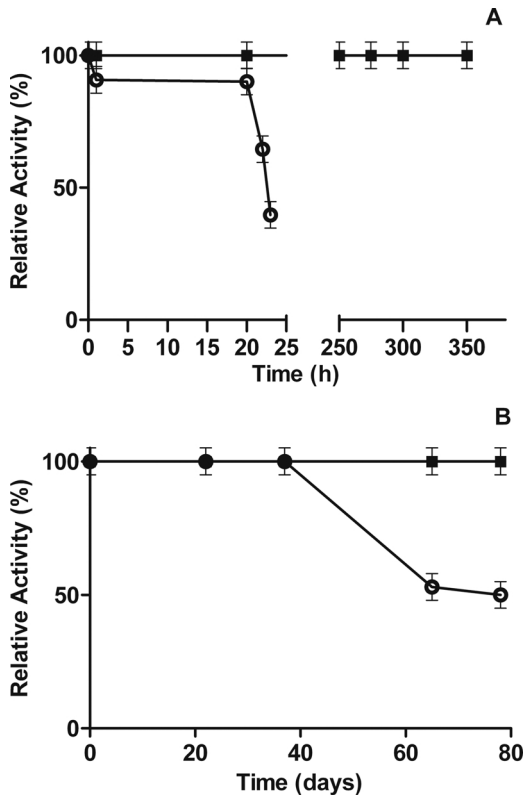

Fig. 2. Biocatalysts stability. Ni^{2+}-*La*NDT (white circles) and Ni^{2+}-Gx-*La*NDT (black squares). A) Reusability using floxuridine biosynthesis as standard reaction. Biocatalysts were filtered at the end of each 1h-cycle and washed using buffer Tris-HCl (20 mM, pH 7.0). B) Storage stability was evaluated storing biocatalysts at 4 °C for several days, being defined as the relative activity of floxuridine conversion between the first and the successive reactions.

recycling in flow and batch reactors, respectively (López-Gallego et al., 2005; Tapias et al., 2016).

Until now, the enzymatic biocatalysis of nucleoside analogues has been accomplished by employing whole cells of microorganisms (Fernández-Lucas et al., 2008; Britos et al., 2012; Rivero and Trelles, 2012; Cappa et al., 2014), soluble or immobilized cell extracts (Britos et al., 2016) or recombinant isolated enzymes (Fernández-Lucas et al., 2013; Fresco-Taboada and Terreni, 2016).

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

Support	Retained activity[a,b] (%)	Operational stability[b] (h)	Floxuridine (g)[c]
IDA-Ag	89	20	1.8
IDA-Gx-Ag	50	300	13.2

[a] Referred to reaction of floxuridine biosynthesis using free NDT.
[b] Reaction conditions: 50 mg derivative in 1 ml of Tris-HCl buffer (20 mM, pH7) containing 6 mM dThd and 2 mM 5FUra at 30 °C for 1 h.
[c] Grams of floxuridine obtained by 1 g of biocatalyst.

The aim of this work was to obtain an efficient biocatalyst through the molecular cloning and expression of a novel and stabilized NDT with high activity in floxuridine biosynthesis.

The *ndt* gene was amplified by PCR from *Lactobacillus animalis* (**ATCC 35046**) genomic DNA, cloned into plasmid pET-28b (+) and sequenced, showing 99% identity with respect to nucleoside deoxyribosyltransferase from *Lactobacillus buchneri* (GenBank accession no. **GI 331700395**). The best induction condition was achieved after 22 h at 19 °C in 2YT medium, in which a protein of 27 kDa appeared mainly in the soluble fraction after sonication, showing that this enzyme does not form inclusion bodies (Fig. 1). It is noteworthy that the molecular weight of the induced protein was consistent with the one previously reported by gradient SDS-PAGE for 2′-*N*-deoxyribosyltransferases. Enzyme purification from the supernatant was performed by IMAC chromatography, optimizing some parameters such as immobilization buffer, being sodium chloride (200 mM) and imidazole (10 mM) the selected concentrations. The best condition to elute nonspecifically bound proteins was tris-(hydroxymethyl) aminomethane–HCl (Tris-HCl) buffer 20 mM, pH 7.0, supplemented with sodium chloride (200 mM) and imidazole (10 mM). Finally, purified protein was efficiently eluted by adding Tris-HCl buffer supplemented with sodium chloride (200 mM) and imidazole (25 mM). After the purification process, 1.4 mg/mL of pure NDT was obtained.

