Journal of Molecular Catalysis B: Enzymatic 114 (2015) 58–64

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

New biocatalysts for one pot multistep enzymatic synthesis of pyrimidine nucleoside diphosphates from readily available reagents

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ARTICLE INFO

Article history:
Available online 25 December 2014

Keywords:
Nucleoside 5′-monophosphate
Nucleoside 5′-diphosphate
Multistep enzymatic synthesis

ABSTRACT

Since the preparation of nucleoside 5′-diphosphates by classical methodologies is complex, multistep enzymatic systems were explored to synthesize pyrimidine nucleoside 5′-diphosphates starting from readily available reagents. Different strategies were combined to prepare uridine- and thymidine 5′-diphosphates as ribo- and deoxyribonucleoside models, respectively. For uridine 5′-diphosphate synthesis, conversions between 38 and 66% were achieved, using a simple methodology that involves commercial yeast extract as biocatalyst and biocatalytically *in situ* prepared uridine 5′-monophosphate. *Corynebacterium ammoniagenes* ATCC 19350 was used for the first time as biocatalyst to synthesize uridine 5′-monophosphate from uracil and orotic acid while *Raoultella planticola* was the selected biocatalyst for uridine 5′-monophosphate synthesis from uridine. The overall performances of all the tested approaches were similar but the use of uracil leads to a more suitable and cheaper process. Alternatively, for thymidine 5′-diphosphate synthesis two consecutive one pot multistep enzyme systems were assayed. In the first biotransformation, 2′-deoxyribose 5-phosphate was formed from glucose by *Erwinia carotovora* whole cells followed by the action of phosphopentomutase and thymidine phosphorylase affording thymidine in 85% conversion relative to 2′-deoxyribose 5-phosphate. Finally, in the second one pot reaction, the nucleoside was converted to thymidine 5′-diphosphate by the combined action of *Escherichia coli* BL21 *pET22b-phoRp* and *Saccharomyces cerevisiae*.

1. Introduction

Since 1985, when the anti-HIV activity of AZT was discovered, many nucleoside drugs have been evaluated. Nucleoside analogs (NAs) can act as chain terminators after being incorporated into growing DNA/RNA strands and/or inhibit viral polymerases by competition with the natural nucleoside 5′-triphosphate substrate [1]. Purine nucleoside analogs such as abacavir [2], fludarabine [3] and amdoxovir [4] as well as pyrimidine ones such as stavudine [5], zidovudine [6] and lamivudine [7] are widely used in clinical treatments.

Phosphorylated nucleosides are essential metabolites in numerous biochemical processes. Natural and modified nucleoside 5′-monophosphates (NMPs) are used as active pharmacological components [8] and as flavor enhancers in the food industry [9]. For example, sodium deoxyribonucleotides are adjuvant in the treatment of many types of diseases, such as hepatitis, leukemia and anemia [10]. Deoxynucleoside 5′-monophosphates (dNMPs) are also key precursors for the production of deoxynucleoside 5′-diphosphates (dNDPs) and deoxynucleoside 5′-triphosphates (dNTPs), being the last ones the essential substrates for polymerase chain reaction (PCR) [11]. Regarding arabinonucleosides, 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate (fludarabine monophosphate, 5′-FAraAMP) is widely used in the treatment of chronic lymphocytic leukemia therapies [12], acting as a hydrophilic prodrug.

Many compounds containing nucleoside 5′-diphosphates (NDPs) play also important roles in biologic systems [13] *e.g.* sugar nucleoside diphosphates act as the glycosyl donors of glycosyltransferases in the synthesis of oligosaccharides and glycoconjugates [14].

As a consequence, the development of suitable and efficient methodologies for the synthesis of nucleoside mono- and diphosphates has been the target of various chemical and enzymatic

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http://dx.doi.org/10.1016/j.molcatb.2014.12.004
1381-1177/

synthetic efforts. In general terms, chemical methods require multistage and harsh reaction conditions, leading to pollution and increased time consumption [15–17]. Moreover, the chemical synthesis of deoxyribonucleosides (dNs) is particularly complex due to the absence of the anchimeric effect [18]. In contrast, enzymatic methods proceed in a highly regio and stereo controlled manner avoiding protection and deprotection steps then, efficient and environmentally clean reactions occur.

Enzymatic one-pot multistep reactions, which mimic the efficiency of biosynthetic pathways, are gaining much attention [19]. In this methodology the biotransformation occurs in one vessel, sequentially or simultaneously, generating less waste formation by avoiding downstream operations [20]. The application of enzymes in multistep processes employing recombinant whole-cell expressing multiple enzymes or sequential bio- or chemocatalysed transformations provides efficient pathways for the synthesis of complex compounds in a unique operation [21].

