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BIOCATALYSIS

Magnetic chitosan beads for covalent immobilization of nucleoside 2′-deoxyribosyltransferase: application in nucleoside analogues synthesis

Jesús Fernández-Lucas · Ruth Harris · Iria Mata-Casar · Angeles Heras · Isabel de la Mata · Miguel Arroyo

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Abstract Cross-linked magnetic chitosan beads were prepared in presence of epichlorohydrin under alkaline conditions, and subsequently incubated with glutaraldehyde in order to obtain an activated support for covalent attachment of nucleoside 2′-deoxyribosyltransferase from *Lactobacillus reuteri* (*Lr*NDT). Changing the amount of magnetite (Fe_3O_4) and epichlorohydrin (EPI) led to different macroscopic beads to be used as supports for enzyme immobilization, whose morphology and properties were characterized by scanning electron microscopy, spin electron resonance (ESR), and vibrating sample magnetometry (VSM). Once activated with glutaraldehyde, the best support was chosen after evaluation of immobilization yield and product yield in the synthesis of thymidine from 2′-deoxyuridine and thymine. In addition, optimal conditions for highest activity of immobilized *Lr*NDT on magnetic chitosan were determined by response surface methodology (RSM). Immobilized biocatalyst retained 50 % of its maximal activity after 56.3 h at 60 °C, whereas 100 % activity was observed after storage at 40 °C for 144 h. This novel immobilized biocatalyst has been successfully employed in the enzymatic synthesis of 2′-deoxyribonucleoside analogues as well as arabinosyl-nucleosides such as vidarabine (ara-A) and cytarabine (ara-C). Furthermore, this is the first report which describes the enzymatic synthesis of these arabinosyl-nucleosides catalyzed by an immobilized nucleoside 2′-deoxyribosyltransferase. Finally, the attached enzyme to magnetic chitosan beads could be easily recovered and recycled for 30 consecutive batch reactions with negligible loss of catalytic activity in the synthesis of 2,6-diaminopurine-2′-deoxyriboside and 5-trifluorothymidine.

Keywords Nucleoside 2′-deoxyribosyltransferase · *Lactobacillus reuteri* · Immobilization · Chitosan · Magnetic

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J. Fernández-Lucas · I. Mata-Casar · I. de la Mata · M. Arroyo (✉)
Departamento de Bioquímica y Biología Molecular I. Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, c/José Antonio Novais 2, 28040 Madrid, Spain
e-mail: arroyo@bbm1.ucm.es

J. Fernández-Lucas
Departmento de Farmacia y Biotecnología, Facultad de Ciencias Biomédicas, Universidad Europea, Urbanización El Bosque, c/Tajo s/n, 28670 Villaviciosa de Odón, Madrid, Spain

R. Harris · A. Heras
Departamento de Química Física II. Facultad de Farmacia. Instituto de Estudios Biofuncionales, Universidad Complutense de Madrid, Paseo Juan XXIII n°1, 28040 Madrid, Spain

Introduction

The major drawbacks of the application of enzymes in industry are their low thermostability and recycling. Enzyme immobilization may overcome these cost-effective problems by enhancing protein stability, facilitating separation of products, and allowing biocatalyst reuse in various reactor configurations. There are plenty of methods for enzyme immobilization, but covalent binding to solid supports is actually the best approach from an industrial point of view, since immobilized biocatalysts are easy to separate from the reaction medium, enzyme desorption from the support is prevented and tight attachment to the carrier may promote a strong stabilization effect under a

wide range of experimental conditions [25]. Many polymeric supports including natural biopolymers and synthetic materials have been prepared in different geometric forms and chemically activated for covalent immobilization of enzymes [30]. Among natural biopolymers, chitin and chitosan are promising supports for enzyme immobilization that have allowed the preparation of many useful biocatalysts [3, 5, 20].

Chitin is the major constituent of the shells of crustaceans, the exoskeletons of insects, and the cell walls of fungi, being the second most abundant biopolymer in nature after cellulose, and one of the most renewable organic resources. Chemically, chitin is a linear polysaccharide composed by $\beta-(1 \rightarrow 4)$ linked residues of N-acetyl-2-amino-2-deoxy-D-glucose (or N-acetyl-D-glucosamine) which can be partially deacetylated. In this sense, chitosan can be considered as deacetylated chitin with at least 60 % of 2-amino-2-deoxy-D-glucose residues in the biomacromolecule. Chitin is obtained at relative low cost from wastes of seafood processing industry, after deproteinization of raw shellfish material (mainly crabs, shrimps, lobsters, and krills) with dilute NaOH solution and decalcification with dilute HCl solution. The obtained chitin is randomly *N*-deacetylated to chitosan by treatment with a 40–45 % NaOH solution, followed by purification procedures. In contrast to chitin, the presence of free amine groups along the chitosan chain allows this biopolymer to be dissolved in diluted aqueous acidic solutions at $pH < 6.5$ due to the protonation of these groups, rendering one of the few found cationic polyelectrolytes in nature. Both the solubility in acidic solutions and aggregation with polyanionic compounds impart chitosan with excellent gel-forming capacity [4]. Apart from these chemical and biological properties, chitosan actually offers an extraordinary potential for many biotechnological and medical applications since it is also highly biocompatible, non-toxic, and biodegradable [16, 31]. As enzyme immobilization support, chitosan is commonly manufactured as beads which are prepared by the neutralization method [20]. This strategy is based on the fact that chitosan dissolves readily in dilute solutions of most organic acids to form viscous solutions that precipitate into an alkaline solution. Afterwards, enzyme immobilization is achieved by binding onto gel surface by adsorption, reticulation or covalent binding.

Recently, enzyme binding to magnetic supports has appeared as an alternative method that renders immobilized biocatalysts which are easily separated from the reaction medium and could be employed in fluidized bed reactors which are stabilized by applying a magnetic field. Magnetic supports can be manufactured from inorganic materials, but natural or synthetic polymers are preferred, since they have a wider variety of functional groups which can be tailored for selective binding. As a matter of fact, magnetic chitosan supports have recently received great attention for immobilization of many enzymes with potential biotechnological applications in food industry [35], intermediate synthesis [15], biosensors [19, 21], as well as for removal of contaminants from wastewaters [7, 33].