In order to determine the NDT activity of *L. animalis* (*La*NDT), hydrolysis assays were performed using thymidine (dThd) and deoxyadenosine (dAde) at 30 °C in Tris-HCl buffer (20 mM, pH 7.0). The recombinant enzyme exhibited hydrolytic activity for both purine and pyrimidine nucleosides, suggesting that the novel *La*NDT obtained should be classified as NDT type II, as reported by Britos and col. (Britos et al., 2012). Besides, the purified *La*NDT was used to biosynthesize 5-fluorouracil-2′-deoxyriboside (FdUrd), an anticancer compound known as floxuridine. A yield of 77% was obtained with a substrate ratio 6:2 dThd and 5-fluorouracil (5FUra) after 1 h of reaction (data not shown). The stabilization of the recombinant enzyme was carried out using affinity immobilization based on the site-selective interaction between the poly-His-tag fused to protein N-terminus and the chelates groups of the support (see supplementary appendix). However, the enzyme was reversibly bound to this carrier, therefore in order to make the enzyme immobilization irreversible and taking into account that real stabilization are often achieved when the enzyme is bound to a rigid support through an intense multipoint covalent (Barbosa et al., 2013), we decided to use a hetero-functional support activated with nickel chelates to selectively immobilized the enzyme and with glyoxyl groups to promote the irreversible multivalent attachment (Ni^{2+}-Gx). In this way, recombinant NDT was immobilized on Ni^{2+}-Gx through three reaction steps 1) affinity binding between the his-tag of the enzyme and the metals on the support, 2) covalent multi-valent attachment at alkaline pH by lysine residues attachment to the glyoxyl groups of the carrier and 3) mild reduction in order to turn the reversible Schiffs base formed into irreversible (secondary amino) bonds and transform the remaining aldehyde groups into inert hydroxyl moieties. Remarkably, floxuridine yield was not affected significantly when the biocatalyst obtained by