Bacteria contain enzymes from salvage pathways capable to synthesize purine nucleosides from pyrimidine ones. This process, called transglycosylation, is a one-pot cascade reaction based on the action of nucleoside phosphorylases (NPs) that catalyze the phosphorolysis of the glycosidic bond. Due to the small number of commercially available NPs bacteria whole cells have also been extensively used with this purpose [22]. This methodology requires pyrimidine nucleosides and inorganic orthophosphate as starting materials and proceeds through the formation of the corresponding α-furanose 1-phosphate. A limitation of this approach is the need of the corresponding pyrimidine analogs. A way to overcome this problem is to employ furanose 1-phosphate intermediates as starting materials, but unfortunately their availability is limited due to synthetic and stability problems [23]. Alternatively, these compounds can be enzymatically obtained from furanose 5-phosphates employing phosphopentomutase (PPM, E.C. 5.4.2.7), an enzyme of the pentose pathway. Therefore, a strategy to prepare nucleosides that involves the use of furanose 5-phosphate and the combined action of PPM and one NP was performed [24,25]. In this process, furanose 5-phosphates synthesis is a key step. Two biocatalysed approaches were employed in our laboratory to prepare these compounds. Ribose- and 2′-deoxyribose 5-phosphates (R5P and dR5P, respectively) were prepared using *Enterobacter aerogenes* and *Raoultella planticola* whole cells, containing non-specific acid phosphatases (NSAPs), starting from the corresponding sugar and disodium acid pyrophosphate (PPi) as phosphate donor [26]. On the other hand, dR5P was obtained using *Erwinia carotovora* whole cells containing dR5P aldolase (DERA) as biocatalyst for the aldol addition between acetaldehyde and D-glyceraldehyde 3-phosphate (G3P) that can also be *in situ* generated by glycolytic intermediates [27]. Previously, Horinouchi et al. [28] reported the synthesis of deoxyinosine from adenine using recombinant phosphatase-negative *Escherichia coli* 10B5 cells expressing DERA from *Klebsiella pneumoniae*, PPM and purine nucleoside phosphorylase (PNP).

In addition to the NP biocatalysed strategies mentioned before, N-deoxyribosyltransferases (NdRTs) present in most *Lactobacillus* strains, which transfer glycosyl residues to acceptor bases, have also been used in the synthesis of pyrimidine 2-deoxynucleosides [29].

Regarding natural NMPs, they are usually obtained by microbial fermentation from *Penicillium citrinum* and from hydrolysates of nucleic acids [30]. However, this process is rather complicated and time-consuming. Methods for the production of modified NMPs are based on either chemical or enzymatic phosphorylation of the corresponding nucleosides being the enzymatic phosphorylation considered as a green advantageous alternative [31]. In bacteria, each NMP formation is catalyzed by its respective nucleoside kinase (NK) that transfers a phosphate group from a nucleoside triphosphate to the 5′ position of a nucleoside [32]. Thymidine kinase, which usually uses thymidine or deoxyuridine as substrates, is a ubiquitous enzyme but other NKs are only found in certain organisms [33]. Some dNMPs were synthesized using N-deoxyribosyltransferase II, deoxycytidine kinase and acetate kinase overexpressed in *E. coli* BL21 (DE3) in a one-pot reaction system. Thymidine and GTP were used as deoxyribose and phosphate donors, respectively, and acetyl phosphate was added to regenerate GTP [31].

Particularly, uridine 5′-monophosphate (UMP) is the precursor of pyrimidine nucleotides-related oligosaccharides, which are the focus of many studies [34]. A successful procedure was developed to produce UMP from orotic acid by *Corynebacterium ammoniagenes* ATCC 6872 using a one-pot two-step biocatalytic process. This strain has strong ATP regeneration activity and an adequate 5-phosphoribosyl-1-pyrophosphate supply [35]. Two enzymes catalyze the synthesis of UMP from orotic acid, orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) and orotidine 5′-monophosphate decarboxylase (ODCase, EC 4.1.1.23), which are the last two enzymes in the *novo* synthesis pathway of UMP.

To avoid the use of NTP as phosphate source, natural and modified nucleosides as well as sugars, were subjected to phosphorylation using NSAPs and PPi as phosphate donor [36,37]. In particular, intact cells of recombinant *E. coli* cells carrying *E. aerogenes* and *R. planticola* acid phosphatases showed to be efficient catalysts [26].

Regarding the synthesis of NDPs, nucleoside monophosphate kinases (NMPKs), enzymes that catalyze the phosphorylation of NMPs using ATP as the main phosphoryl group donor, were employed. While in eukaryotes the formation of UDP and CDP is catalyzed by a single bifunctional UMP/CMP kinase, the formation of UDP and CDP in bacteria is catalyzed by UMPK and CMPK respectively which, having no eukaryotic counterparts, represent attractive therapeutic targets [38].

Previous reports have demonstrated that *Saccharomyces cerevisiae* has not only unspecific UMPK/CMPK but also adenylate, guanylate, thymidylate, deoxyguanylate and deoxyadenylate monophosphate kinases [11,39,40]. ATP and dATP are the best phosphate donors but GTP, dGTP, dCTP and dTTP can also be used [41]. Moreover, in yeast, continuous ATP production and regeneration could be achieved employing glucose as the energy source [42]. Therefore, *S. cerevisiae* whole cell could be used as biocatalyst to produce a broad spectrum of NDPs.

The aim of this work was to design different environmentally friendly one pot multistep enzyme systems to synthesize pyrimidine NDPs starting from readily available reagents. Different strategies were explored to prepare UDP (Fig. 1) and TDP (Fig. 2) as ribo- and deoxyribonucleoside models, respectively.