In this work, covalent immobilization of nucleoside 2′-deoxyribosyltransferase (EC 2.4.2.6) from *Lactobacillus reuteri* in glutaraldehyde-activated magnetic chitosan beads is described, as well as the application of the obtained immobilized biocatalyst in the enzymatic synthesis of nucleoside analogues. These molecules are extensively used for antiviral and anticancer therapy due to their ability to act as reverse transcriptase inhibitors or chain terminators in RNA or DNA synthesis [1, 10]. Biocatalysis is a promising and eco-friendly alternative to traditional chemical synthesis of nucleoside analogues [23, 27], and the choice of one-step enzyme-catalyzed transglycosylations performed by nucleoside 2′-deoxyribosyltransferases [14] is actually far more advantageous than the use of nucleoside phosphorylases, which needs the two-step performance of both pyrimidine and purine nucleoside phosphorylases in the reaction [22].

Materials and methods

Materials

Adenine, thymine, thymidine, magnetite (Fe_3O_4), epichlorohydrin, and glutaraldehyde were purchased from Sigma. Cytosine and uracil were purchased from Aldrich. Chitosan (644 kDa mean molecular weight, and 90 % deacetylating degree) was supplied by Primex (Iceland). 2′-Deoxyadenosine, 2′-deoxyuridine, and arabinosylnucleosides were a gift from Pro.Bio.Sint (Varese, Italy). 2′-Fluoro-2′-deoxyuridine was kindly donated by Rasayan Inc. (Encinitas, CA, USA). Bovine serum albumin (BSA) was supplied by Serva (Germany).

2′-Deoxyribosyltransferase production

Recombinant NDT from *Lactobacillus reuteri* was produced from recombinant *E. coli* CECT 7435 and purified as described [12]. The specific activity of the pure enzyme was 103 IU/mg of protein in the standard synthetic reaction of 2′-deoxyadenosine from 2′-deoxyuridine and adenine according to the standard assay described elsewhere [12].

Preparation of activated magnetic chitosan beads

Magnetic chitosan beads were prepared by a phase-inversion technique according to a modified protocol described

elsewhere [7]. Magnetite (75 and 150 mg) was added to 10 mL of 3.0 % (w/v) powder chitosan dissolved in 4.0 % (v/v) acetic acid solution, and stirred until a homogenous suspension with different Fe_3O_4:chitosan (w:w) weight ratios were obtained (1:4 and 1:2). Three milliliters of the resulting chitosan suspension containing magnetite were transferred into a syringe and introduced into a sodium hydroxide solution (2.0 M, 50 mL) dropwise while stirring continuously with a magnetic stirrer. Then, stirring was continued for 2.0 h for curing, and afterwards the formed magnetic chitosan beads were filtered and washed twice with 50 mL of distilled water. Cross-linking was attained by immersing the magnetic chitosan beads (2.5 g) into 25 mL sodium hydroxide solution (1.0 M) containing epichlorohydrin (2.0 or 5.0 %) at 50 °C for 2.0 h while stirring with a magnetic stirrer at 200 rpm. The resulting cross-linked chitosan beads were stirred in 50 mL refrigerated cold ethanol for 30 min, and then washed extensively with distilled water to remove any possible impurities. Activation of the support was accomplished by contacting 1.0 g magnetic chitosan beads with 4.0 mL potassium phosphate buffer 0.1 M pH 7.0 containing 2.5 % (*w/v*) glutaraldehyde during 2.0 h at 25 °C, and then washed extensively with distilled water to remove the excess of the activating agent. Finally, the wet activated beads were stored at 4 °C prior to use.

Properties of magnetic chitosan beads

Electron spin resonance (ESR) spectroscopy was carried out with a Bruker ESP 300E spectrometer at room temperature (see the Online resource 1 for further details). The magnetization of magnetic chitosan beads were measured on a vibrating sampling magnetometer (VSM) 7304 (LakeShore Co.) at room temperature. The shape and surface of beads were observed by scanning electron microscopy (SEM). The samples were examined using a scanning electron microscope (JEOL JSM-6400, Tokyo, Japan).

Determination of oxirane content on chitosan supports

Analysis of oxirane groups were performed as described elsewhere [2]. The release of hydroxyl groups was followed by titration with 0.1 M hydrochloric acid. Oxirane in solution was determined as follows: the oxirane-containing chitosan support (100 mg) was added to 15 mL of 1.3 M sodium thiosulphate solution at pH 7.0 (by addition of hydrochloric acid) until the reaction was over. The amount of oxirane present in the solution was then calculated from the amount of hydrochloric acid needed to maintain neutrality.

Immobilization of bovine serum albumin (BSA) on magnetic chitosan beads

Glutaraldehyde-activated magnetic chitosan beads (100 mg of wet weight) were transferred into 0.3 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing bovine serum albumin (BSA) at 0.1 mg/mL. While orbital shaking at 350 rpm, protein was maintained in contact with the activated support for 5 h at 25 °C, and finally beads were filtered from the reaction medium. The residual BSA found in the filtrate and washing solutions was determined by the Coomassie Blue method [9].

Immobilization of nucleoside 2′-deoxyribosyltransferase on magnetic chitosan beads

Immobilization process was performed as described above, but using enzyme solutions at different concentrations (from 0.06 to 0.3 mg/mL) in 0.1 M potassium phosphate buffer pH 7.0. In order to analyze the efficiency of the immobilization process, immobilization yield was calculated. Immobilization yield was considered as the relative amount of bound protein to the support with respect to the initial amount of enzyme prior to the immobilization process, and it was calculated as the difference between protein content in the initial solution offered to the support and residual protein found in the filtrate and washing solutions obtained after the immobilization process. All protein determinations were carried out by the Coomassie Blue method [9].

General procedure for the enzymatic synthesis of thymidine

Synthesis of thymidine from 2′-deoxyuridine and thymine catalyzed by immobilized *Lr*NDT was established as standard reaction during the optimization of the immobilization process. Enzymatic activity of immobilized enzyme was measured using one bead of immobilized biocatalyst which was added to 175 µl solution containing 16 mM 2′-deoxyuridine and 16 mM thymidine in 50 mM MES buffer pH 6.5. Reaction mixture was incubated at 40 °C for 10 min at 350 rpm orbital shaking. After this reaction time, 50 µl aliquot of the assay mixture was withdrawn, mixed with 50 µl of cold methanol (4 °C), and further heated at 95 °C for 5 min. Then, this solution was centrifuged at 9,000× *g* for 2 min, and the supernatant was analyzed by HPLC to quantitatively measure the reaction products as described below in the analytical methods. All determinations were carried out by triplicate and the maximum error was below 5 %.