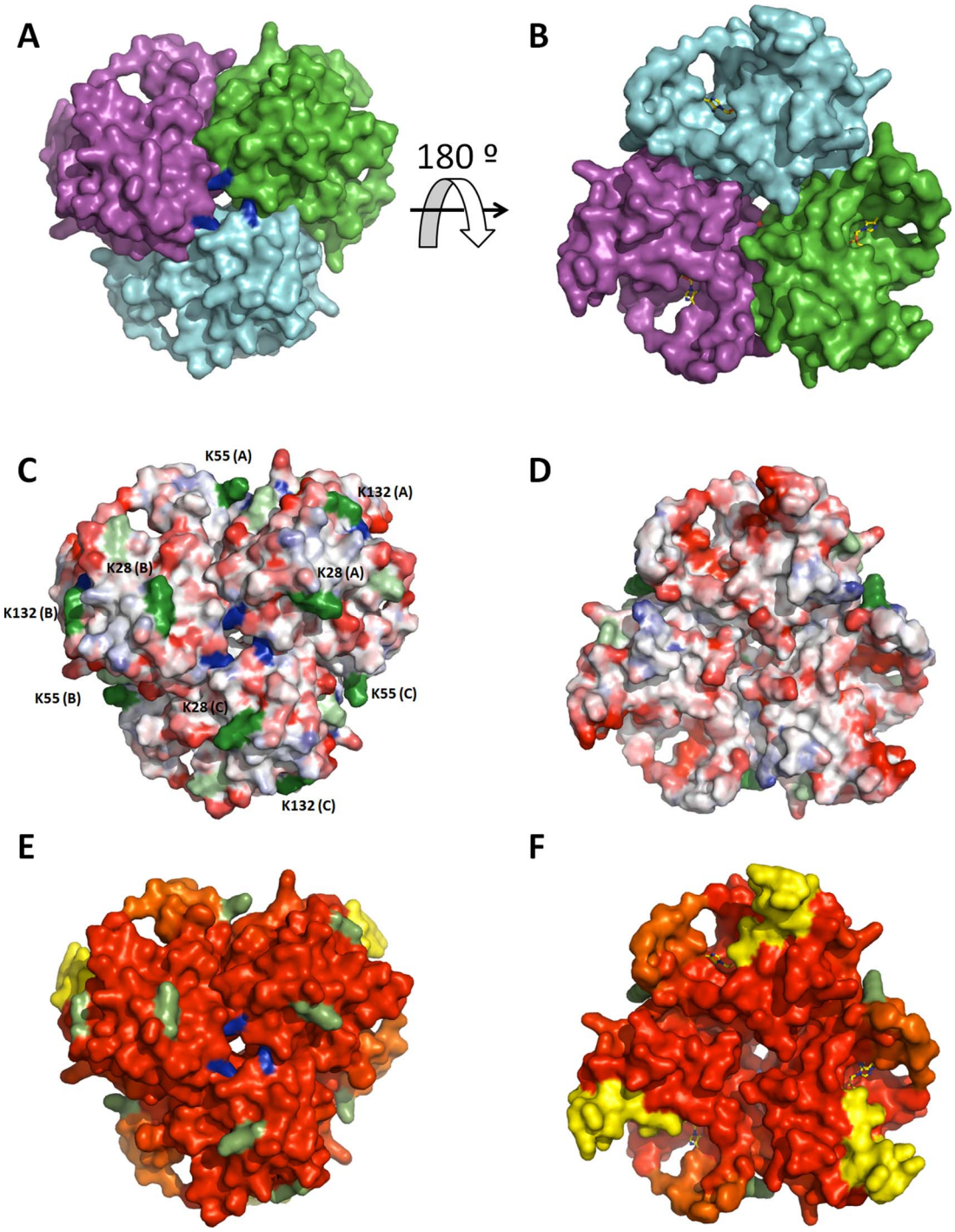

Fig. 3. Structural modeling and representation of different physic-chemical properties of *La*NDT. The multimeric model is formed by three subunits colored in purple, green and cyan. The immobilization face with the N-terminus colored in blue (A, C and E), and the active site face with the substrate bound (yellow sticks) extracted from the template protein, PDB: 1SG2 (B, D and F). Electrostatic surface of the trimmer represents the basic regions in blue, the acid ones in red (C, D). Local flexibility of the structural model (E and F) calculated shown rigid and flexible residues that are colored in red and yellow respectively. Orange color represents moderately stable regions. In B-D, the N-terminus of each subunit are colored in blue while lysines of each subunit with pKa lower than 10.5 and clearly exposed to the medium are colored in green (K28, K55 and K132). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

affinity immobilization (Ni^{2+}-*La*NDT) was assayed, but a decrease of around 50% of enzymatic activity was observed when Ni^{2+}-Gx-*La*NDT derivative was used. However, the reusability test of the covalent biocatalyst Ni^{2+}-Gx-*La*NDT showed 300 h of successive batches, without significant loss of its initial activity, while the biocatalyst Ni^{2+}-*La*NDT maintained its activity only for 20 reuses, and then its activity drastically decreased until been completely inactivated after 22 h (Fig. 2A). In this way, although the covalent immobilization has affected the enzymatic activity, it is possible to obtain a robust biocatalyst with improved stability that allowed to significantly increase the productivity of the bioprocess (Table 1).

Likewise, when storage stability at 4 °C was evaluated, Ni^{2+}-Gx-*La*NDT retained full activity for more than 78 days, whereas Ni^{2+}-*La*NDT was inactive after 65 days (Fig. 2B). Moreover, it was found that Ni^{2+}-Gx-*La*NDT biocatalyst was stable after 250 h of incubation at 50 °C, a condition in which the ionic biocatalyst released part of its

Table 2
Biocatalysts based on stabilized NDTs.