2. Materials and methods

2.1. Chemicals and microorganisms

Reagents and substrates were purchased from Sigma–Aldrich and ICN. The culture media components were obtained from Anedra, Britania, and Sigma–Aldrich. Solvents for qualitative and quantitative analyses were from Sintorgan and Biopack. Thymidine phosphorylase (TP) was purchased from Sigma–Aldrich and PPM was kindly supplied by *Dra. Marisa Taverna Porro* [25]. *C. ammoniagenes* ATCC 19350, *E. carotovora* ATCC 33260, *R. planticola* ATCC 33531, *E. aerogenes* ATCC 13048 and *E. coli* BL21 (DE3) ATCC 47092 were from the Colección Española de Cultivos Tipo, Universidad de Valencia (Spain).

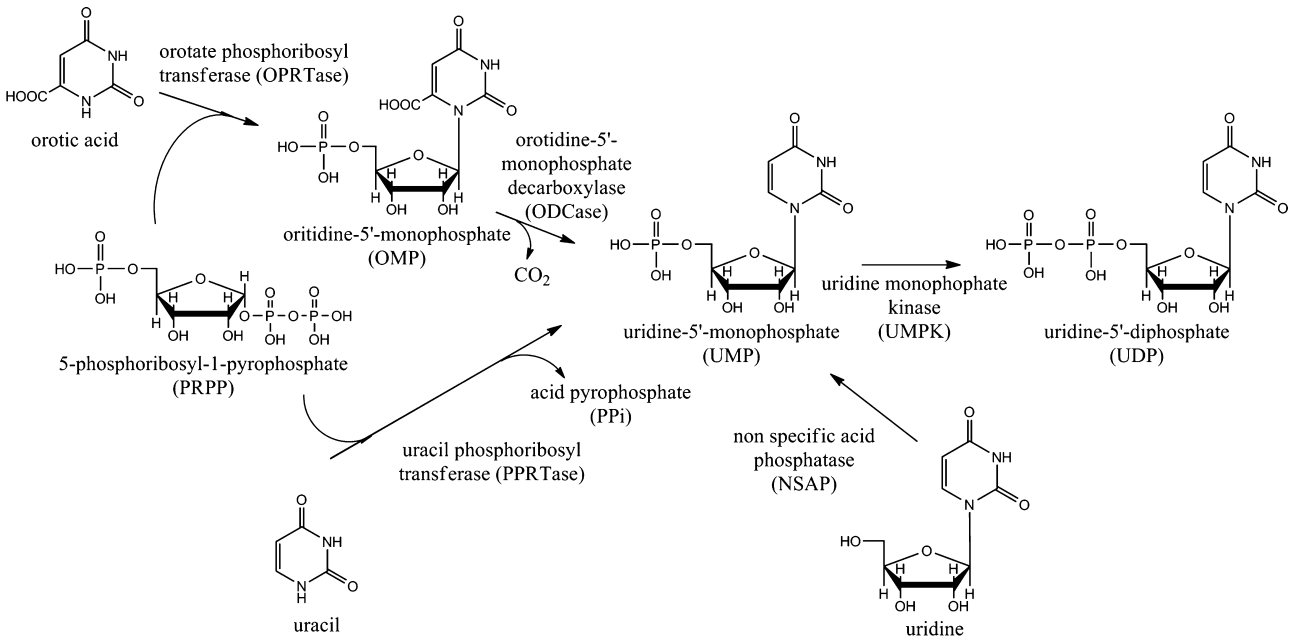

Fig. 1. Strategies for one pot UDP biocatalysed synthesis from uracil, orotic acid and uridine.

2.2. UMP synthesis

2.2.1. Biotransformation from orotic acid

2.2.1.1. Biocatalyst preparation. C. ammoniagenes was cultured in liquid medium (CM1) with the following composition: 2% (w/v) glucose, 1% (w/v) tryptone, 1% (w/v) yeast extract, 0.3% (w/v) NaCl, pH 7.2 at 30 °C and 200 rpm for 30 h.

The culture broth (30 mL) was added to 270 mL of liquid media (CM2) with the following composition: 8% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) KH_2PO_4, 1% (w/v) K_2HPO_4, 1% (w/v) $MgSO_4{\cdot}7H_2O$, 0.01% (w/v) $CaCl_2$, 0.002% (w/v) $MnSO_4$, pH 7.2 at 30 °C and 200 rpm. After 24 h, the culture was centrifuged at 4 °C and 5000 rpm and stirred 15 min and the pellet was used as biocatalyst.