Substrate specificity of immobilized 2′-deoxyribosyltransferase

Enzymatic activity was measured using 14.3 μg of (free or immobilized) enzyme which was added to 1.4 mL solution containing 10 mM 2′-deoxyribonucleoside (sugar donor) and 10 mM base acceptor in 50 mM MES buffer pH 6.5. Reaction mixture was incubated at 60 °C for 5 min at 350 rpm orbital shaking. After this reaction time, 50 μl aliquot of the assay mixture was withdrawn, mixed with 50 μl of cold methanol (4 °C), and further heated at 95 °C for 5 min. Then, this solution was centrifuged at 9,000× *g* for 2 min, and the supernatant was analyzed by HPLC to quantitatively measure the reaction products as described below in the analytical methods. All determinations were carried out by triplicate and the maximum error was below 5 %. In such conditions, one international activity unit (IU) was defined as the amount of enzyme producing 1 μmol/min of 2′-deoxyriboside product under the assay conditions described above. The recovery (also called retained activity) was calculated as the relative activity of the immobilized biocatalyst with respect to the same amount of native soluble enzyme which contains the enzymatic derivative under the same standard assay conditions.

Thermal inactivation of immobilized 2′-deoxyribosyltransferase

Samples of 17 mg of immobilized 2′-deoxyribosyltransferase derivative were incubated at 40 and 60 °C in 200 μl of 50 mM MES buffer pH 6.5 at different storage times. Then, the biocatalyst was filtrated and assayed for synthesis of thymidine from 2′-deoxyuridine and thymine as described above. Experimental plots of residual activity versus storage time were fitted to exponential decays by using the SigmaPlot 11.0 program (Systat Software Inc., Point Richmond, CA, USA).

Enzymatic synthesis of 2′-deoxyribonucleosides and arabinosyl-nucleosides analogues

Enzymatic synthesis of nucleoside analogues from 2′-deoxyuridine and non-natural bases (2,6-diaminopurine and trifluorothymine) were carried out in 50 mM MES buffer pH 6.5 at 40 °C and 350 rpm orbital shaking for 2 h. On other hand, other nucleoside analogues were synthesized from non-natural 2′-deoxyribonucleosides (2′-fluoro-2′-deoxyuridine and ara-U) and natural bases (cytosine and adenine) in 50 mM MES buffer pH 6.5 at 40 °C and 350 rpm orbital shaking for different reaction times. Once every reaction was finished, 50 μl aliquot of the assay mixture was withdrawn, mixed with 50 μl of cold methanol (4 °C), and further heated at 95 °C for 5 min. Then, this solution was centrifuged at 9,000× *g* for 2 min, and the supernatant was analyzed by HPLC to quantitatively measure the reaction products as described below in the analytical methods. All determinations were carried out by triplicate and the maximum error was below 5 %.

Recycling of immobilized 2′-deoxyribosyltransferase

Immobilized biocatalyst (17 mg) was evaluated for 2′-deoxyribosyltransferase activity after repeated performance of the same biocatalyst in the synthesis of 2,6-diaminopurine-2′-deoxyriboside and 5-trifluorothymidine. Immobilized biocatalyst was added to a 100 μl solution containing 1 mM 2′-deoxyuridine and 1 mM of the corresponding nucleobase concentration in 50 mM MES buffer pH 6.5. After enzymatic reaction for 2 h at 40 °C and 350 rpm shaking, 50 μl aliquot of the assay mixture was withdrawn, mixed with 50 μl of cold methanol (4 °C), and further heated at 95 °C for 5 min. Then, this solution was centrifuged at 9,000× *g* for 2 min, and the supernatant was analyzed by HPLC to quantitatively measure the reaction products as described below in the analytical methods. Then, the recovered immobilized enzyme was washed three times with freshly prepared 50 mM MES buffer pH 6.5, and then used for another conversion cycle.

Analytical methods

The production of nucleosides was quantitatively measured by HPLC (Agilent 1100 series). Reactions were analyzed with a LUNA C18(2) column 5 μm, 250 × 4.6 mm (Phenomenex, USA) using the following mobile phase: (i) a lineal gradient (time = 10 min) from 0.1 M trimethylammonium acetate to 90/10 (v/v) 0.1 M trimethylammonium acetate/acetonitrile, (ii) 10 min: 90/10 (v/v) 0.1 M trimethylammonium acetate/acetonitrile. The flow rate was fixed at 1 ml/min (180 bar pressure) and the UV detector was set at 254 nm. Retention times for the different bases were (hereafter abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature): adenine (Ade), 10.14 min; uracil (Ura), 5.41 min; cytosine (Cyt), 4.14 min; thymine (Thy), 9.68 min; 2,6-diaminopurine (2,6-DAP), 8.71 min; 5-trifluorothymine (5-tFThy), 12.6 min. Retention times for the different natural nucleosides were as follows: 2′-deoxyuridine (dUrd), 9.16 min; 2′-deoxyadenosine (dAdo), 15.50 min; 2′-deoxycytidine (dCyd), 8.22 min; thymidine (dThd), 13.25 min. Retention times for the different non natural nucleosides were as follows: 2,6-diaminopurine-2′-deoxyriboside (2,6-DAPdRib), 14.29 min; 5-trifluorothymidine (5-tFdThd), 18.0 min; 2′-fluoro-2′-deoxyuridine (2′-FdUrd), 10.3 min; 2′-fluoro-2′-deoxycitydine (2′-FdCyd), 8.7 min; ara-uracil (ara-U), 8.68 min; and ara-adenine (ara-A), 13.9 min.