NDT Source	Biocatalyst[a]	Support	Specific Activity (U/g)[b]	References
L. animalis	R	Hetero-functional (Ni^{2+}-Gx)	356	This study[c]
L. animalis	N	DEAE-agarosa	162	Britos et al. (2016)
L. reuteri	R	EC-EP	1.46	Fernández-Lucas and Arroyo (2011)

[a] Recombinant enzyme (R) and native enzyme (N).
[b] Enzyme activity (U) was calculated as μmol/min.
[c] Reaction conditions for floxuridine biosynthesis: 50 mg of derivative (Ni^{2+}-Gx-*La*NDT) in 1 ml reaction volume; 6 mM dThd, 2 mM 5FUra, Tris-HCl buffer (20 mM, pH 7), 30 °C, 1 h.

enzymatic charge during the first 6 h of incubation (data not shown). These results evidence the greater stabilization reached using site-selective and multi-valent covalent immobilization, in which the multipoint attachment avoided the enzyme leakage at 50 °C and increased both storage and operational stability.

Structural modeling of *La*NDT (Arnold et al., 2006; Benkert et al., 2011; Biasini et al., 2014) allowed us understanding from a rational point of view the effect of the immobilization on some of the physic-chemical properties of the immobilized NDT (Fig. 3). In the model structure, the three N-terminus of each subunit colocalize in the same face of the multimeric assembly, which suggests that one orientation will prevail over the others when the enzyme is immobilized through the coordination chemistry between its histidine tags and the metal chelates at the carrier surface. Then, the presence of aldehyde groups on the same surface will promote a multivalent and covalent attachment through the exposed lysine residues located in the same face as the N-terminus. Out of the 10 lysines per subunit, 5 lysines (K17, K28, K55, K132 and K149) located in the immobilization face were fairly exposed to the media. Moreover, the apparent pKa value of all these lysine residues has been estimated to be lower than 10.5, which guarantees their reactivity under alkaline conditions (pH10.0) during the post-immobilization process on the hetero-functional carriers once the enzyme has been oriented.

Finally, the orientation of *La*NDT multimer on Ni^{2+}-Gx-support seems to be optimal for substrate binding and catalysis since the immobilization face is just the face opposite the active site region, allowing the free diffusion of the substrates to the active centers and leaving flexible the active site loops (Q81-T91) that are required for the catalysis. Additionally, such orientation enables the establishment of covalent bonds with K56 and the support surface that may rigidify a moderately flexible region (E47-F55), which may contribute to increasing the global thermal stability of the protein once it is immobilized and the covalent and irreversible bonds have been promoted.

The environmental factor (E-factor) is a measure of the environmental impact caused by industries. E-factor values are around 25–100 for pharmacological compounds. Furthermore, Carbon efficiency (C-efficiency) and atom economy (A-economy) were designed as parameters to evaluate the efficiency of chemical synthesis. In this work, an E-factor of 3.53 was obtained for floxuridine biotransformation catalyzed by Ni^{2+}-Gx-*La*NDT, showing mass utilization efficiency and a significant decrease of waste production.

Besides, a C-efficiency of 64% and an A-economy of 66% were obtained, according with those results reported by Cappa and col (Cappa et al., 2014), which imply a positive effect on atom recovery and good process performance.

In this work a highly efficient biocatalyst based on a novel NDT immobilized on a hetero-functional support that allowed the orientation and rigidification of this enzyme, was developed. This stabilized enzymatic biocatalyst showed a specific activity of 356 μmol/min.g of protein in floxuridine biosynthesis after one hour of reaction which is significantly higher than that reported in previous works where NDT was used as immobilized biocatalysts (Table 2). Besides, the obtained biocatalyst shows an improved productivity compared to the floxuridine biosynthesis previously reported where whole cells or cell extracts immobilized by different methodologies were used.

Therefore, this biocatalyst meets the requirements of high activity, stability, and short reaction times needed for low-cost production in a future preparative application.

Finally, a smooth and green bioprocess has been designed for obtaining antitumoral compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jbiotec.2018.01.011.

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