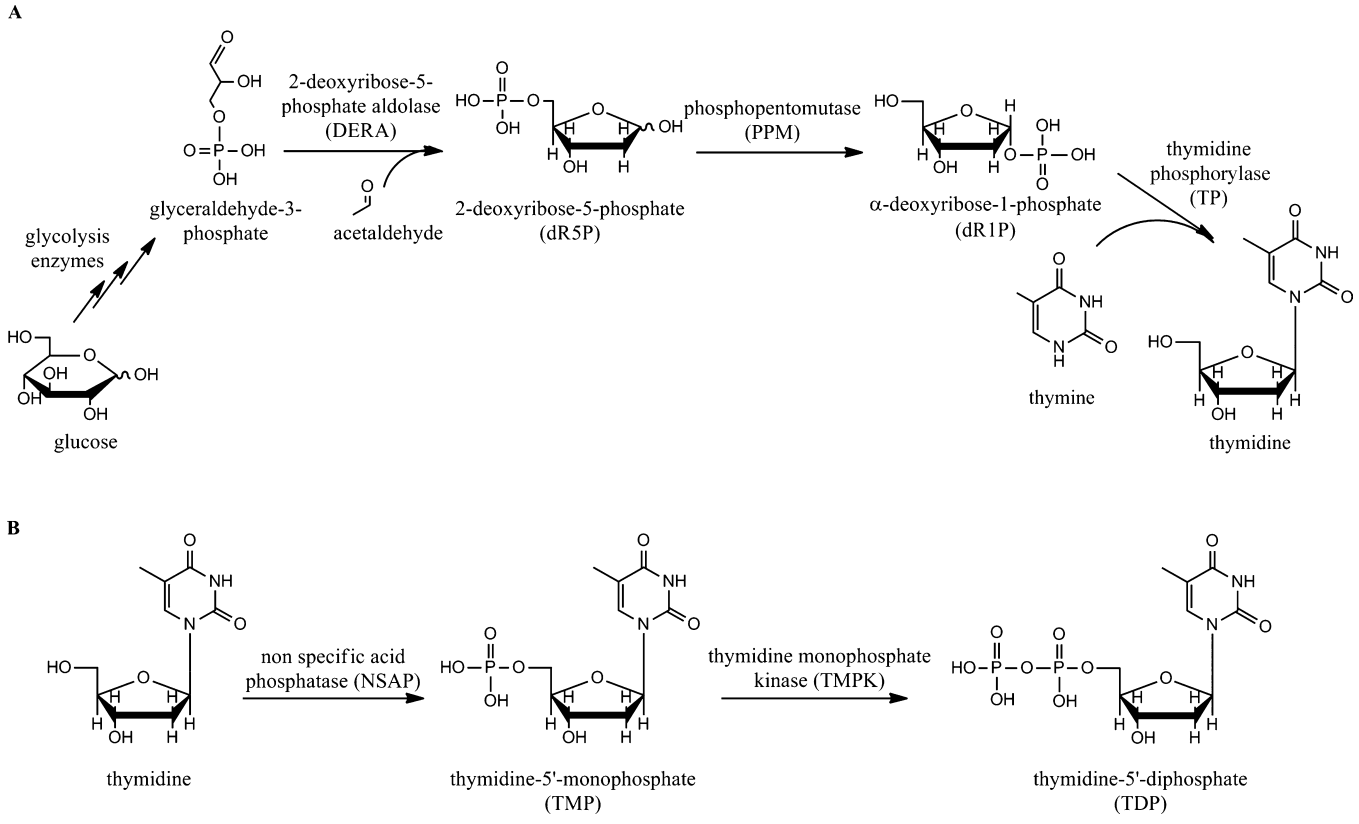

Fig. 2. TDP enzymatic synthesis (A) thymidine from glucose (B) TDP from thymidine.

2.2.1.2. Standard biotransformation. The reaction mixture (RMI) comprising: 20% (w/v) biocatalyst, 38.5 mM orotic acid, 333 mM glucose, 134 mM KH_2PO_4, 134 mM K_2HPO_4, 20 mM $MgCl_2$, 0.6% (v/v) triton X-100 and 50 mM Tris–HCl buffer pH 7.5 was stirred at 200 rpm and 30 °C. Samples were heated in boiling water for 1 min, centrifuged at 10,000 rpm for 3 min and the supernatants were analyzed by TLC and HPLC.

An alternative reaction mixture was assayed using sodium phosphate salts instead of potassium ones (RMII).

2.2.2. Biotransformation from uracil

2.2.2.1. Biocatalyst preparation. C. ammoniagenes was cultured in liquid medium (CM3) with the following composition: 5% (w/v) glucose, 1% (w/v) tryptone, 1% (w/v) yeast extract, 0.25% (w/v) NaCl, 0.3% (w/v) urea, pH 7.2 at 30 °C and 200 rpm. After 24 h, this culture broth (30 mL) was added to 270 mL of an induction medium (CM4) with the following composition: 15% (w/v) glucose, 0.5% (w/v) KH_2PO_4, 0.5% (w/v) K_2HPO_4, 0.5% (w/v) $MgSO_4{\cdot}7H_2O$, 0.002% (w/v) $MnSO_4{\cdot}6H_2O$, 0.002% (w/v) $FeSO_4{\cdot}7H_2O$, 0.001% (w/v) $ZnSO_4{\cdot}7H_2O$, 0.2% (w/v) urea, 0.1% (w/v) sodium glutamate, 0.0015% (w/v) β-alanine, 0.002% (w/v) cysteine, 0.002% (w/v) threonine, 0.005% (w/v) nicotinic acid, 0.005% (w/v) vitamin B1·HCl, 100 μg/L biotin, pH 7.2, 30 °C and 200 rpm. After 24 h, the culture was centrifuged at 4 °C and 5000 rpm for 15 min and the pellet was used as biocatalyst.