Results and discussion

Characterization of magnetic chitosan beads

Immobilized enzyme bioreactors based on magnetizable particles or beads under the influence of an external magnetic field show several advantages [7], including a rapid recovery of the immobilized enzyme from the reaction mixture. A physical characterization of magnetic particles is essential in order to establish those optimal conditions in the bioreactor. In this work, magnetic chitosan beads were prepared by phase inversion technique in the presence of epichlorohydrin under alkaline conditions. Firstly, solid chitosan was dissolved in a diluted acidic solution (in absence or presence of different amounts of magnetite) to form viscous solutions which precipitated as macroscopic beads into a 2.0-M NaOH solution when added dropwise. Afterwards, these chitosan beads were subjected to cross-linking with an epichlorohydrin solution at different concentrations (2.0 or 5.0 %). As a result of this process, different wet beads were obtained (Table 1) whose average size was approximately 2–3 mm. SEM images (Fig. 1) of both magnetic and bare chitosan beads (hereafter MC and BC beads, respectively) showed their morphology, which displayed spherical or pear shape. In the case of chitosan beads obtained with magnetite, the observed morphology suggests that iron oxide is embedded in the cross-linked chitosan matrix. Likewise, chitosan cross-linking with 5 % epicholorohydrin showed the appearance of many breakages in the surface (Fig. 1b, d, f), whereas a lower reticulation degree prevented the formation of such holes and the beads showed a smooth surface (Fig. 1a, c, e). An important parameter for the practical application of magnetic beads is their magnetization. In this sense, magnetic properties of such beads were confirmed with electron spin resonance spectroscopy (ESR) at room temperature (Fig. 2). When measuring intensity versus magnetic field (Gauss), bare chitosan beads showed a negative high intensity ferromagnetic resonance signal at 9,400 G magnetic field, and a positive low intensity ferromagnetic resonance signal at 8,300 G magnetic field. Such signals were more intense when magnetite was embedded in chitosan beads. In addition, these magnetic chitosan beads spectra showed a typical signal at lower fields which were not present in the bare beads. In this sense, maxima at 2,560 and 2,800 G were found in ESR spectra of magnetic chitosan beads prepared with a 1:4 magnetite:chitosan weight ratio (MC1 and MC2) and 1:2 weight ratio (MC3 and MC4), respectively. In literature, such magnetic field values may vary up to 20,000 G for various applications [7], indicating that our chitosan magnetic beads need less magnetic intensity to excite all the dipole moments. The *g*-factor (or spectroscopic manifestation of the magnetic moment) can be considered as a key characteristic of a magnetic material. Moreover, measurement of the *g*-factor for an unknown signal can be a valuable aid in the identification of a signal (see Online resource 1). In this study, the *g*-factor was calculated from the ESR spectra and found to be 2.51 for magnetic chitosan beads containing a lower amount of magnetite, whereas *g*-factor was 2.75 when magnetite content was higher (Table 1). In literature, *g*-factor for Fe(III) is ranged from 1.4 to 3.1 for low-spin, and ranged from 2.0 to 9.7 for high-spin complexes [32]. To further characterize the magnetic properties of MC beads, magnetization (*M*, emu/g) was measured when applied magnetic field was changed. Magnetization curves for all MC beads series are shown in Fig. 3, and saturation magnetization intensities (SMI) calculated from the hysteresis loops are summarized in Table 1. In this sense, SMI values of the supports were influenced by the amount of magnetite, as well as by the epichlorohydrin concentration, employed during their preparation. The highest SMI values were obtained with MC1 beads (3.35 emu/g) and MC3 beads (5.07 emu/g), which were prepared with increasing amounts of iron oxide (25 and 50 % embedded magnetite, respectively), but lower reticulation degree (cross-linked

Table 1 Properties of magnetic chitosan beads

Magnetic chitosan bead	Fe_3O_4:chitosan weight ratio (w:w)[a]	Conditions for chitosan cross-linking with epichlorohydrin[b] (%)	*Hr* (Hz)	*g*	SMI (emu/g)	Wet weight (mg/particle)[c]	Oxirane content (μmol/g)[d]
MC1	1:4	2	2800	2.51	3.35	13.8 ± 1.3	152
MC2	1:4	5	2800	2.51	0.69	14.0 ± 1.9	95
MC3	1:2	2	2560	2.75	5.07	15.7 ± 1.3	153
MC4	1:2	5	2560	2.75	1.03	15.5 ± 1.1	99

[a] See "Materials and methods" section for more details referred to magnetite:chitosan weight ratio and epichlorohydrin concentration employed in cross-linking

[b] *SMI* saturation magnetization intensity

[c] Mean value of ten weight determinations

[d] Oxirane content on supports were determined according to "Materials and methods"

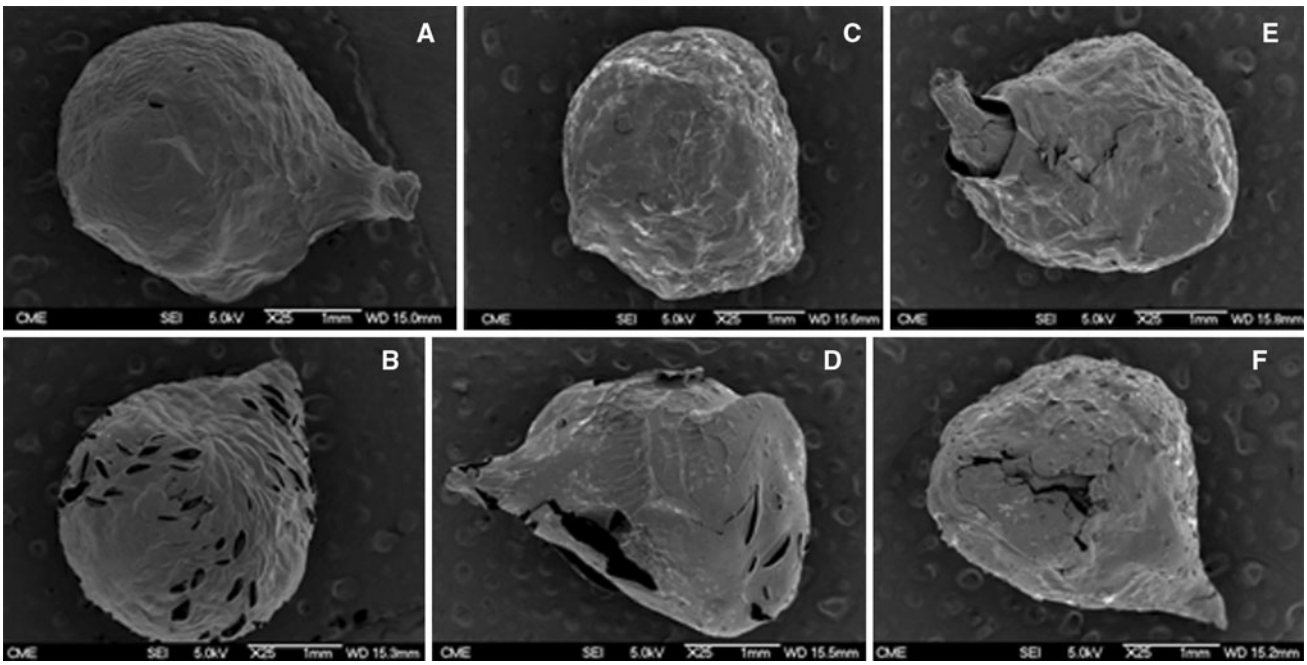


Fig. 1 SEM images of the surface morphology and structure of bare and magnetic chitosan beads prepared with different magnetite:chitosan weight ratios and crosslinked with epichlorohydrin at different concentrations: **a** 1:4 ratio and 2 % epichlorohydrin (MC1 beads); **b** 1:4 ratio and 5 % epichlorohydrin (MC2 beads); **c** 1:2 ratio and 2 % epichlorohydrin (MC3 beads); **d** 1:2 ratio and 5 % epichlorohydrin (MC4 beads); **e** no magnetite and 2 % epichlorohydrin; **f** no magnetite and 5 % epichlorohydrin

chitosan with 2 % epichlorohydrin). Interestingly, the SMI result obtained with MC4 beads (1.03 emu/g) was lower than the one obtained for MC1 even though the support contained a higher amount of magnetite, suggesting that a higher reticulation degree of the beads could diminish their magnetic properties. When a magnetic field was applied, magnetic beads could joined to each other and be stirred without the help of a conventional magnetic stirrer (see Online Resource 2).