2.2.2.2. Standard biotransformation. The reaction mixture (RMIII) comprising: 20% (w/v) biocatalyst, 20 mM uracil, 277 mM glucose, 140 mM sodium phosphate buffer pH 7.2 and 1.5% (v/v) xylene, was stirred at 200 rpm and 30 °C. Samples were heated in boiling water for 1 min, centrifuged at 10000 rpm for 3 min and the supernatants were analyzed TLC and HPLC.

2.3. NMP synthesis

The reaction mixture (RMIV, 1 mL) consisting of 100 mM sodium acetate buffer pH 4.0, 260 mM disodium acid pyrophosphate (PPi), 1 mM $MgSO_4{\cdot}7H_2O$, 50 mg/mL wet weight of biocatalyst (*R. planticola*, *E. aerogenes* or *E. coli* over-expressing the corresponding NSAPs) obtained as previously reported [26] and 74.5 mM nucleoside was stirred at 40 °C and 200 rpm. At different reaction times, aliquots (70 μL) were taken, reaction stopped by the addition of 1.5 μL 2 N HCl and after centrifugation, the supernatants were stored at −20 °C until HPLC analysis.

2.4. NDP synthesis

2.4.1. Biocatalyst preparation

Yeast was cultured in liquid media with the following composition: 1% (w/v) casein peptone, 0.5% (w/v) yeast extract, 0.5% (w/v) glucose, 0.5% (w/v) NaCl, pH 7.2 at 37 °C and 200 rpm. After 24 h, the culture was centrifuged at 4 °C and 5000 rpm for 10 min. The pellet was transferred to a petri dish and was dried by air stream. The resulted solid was disrupted mechanically and used as biocatalyst.

2.4.2. Standard biotransformation

The reaction mixture (RMV) comprising: 100 mM glucose, 20 mM $MgSO_4{\cdot}7H_2O$, 4 mM NMP (N: uridine, thymidine, cytidine, inosine, guanosine, fludarabine or adenine arabinoside), 10% (w/v) of biocatalyst and 0.4 M sodium phosphate buffer pH 7.0 was stirred at 200 rpm and 37 °C. Samples were heated in boiling water for 1 min, centrifuged at 10,000 rpm for 3 min and the supernatants were analyzed by HPLC.

2.5. One pot multistep enzyme systems

2.5.1. Synthesis of UDP from orotic acid

In order to carry out one pot reaction, to 1 mL of RMI obtained after 24 h reaction as in Section 2.2.1.2, were added 310 μL of 752 mM sodium phosphate buffer pH 7.0, 23.6 mg of glucose and 6.5 mg of $MgSO_4{\cdot}7H_2O$. Similarly, after 10 h reaction, to 1 mL of RMII, were added 238 μL of 1.04 M sodium phosphate buffer pH 7.0 and 6.1 mg of $MgSO_4{\cdot}7H_2O$. In this case it was not necessary the addition of glucose because the dilution generated the appropriate amount for the reaction. These new reaction mixtures were stirred at 200 rpm and 37 °C and samples were treated as mentioned in Section 2.4.2.

2.5.2. Synthesis of UDP from uracil

After 24 h reaction, 1.4 mL of 600 mM sodium phosphate buffer pH 7.0, 29.8 mg of glucose and 11.8 mg of $MgSO_4{\cdot}7H_2O$ were added to 1 mL of RMIII (Section 2.2.2.2). This new reaction mixture was stirred at 200 rpm and 37 °C and samples were treated as mentioned in Section 2.4.2.

2.5.3. Synthesis of UDP from uridine

UMP was obtained using uridine as substrate and *R. planticola* wild type as biocatalyst at 24 h using the methodology described in Section 2.3. In order to adapt this mixture to the standard conditions for UDP synthesis, 38 μL of NaOH 10 N, 223 μL of distilled water, 20.4 mg of glucose and 5.6 mg of $MgSO_4{\cdot}7H_2O$ were added to 870 μL of RMIV. This new reaction mixture was stirred at 200 rpm and 37 °C and samples were treated as mentioned in Section 2.4.2.

2.5.4. Synthesis of TDP from glucose

2.5.4.1. Thymidine synthesis from glucose. A solution containing 14.1 mM dR5P, obtained from glucose and acetaldehyde as previously reported [27], was centrifuged at 5000 rpm for 10 min and the supernatant was lyophilized. The resultant solid was resuspended in 1 mL of 80 mM Tris–HCl buffer pH 8.0. To 0.25 ml of this solution (containing 3.6 mM dR5P) was added 0.75 mL of the reaction mixture comprising: 6 mM thymine, 1 mM $MnCl_2$, 0.2 M β-mercapthoethanol, 0.048 mg PPM, 35 U TP and 80 mM Tris–HCl buffer pH 8.0. The mixture was stirred at 160 rpm and 45 °C, samples were treated with 17% (v/v) HCl 2 N, centrifuged at 10,000 rpm for 3 min and the supernatants were analyzed by HPLC.

2.5.4.2. TDP synthesis from thymidine. The reaction mixture obtained after 25 min according to Section 2.3 using *E. coli* BL21 *pET22b-phoRp* whole cells as biocatalyst and thymidine, was used to synthesize TDP, following the conditioning of the medium as mentioned in Section 2.5.3.

2.6. Analytical methods

2.6.1. Nucleosides and nucleotides analysis

Qualitative analysis of nucleosides and nucleotides was performed by TLC using Silicagel 60 F254 plates (Merck, Rahway, NJ, USA and 6.6/5.0/1.0 (v/v/v) n-propanol/NH_4OH/H_2O as mobile phase.

HPLC analysis was performed using a C18 column (150 × 4 mm) at a flow rate of 0.9 mL/min. The UV detector was set at 254 nm, and the column was operated at room temperature. The operating conditions were as follows:

- For UMP synthesis using *C. ammoniagenes* as biocatalyst and for UDP and TDP synthesis: 15 min 100% 100 mM triethylammonium

Table 1
Biocatalysed synthesis of UMP by *C. ammoniagenes* ATCC 19350 from orotic acid (RMI and RMII) and uracil (RMIII).

Time (h)	UMP conversion mM (%)		
	RMI	RMII	RMIII
0	3.4 (9)	1.2 (3)	2.4 (12)
2	4.5 (12)	2.4 (6)	7.2 (36)
4	5.2 (14)	3.0 (8)	7.5 (38)
8	5.8 (15)	3.4 (9)	8.7 (44)
10	6.0 (16)	5.0 (13)	9.3 (46)
24	5.2 (14)	4.3 (11)	10.0 (48)

acetate buffer (TEAA) pH 7.0, 2 min to 98/2 (v/v) TEAA/acetonitrile (ACN) and 10 min 98/2 (v/v) TEAA/ACN.
- For NMP synthesis using *R. planticola* as biocatalyst: 16 min 98/2 (v/v) TEAA/ACN.
- For thymidine production: 15 min 90/10 (v/v) water/ACN.

2.6.2. Glucose quantification

The quantitative determination of glucose in the reaction mixtures was performed using an enzymatic analytical kit usually employed for the quantification of glucose in serum (Glicemia enzimática, cod. 1400101, Wiener lab Group, Rosario, Argentina). Samples of 40 μL were added to 160 μL of reagent on 96 wells microplates and absorbance was measured at 490 nm using a microplate reader 340 ASYS UVM. The methodology presented linearity in the concentration range 0–4.5 g/L glucose and the minimum detection limit was 0.0054 g/L.

2.6.3. Inorganic phosphate quantification

The inorganic phosphate (Pi) in the reaction mixtures was quantified using the ammonium molybdate method [43]. Samples of 20 μL were added to 120 μL of reagent in 96 wells microplates and absorbance was measured at 660 nm using the microplate reader mentioned above.

3. Results and discussion

3.1. UMP synthesis

3.1.1. UMP synthesis from orotic acid

In order to synthesize UMP from readily available reagents using microorganisms as biocatalysts, *C. ammoniagenes* ATCC 19350 was selected from our bacteria collection. Different strains of this species had been used for the production of nucleoside 5′-monophosphates [44,45] and for adenosine 5′-triphosphate (ATP) regeneration [35,46].

To carry out the biotransformation, the reaction mixture (RMI) containing the biocatalyst prepared as in Section 2.2.2.2, orotic acid as substrate, glucose as precursor for ATP synthesis, potassium phosphate as Pi source, Mg^{2+} as enzyme cofactor [47] and triton X-100 as poranting agent were used. The reaction was performed in a heterogeneous medium due to the low water solubility of orotic acid (1.7 mg/mL). As this starting material is converted to orotidine 5′-monophosphate (OMP) by orotate phosphoribosyltransferase (OPRTasa) and subsequently to UMP by decarboxylation, the solid fraction became soluble (Fig. 1). Regarding the CO_2 generated by OMP decarboxylation, the use of an efficient buffer system is needed to keep the pH around 7.5 and KOH was added when necessary. The results obtained for this biotransformation are shown in Table 1.

The effect of the phosphate buffer cation was also analyzed. Sodium salts were used instead of potassium (RMII) and the results are also shown in Table 1. UMP conversions were lower than those achieved with potassium salts.

Table 2
Nucleoside-5′-diphosphate (NDP) synthesis by *Saccharomyces cerevisiae*.

NDP	UDP	TDP	CDP	IDP	GDP	FAraADP	AraADP
Conversion[a] (mM)	2.1	0.40	0.24	0.52	0.48	0.40	0.17

[a] Reaction time: 90 min.

3.1.2. UMP synthesis from uracil

Considering the low cost of uracil and that it is a precursor in the biosynthesis of UMP, the use of this starting material was also assessed. To metabolize uracil, *C. ammoniagenes* ATCC 19350 requires the enzyme uracil phosphoribosyltransferase (PPRTasa) which only accepts uracil and PRPP to produce UMP (Fig. 1). Since this pathway does not involve a decarboxylation step, the pH remained constant during all the biotransformation. The obtained results are shown in Table 1.

The maximum conversion of UMP (48%) was obtained at 24 h, being higher compared to previously tested biotransformations which used orotic acid as substrate. One of the advantages of this biotransformation is that uracil is highly soluble in water (10 mg/mL), therefore the reaction is carried out in an homogeneous system. The biotransformation proceeds without the addition of Mg^{2+} [48] since the enzymes do not require such cofactor and adittionaly UMP does not act as an inhibitor.