Immobilization of recombinant 2′-deoxyribosyltransferase on activated chitosan beads

Epichlorohydrin shows epoxy and chloride groups (being the latter one more reactive), whereas chitosan has amine and hydroxyl groups, the former being more reactive than hydroxyl. As a consequence, epichlorohydrin displays a cross-linking effect on chitosan (Scheme 1), but it may also react with hydroxyl or amine groups of the biopolymer to generate reactive epoxide groups which can be used for enzyme immobilization as described in literature [7]. As a matter of fact, some remnant epoxide groups in our cross-linked chitosan supports were still available to react with free amines from amino acid residues, as observed in Table 1. However, when immobilization of bovine serum albumin (BSA, a standard protein with many lysine residues) was performed with all these supports at different alkaline pH values, no protein could be covalently linked to any support. Thus, another approach was followed taking into account that only few chitosan amine groups react with epichlorohydrin under mild basic conditions [34] such as the ones described in our protocol and then, many intact amine groups on cross-linked chitosan beads could be activated with glutaraldehyde. This bi-functional reagent allows the generation of aldehyde groups in the support that can react with amine groups (terminal and in lysine residues) of the protein surface to form Schiff bases, thus leading to the covalent immobilization to the carrier (Scheme 1). Moreover, immobilization on supports activated with glutaraldehyde is usually performed at a pH range from 7.0 to 8.5, since these aldehyde groups are very unstable at higher pH values [24]. Despite the low reactivity of ε-amino of lysine residues at neutral pH value, BSA immobilization was successfully carried out at pH 7.0. In fact, those supports prepared with the lowest epichlorohydrin concentration (GA-MC1 and GA-MC3) linked at least 0.45 mg BSA per gram of wet support after 3.5 h of immobilization time, whereas loading capacity was slightly diminished to 0.37 mg BSA per gram of wet support employing cross-linked chitosan with higher reticulation degree (GA-MC2 and GA-MC4). Such difference could be explained as hindered access of the protein to aldehyde groups of the support. These preliminary results encouraged us to proceed with the immobilization of nucleoside 2′-deoxyribosyltransferase from *L. reuteri* (*Lr*NDT) in the same conditions, which were very

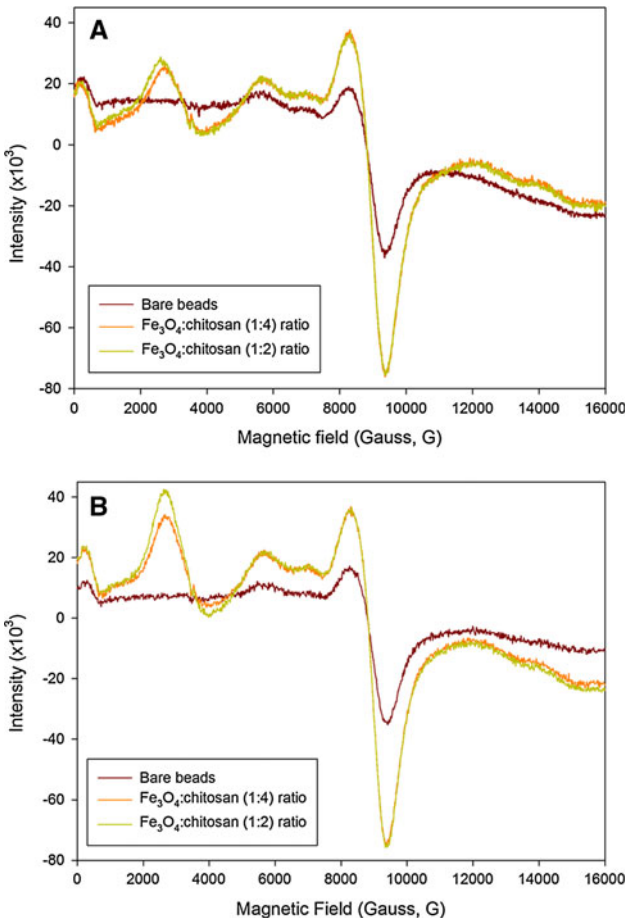


Fig. 2 ESR spectra of bare and magnetic chitosan beads prepared with different magnetite:chitosan weight ratios and crosslinked with epichlorohydrin at different concentrations: **a** 2 % epichlorohydrin; **b** 5 % epichlorohydrin

convenient due to enzyme instability at alkaline pH [12]. As a result of this strategy, several immobilized *Lr*NDT biocatalysts were prepared by using cross-linked chitosan with lower reticulation degree and increasing the amount of enzyme in the immobilization process. As shown in Table 2, immobilization yield was diminished when the enzyme concentration of the solution offered to the carrier was increased, indicating that the maximum enzyme loading capacity of the support was approximately 0.7 mg of *Lr*NDT per gram of wet support. In all cases, the specific enzymatic activity reached a value of 1.8–2.1 IU/g of wet carrier, and yield in nucleoside was approximately 18–20 % at 5 min reaction time.

Optimum pH and temperature of immobilized 2′-deoxyribosyltransferase

Taking into account the morphology, magnetic properties and loading capacity of the support, MC*Lr*NDT4 biocatalyst (immobilized enzyme on GA-MC3 support prepared with the lowest amount of enzyme in the immobilization process) was chosen for further functional characterization. In this sense, optimal conditions for best performance were studied by using the response surface methodology (RSM), which allows the reduction of the number of experiments without neglecting the interaction between the various parameters which affect the activity [8]. A central composite design (CCD) of experiments was used to determine the effects of independent variables (pH and temperature) on the dependent variable (immobilized enzyme activity). The process variables used in the experimental design, coded levels, results obtained for the activity of the immobilized enzyme, and analysis of variance are shown in the Online resource 1. The goodness of fit of the model was checked using the coefficient of determination ($R^2 = 0.882$), indicating that 11.8 % of the total variation was not explained by the model. RSM yielded the following regression equation:

$$Y = 2.78417 + 0.2962 \times T + 0.0216 \times \text{pH} - 0.0727143 \times T^2 - 0.0068 \times T \times \text{pH} - 0.124571 \times \text{pH}^2$$

where Y is the predicted response for biocatalyst activity in the synthesis of thymidine (IU/g), and pH and T are the coded values for pH and temperature. The RSM for immobilized enzyme activity as a function of pH and temperature is depicted in Fig. 4. These results confirmed that highest nucleoside 2′-deoxyribosyltransferase activity displayed by immobilized biocatalyst occurred at 60 °C and pH 6.5.