3.1.3. UMP synthesis from uridine

With the aim of evaluating an alternative methodology for pyrimidine NMP synthesis, bacteria of the genus Enterobacteriaceae from our strain collection with proved NSAP activity [26] were tested as biocatalysts. NSAPs exhibit the maximum specific phosphatase activity at acidic pHs, then, sodium acetate buffer pH 4.0 was the biotransformation medium. Two new biocatalysts *E. aerogenes* (ATCC 13048) and *R. planticola* (ATCC 33531), were selected and assessed for the synthesis of different nucleoside monophosphates. In all cases only 5′-monophosphates were obtained, what suggests that the NSAPs contained in the selected microorganisms belong to class A which are known to possess high regioselectivity [49]. Regarding UMP, *R. planticola* afforded the best conversion (5.6 mM in 24 h) and was later used for the multienzymatic synthesis of UDP.

3.2. NDP synthesis from NMP

S. cerevisiae have been an important source of different NMPKs [50], enzymes involved in the biosynthesis of NDP from NMP. To optimize this biotransformation, the synthesis of UDP from commercial UMP was assessed [41].

Thus, starting from commercial Baker's yeast, the biocatalyst was prepared as Section 2.4.1. The presence of Mg^{2+} in the reaction medium was essential for the activity of key metabolic enzymes, such as phosphofructokinase and pyruvate kinase, and also to form the Mg^{2+} ATP salt, phosphate donor required by the UMPK for optimal activity [40,51]. Different glucose concentrations and reaction times were tested determining that UDP conversion increased from 30 to 52% at 90 min when glucose rises from 10 to 100 mM using potassium phosphate buffer. Jong and Campbell [52] reported the inhibition exerted by potassium ion on thymidylate kinase (TMPK) from *S. cerevisiae*, thus, on the assumption that similar effect may occur on the activity of UMPK, the use of sodium or potassium buffer was evaluated. It was observed that sodium phosphate buffer impacted slightly positively on conversion affording 58% (2.3 mM) of UDP.

The production of different NDP was performed (Table 2) showing that the used conditions were appropriate for *S. cerevisiae* NMPKs. Low substrate specificity was observed, since arabinoside 5′-diphosphates were also obtained. The preparation of these

Table 3
One-pot UDP synthesis.

Components	RMI_{final} [a]	$MRII_{final}$ [a]	$MRIII_{final}$ [a]	$MRIV_{final}$ [a]	$RMV_{initial}$ [b]
UMP	5.2 mM	5 mM	10 mM	5.6 mM	4 mM
Glucose	0	128	74	0	100 mM
Inorganic phosphate	281 mM (K^+)	258 mM (Na^+)	121 mM (Na^+)	514 mM (Na^+)	400 mM (Na^+)
Mg^{2+}	20 mM	20 mM	0	0.2 mM	20 mM
Others	Tritón 0.6% Orotic acid 33 mM	Tritón 0.6% Orotic acid 33 mM	Xilene 1.6% Uracil 10.4 mM	Uridine 69 mM	0
Buffer	Tris–HCl 50 mM	Tris–HCl 50 mM	Sodium phosphate 121 mM	Sodium acetate 100 mM	Sodium phosphate 400 mM
pH	7.5	7.5	7.2	4.0	7.0
UDP[c]	1.54 mM	1.72 mM	2.64 mM	2.40	2.30 mM

[a] Composition of the different reaction mixtures containing UMP at the corresponding final reaction times.
[b] Initial composition of the reaction mixture for UDP synthesis starting from commercial UMP.
[c] UDP concentration afforded for the different one pot systems.

compounds by methodologies involving yeast NMPKs has not been previously reported.

3.3. One pot multistep enzyme systems for UDP synthesis

UDP production, using one pot procedures, was also assessed by adapting the reaction mixtures containing biocatalytically synthesized UMP as described above.

3.3.1. Orotic acid as substrate

In order to adequate the reaction mixture for UMP synthesis from RMI, the final conditions (24 h) (MRI_{final}, Table 3) were compared to the requirements for UDP production ($RMV_{initial}$, Table 3). Glucose and sodium phosphate buffer pH 7.0 was added to reach 100 and 400 mM respectively, obtaining in this way also the optimal concentration of UMP (4 mM). UDP conversion reached 38% (1.54 mM) after 90 min reaction.

In a similar way, yeast biotransformation was evaluated using RMII after 10 h reaction. Phosphate concentration was corrected (from 258 mM to 400 mM), achieving also in this way suitable concentrations of UMP and glucose (4 mM and 103 mM, respectively). UDP conversion reached 43% (1.72 mM) after 90 min reaction.

3.3.2. Uracil as substrate

The final RMIII reaction mixture composition ($MRIII_{final}$, Table 3) is quite compatible with that required for UDP synthesis ($RMV_{initial}$, Table 3). In addition to buffer and UMP correction, glucose and $MgSO_4$ were added to reach 100 and 20 mM, respectively. The reaction was carried out for 90 min and 66% UDP conversion, corresponding to 2.64 mM, was obtained suggesting that the use of sodium salts as buffer system and the absence of Tris and Triton favor the reaction.