Thermal inactivation of immobilized 2′-deoxyribosyltransferase

Thermal inactivation of immobilized biocatalyst MC*Lr*NDT4 was studied at two different temperatures (40 and 60 °C) and pH 6.5 (Fig. 5). Immobilized enzyme suffered no loss of enzymatic activity when stored at 40 °C for at least 144 h. On other hand, the obtained experimental deactivation curve at 60 °C was adjusted to a single exponential decay and explained according to a deactivation model proposed by Henley and Sadana [17]. This model involves two different enzymatic states, an active (E) and a deactivated state (E_d), where k_d is the first-order deactivation rate coefficient.

$$E \xrightarrow{k_d} E_d \quad (1)$$

Thus, first-order deactivation rate coefficient ($k_d = 0.0123\ h^{-1}$) and half-life ($t_{1/2} = 56.3$ h) of the immobilized enzyme at 60 °C could be calculated. These results confirmed that covalent attachment of *Lr*NDT to glutaraldehyde-activated chitosan magnetic beads lead to a thermal stabilization of the enzyme at 60 °C. Taking into account that the reported half-life of the free enzyme was

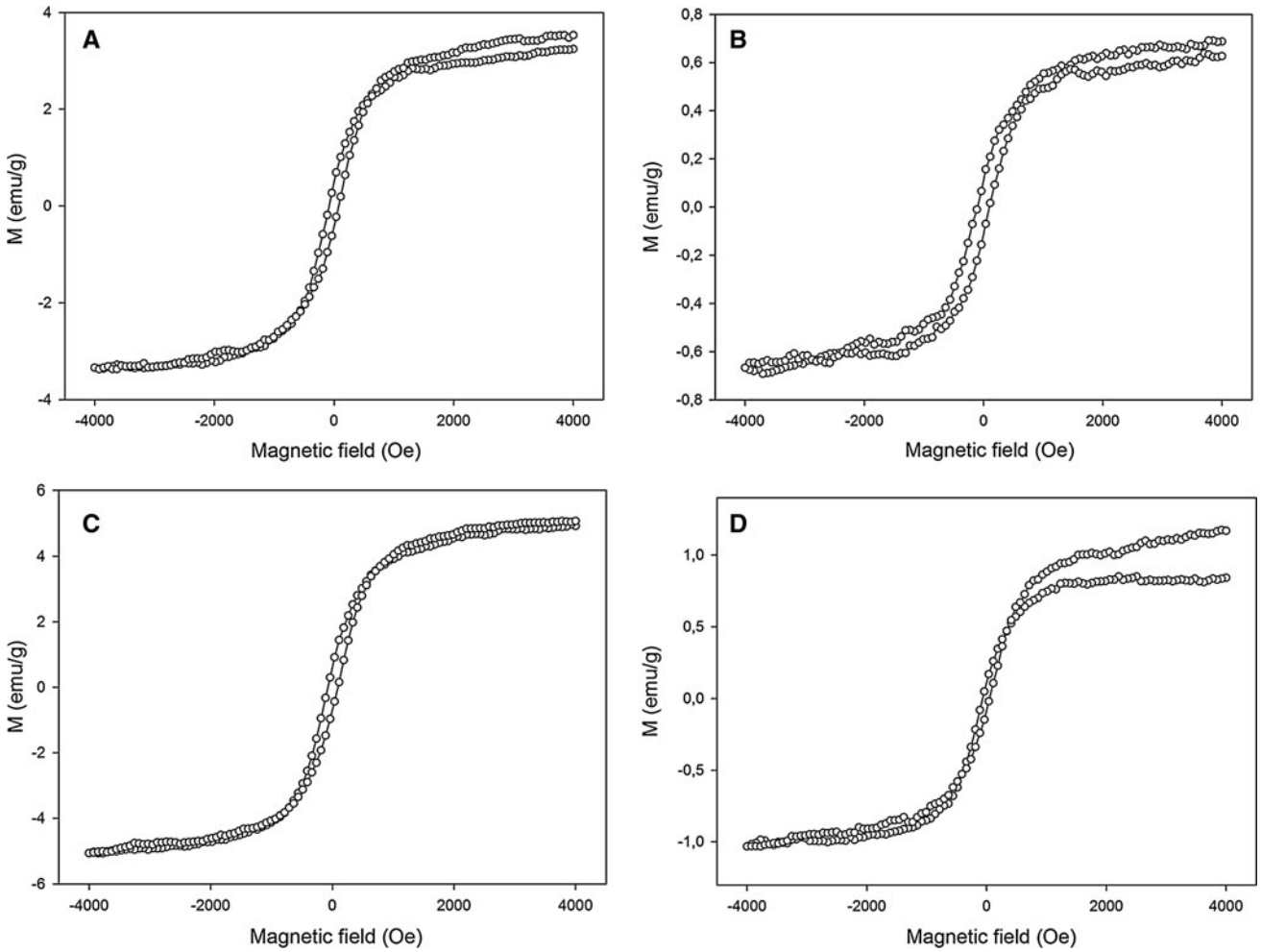
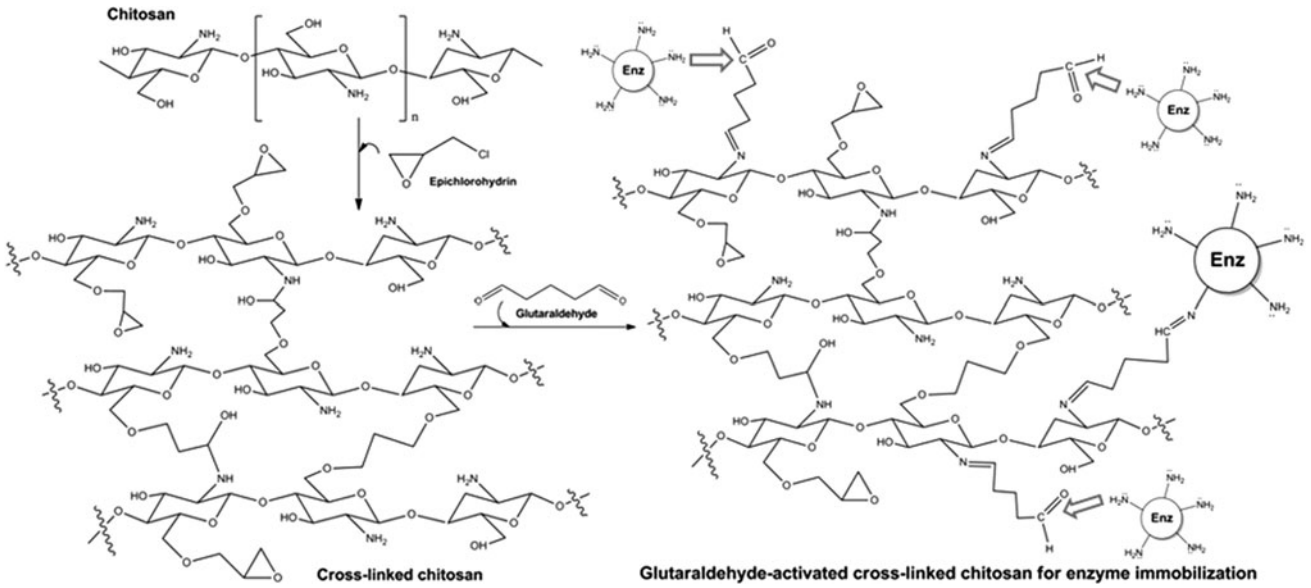