3.3.3. Uridine as substrate

Finally, the reaction medium from UMP synthesis using *R. planticola* as biocatalyst and uridine as substrate ($RMIV_{final}$, Table 3) was adapted for UDP production. The main differences between both reaction media are the buffer composition and pH. Therefore, the influence of acetate in the second reaction was analyzed. Both acetate and sodium phosphate were used as reaction medium for UDP synthesis as in Section 2.4.2, achieving similar conversions. Thus, it was concluded that $RMIV_{final}$ could be used for a consecutive UDP synthesis reaction without affecting the performance of the reaction. Therefore, $RMIV_{final}$ was neutralized with NaOH and the concentration of the rest of the components adapted to fit with $RMV_{initial}$. After 90 min reaction, UDP was obtained in 60% conversion corresponding to 2.4 mM.

As detailed in the above sections, UDP yields were similar independent of the approach used to prepare UMP. Therefore, the factor that seems to be determinant for the selection of the best procedure is UMP source. In this regard, not only the overall performance but also reagents cost, growth mode and production of biocatalysts and the total time required for the whole process should be considered for a complete evaluation.

In the case of reaction with *R. planticola*, microorganism culture was fast (24 h) and easy and the reaction time was 24 h, however it was necessary a considerable excess of uridine, the starting material. For biotransformations with RMI and RMII using *C. ammoniagenes*, microorganism growth was more complex due to the need of two steps, one for growth (30 h) and another one for induction (24 h). The highest yields of UMP were reached at 10 h, generating a longer though cheaper process than the biotransformation with *R. planticola*. In the case of the biotransformation with RMIII the preparation of the biocatalyst involved also two steps (48 h), and the required reaction time was 24 h but the low cost of uracil and the superior yield obtained (48%) justifies the choice of this approach as an advantageous alternative strategy for preparing UDP biocatalytically.

3.4. TDP synthesis from glucose

3.4.1. One pot Thymidine synthesis from glucose

Thymidine synthesis was synthesized by a chemoenzymatic method previously reported [25] using dR5P, a recombinant PPM isolated from *E. coli* and commercial TP. As an alternative strategy to obtain dR5P, whole cells of *E. carotovora* ATCC 33260 containing high DERA activity and glucose as substrate [27] was herein assessed (Fig. 2). The first attempt to obtain thymidine from this reaction mixture was unsuccessful. The main components that could be responsible were the phosphate buffer that inhibits the enzymatic activity of PPM and the remaining acetaldehyde that may cause the inhibition of the involved enzymes. We determined that Pi concentrations up to 25 mM did not cause adverse effects while the presence of acetaldehyde prevented thymidine production. Then, the final reaction medium was lyophilized to completely remove acetaldehyde and the residue re-dissolved to fit PPM and TP media requirements. After 2 h of the addition of thymine, PPM and TP, thymidine was obtained in 85% conversion relative to dR5P. This strategy was also applied to the synthesis of 5-bromo-2′-deoxyuridine achieving 100% conversion after 45 min reaction.

3.4.2. One pot TDP synthesis from thymidine

TMP synthesis was performed using thymidine and *E. coli* BL21 *pET22b-phoRp* as previously reported [26]. This biocatalyst was used instead of *R. planticola* wild type since better conversion was obtained (4.6 mM of TMP after 25 min). This mixture was adapted for the synthesis of TDP using commercial Baker' s yeast as described in Section 3.3.3, obtaining similar yields than those achieved starting from commercial TMP.

4. Conclusions

UDP was obtained using a simple methodology that involves the use of commercial yeast extract and UMP as substrate, achieving 58% conversion at 90 min of reaction. This system was also applied to the synthesis of other nucleoside diphosphates such as TDP, CDP, IDP, GDP, AraADP and FAraADP. Due to the high cost of NMPs, different biotransformations to obtain UMP were tested and further used, without purification, for UDP synthesis. Thus, one-pot multistep enzymatic strategies were developed.

C. ammoniagenes ATCC 19350 was used for the first time as biocatalyst to synthesize UMP from readily available substrates as uracil and orotic acid while *R. planticola* was the selected biocatalyst for UMP synthesis from the corresponding nucleoside. The overall performance of all the tested approaches was similar but the use of uracil leads to a more suitable and cheaper process. Therefore, one pot biotransformation using *C. ammoniagenes* and *S. cerevisiae* consecutively seems to be an advantageous alternative for UDP synthesis achieving conversions between 38 and 66%.

Alternatively, dNDP can also be obtained from glucose and the corresponding base by two consecutive one pot multistep biotransformations. In the first cascade reaction, dR5P is formed from glucose and acetaldehyde by *E. carotovora* whole cells and further isomerized to dR1P by the action of PPM. Subsequently, this intermediate is glycosylated to the corresponding deoxynucleoside by a NP. By using this approach thymidine and 5-bromo-2′-deoxyuridine were obtained in 85% and 100% conversion relative to dR5P, respectively. Finally, in the second reaction the deoxynucleoside is converted to dNDP by the action of *E. coli* BL21 *pET22b-phoRp* and commercial Baker's yeast.

Acknowledgements

ESL and AMI are research members of CONICET, Argentina. This work was supported by Universidad Nacional de Quilmes and Secretaría de Ciencia y Técnica de la Nación, Argentina. We thank Dr. Rodrigo Pontigia for providing the optimized protocol for UDP synthesis.

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