Fig. 3 Magnetic hysteresis loops at room temperature of magnetic chitosan beads prepared with different magnetite:chitosan weight ratios and crosslinked with epichlorohydrin at different concentrations: **a** 1:4 ratio and 2 % epichlorohydrin (MC1 beads); **b** 1:4 ratio and 5 % epichlorohydrin (MC2 beads); **c** 1:2 ratio and 2 % epichlorohydrin (MC3 beads); **d** 1:2 ratio and 5 % epichlorohydrin (MC4 beads)

17.9 min at 60 °C [12], MC*Lr*NDT4 biocatalyst was approximately 190 times more stable than the free enzyme as indicated by its stabilization factor, calculated as the ratio between the soluble and immobilized enzyme half-lives. Moreover, stabilization factor of immobilized enzyme on chitosan magnetic beads was even increased threefold comparing with that reported for the same enzyme covalently attached to epoxy-activated Sepabeads® [13].

Substrate specificity of immobilized 2′-deoxyribosyltransferase

Once MC*Lr*NDT4 was functionally characterized, this immobilized biocatalyst was used for the enzyme-catalyzed synthesis of several natural nucleosides employing different nucleoside donors and cytosine as base acceptor (Table 3). In this sense, transglycosylation activity of MC*Lr*NDT4 was higher with 2′-deoxyuridine (dUrd) as 2′-deoxyribose donor rather than thymidine (dThd), 2′-deoxyinosine (dIno) and 2′-deoxyadenosine (dAdo) which show the same activity but lower transglycosylation activity. This preference is quite similar to those previously described for the soluble enzyme [12] and immobilized enzyme on Sepabeads® [13]. As shown in Table 3, activity recovery of the immobilized enzyme ranged from 12 to 35 %, depending on the sugar donor employed in the transglycosylation reaction. Such activity reduction due to the immobilization process might be related to the high density of aldehyde groups on the surface of magnetic chitosan beads that would have lead to an intense covalent multipoint attachment of the enzyme to the support, hence altering the enzyme conformation and distorting its active site. A similar result was reported when *Lr*NDT was immobilized on highly activated Sepabeads® [13] whose activity recovery was approximately 13 %



Scheme 1 Schematic depiction of chitosan cross-linking by epichlorohydrin, activation by glutaraldehyde and enzyme covalent immobilization to the activated support

Table 2 Immobilization of nucleoside 2′-deoxyribosyltransferase from *Lactobacillus reuteri* on glutaraldehyde-activated magnetic chitosan beads

Glutaraldehyde-activated supports[a]	Magnetic chitosan immobilized biocatalyst	Added mg *Lr*NDT/g wt support	Immobilization yield (%)	Activity (IU/g wet biocatalyst)[b]	Yield in nucleoside (%)[c]
GA-MC1	MC*Lr*NDT1	0.70	100	2.00	19.5
	MC*Lr*NDT2	1.40	50	1.90	18.5
	MC*Lr*NDT3	2.80	25	1.82	17.7
GA-MC3	MC*Lr*NDT4	0.70	100	2.10	20.0
	MC*Lr*NDT5	1.40	50	2.10	20.0
	MC*Lr*NDT6	2.80	24	2.00	19.4

[a] Magnetic chitosan supports described in Table 1, further activated with glutaraldehyde (GA)

[b] Specific activity determined by the general procedure described in the experimental section

[c] Yield of thymidine after 5 min reaction time

employing the synthesis of 2′-deoxyadenosine from 2′-deoxyuridine and adenine as the standard reaction.

Synthesis of non-natural nucleosides catalyzed by immobilized 2′-deoxyribosyltransferase

Taking into account all previous results, non-natural nucleoside synthesis was carried out in the optimal conditions of pH 6.5 for long reaction times (ranging from 2 to 72 h) at 40 °C since thermal stability of immobilized enzyme was guaranteed in such mild conditions (Fig. 5). As shown in Table 4, synthesis of different non-natural nucleosides were successfully catalyzed by MC*Lr*NDT4 biocatalyst, including arabinosyl-nucleosides (ara-A and ara-C) that could not be previously obtained with immobilized enzyme on Sepabeads® [13]. In spite of its low specific activity in non-natural nucleoside synthesis (Table 4), immobilized *Lr*NDT on magnetic chitosan beads demonstrated to be useful in the synthesis of several therapeutic nucleosides: 2,6-diaminopurine-2′-deoxyribosides (active against cancer and viral diseases) [26], 5-trifluorothymidine (known as trifluridine, an antiherpetic agent [11], different 2′-fluoro-2′-deoxyribonucleosides such as 2′-fluoro-2′-deoxyadenosine and 2′-fluoro-2′-deoxycytidine (potent antiviral drug against Borna disease virus replication [6], and arabinosyl-nucleosides such as ara-A (or vidarabine, not only an antiviral drug which is active against herpes simplex and varicella zoster viruses, but a potential precursor of fludarabine and clofarabine [28, 36] and ara-C (or cytarabine, used as chemotherapy agent in the treatment of acute myeloid leukemia [29]. In particular, good yields were obtained in the enzymatic synthesis of 2,6-diaminopurine-2′-deoxyriboside (83 % at 2 h), 5-trifluorothymidine (40 % at 2 h), and 2′-fluoro-2′-deoxycytidine

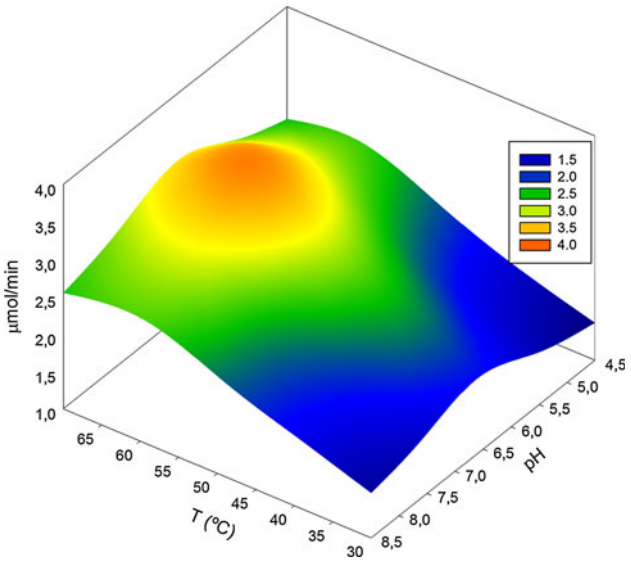


Fig. 4 Response surface plot for the effects of pH and temperature on activity of immobilized nucleoside 2′-deoxyribosyltransferase from *L. reuteri* on glutaraldehyde-activated magnetic chitosan beads (biocatalyst MC*Lr*NDT4)

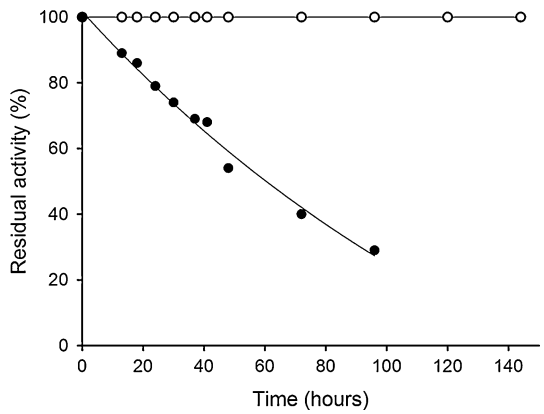


Fig. 5 Thermal inactivation of immobilized biocatalyst MC*Lr*NDT4 at 40 °C (*open circle*) and 60 °C (*closed circle*). At different incubation times, samples of the immobilized enzyme stored in MES buffer pH 6.5 at the indicated temperatures were withdrawn and assayed for activity as described in the experimental section

(67 % at 24 h), whereas low yields (14–17 %) were obtained in the synthesis of arabinosyl-nucleosides at longer reaction times (72 h) (Table 4).

Recycling of immobilized 2′-deoxyribosyltransferase

Recycling of enzymes is one prerequisite to their application in industrial bioreactors. In this sense, immobilized NDT from *Lactobacillus reuteri* on magnetic chitosan beads (derivative MC*Lr*NDT4) could be recycled for at least 30 consecutive batch reactions with negligible loss of catalytic activity in the synthesis of 2,6-diaminopurine-2′-deoxyriboside as well as in the synthesis of

Table 3 Substrate specificity of immobilized nucleoside 2′-deoxyribosyltransferase from *L. reuteri* on glutaraldehye-activated magnetic chitosan beads (biocatalyst MC*Lr*NDT4)

Donor	Acceptor	Activity (IU/g)[a]	Recovery (%)[b]
dUrd	Cyt	15	27
dThd	Cyt	5	12
dIno	Cyt	5	35
dAdo	Cyt	5	21

[a] See "Materials and methods" section for more details referred to standard activity assay for both soluble and immobilized enzyme

[b] Relative activity of the immobilized biocatalyst with respect to the same amount of native soluble enzyme which contains the enzymatic derivative in the same reaction conditions

Table 4 Synthesis of non-natural nucleosides catalyzed by immobilized *Lr*NDT on glutaraldehye-activated magnetic chitosan beads (derivative MC*Lr*NDT4)

Donor	Acceptor	Product	IU/g	Yield (%)
dUrd[a]	2,6-DAP	2,6-DAPdRib	0.16	83
dUrd[a]	5-tFThy	5-tFThd	0.08	40
Ara-U[b]	Ade	Ara-A	1.6×10^{-4}	14
Ara-U[b]	Cyt	Ara-C	1.9×10^{-4}	17
2′-FdUrd[c]	Ade	2′-FdAde	3.2×10^{-4}	10
2′-FdUrd[c]	Cyt	2′-FdCyd	2.2×10^{-3}	67

[a] Reaction conditions: 17 mg of derivative MC*Lr*NDT4 in 100 µL reaction volume; [donor] = [acceptor] = 2 mM; 50 mM MES buffer pH 6.5, reaction time: 2 h, T = 40 °C; 350 r.p.m

[b] Reaction conditions: 32 mg of derivative MC*Lr*NDT4 in 175 µL reaction volume; [donor] = [acceptor] = 0.5 mM in 50 mM MES buffer pH 6.5, reaction time: 72 h, T = 40 °C; 350 r.p.m

[c] Reaction conditions: 17 mg of derivative MC*Lr*NDT4; [donor] = [acceptor] = 0.5 mM in 50 mM MES buffer pH 6.5, reaction time: 24 h, T = 40 °C; 350 r.p.m. See "Materials and Methods" section for more details

5-trifluorothymidine. Similar results were obtained with the same enzyme attached to epoxy-activated Sepabeads® in the synthesis of 2,6-diaminopurine-2′-deoxyriboside [13]. In contrast to our results, immobilized NDT from *Lactobacillus leichmannii* on hydrophobic sepharoses, *k*-carrageenan and poly(acrylamide-co–*N*-acryloxysuccinimide (PAN) decreased its activity after every reaction cycle [18]. In the synthesis of 2′-deoxy-2-thiouridine, best results were obtained with immobilized NDT from *L. leichmannii* on octyl-Sepharose and PAN, which showed 60 % remained activity after 10 cycles in both cases [18].

Conclusions

In conclusion, the present work describes for the first time an extensive characterization of an immobilized biocatalyst

of recombinant 2′-deoxyribosyltransferase from *L. reuteri* (*Lr*NDT) covalently bound to magnetic chitosan beads (MC*Lr*NDT4 biocatalyst). This immobilized biocatalyst has been demonstrated to be active in the synthesis of several therapeutic nucleosides, including arabinosyl-nucleosides. The mild optimal conditions described for MC*Lr*NDT4 activity, its high thermal stability, magnetic properties, recycling, and good product yields at 2-h reaction time, would allow its exploitation for the enzymatic synthesis of 2,6-diaminopurine-2′-deoxyriboside and 5-trifluorothymidine.

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