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REVIEW ARTICLE

Whole Cell Biocatalysts for the Preparation of Nucleosides and their Derivatives

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ARTICLE HISTORY

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Background: Nucleosides constitute an extensive group of natural and chemically modified compounds that display a wide range of structures and activities. Different biocatalysts have been developed for their preparation, but the choice of commercially available enzymes is limited. Therefore, the search of new biocatalysts is particularly attractive. In this sense, microorganisms are a vast source of enzymatic diversity that can be directly used as a whole cell biocatalysts providing a potential cheaper and suitable route for industrial applications.

Methods: This work makes particular emphasis on the following methods: the biocatalyzed whole cell synthesis of nucleosides mediated by phosphorylases, key biocatalyzed steps involved in other chemoenzymatic routes to prepare nucleoside analogues and the transformation of nucleosides in derivatives with particular properties.

Results: The literature covered in this work confirms that biocatalytic procedures that make use of whole cell systems can be successfully applied to obtain a wide variety of nucleoside analogues and their derivatives, providing alternative and complementary routes to traditional chemistry. The direct use of microbial whole cells as biocatalysts affords competitive results since it avoids the cumbersome procedures involved in enzyme isolation and facilitates multienzymatic processes. These biocatalysts also maintain the enzymes in their natural environment, protecting their activities from reaction conditions.

Conclusion: Although the information presented herein shows that these methodologies have reached a high degree of development, it is expected that future contributions of protein engineering and nucleoside metabolism knowledge, among other disciplines, will expand the already wide range of applications in nucleoside chemistry of whole cell biocatalysis.

Keywords: Nucleosides, whole cell biocatalysts, nucleoside phosphates, carbocyclic nucleosides, acylnucleosides, glycosylnucleosides, transglycosylation.

1. INTRODUCTION

Nucleosides and their analogues constitute an extensive group of natural and chemically modified compounds exhibiting a wide range of structures and activities. They are involved in the generation of energy, cell signalling and in the storage and transmission of genetic information, and therefore, they exert their activity on different targets and through diverse mechanisms. One of the most important targets of nucleoside analogues (NAs) is the inhibition of replication and transcription acting in many cases as antiviral and anticancer drugs [1].

As a consequence, many efforts have been done for generating more active and selective drugs, giving rise to a vast family of analogues, being especially explored the modification of the sugar moiety [2].

After the first approval of an antiviral nucleoside (idoxuridine; 5-iodo-2'-deoxyuridine) [3], a long list of analogues has been officially permitted for clinical practices all around the world. Among the most remarkable ones are 3'-azido-3'-deoxythymidine (AZT, zidovudine), 2',3'- dideoxyinosine (didanosine), L-2'-deoxy-3'-thiacytidine (lamivudine), 6-cyclopropylamino-2',3'-didehydro-2',3'-dideoxyguanosine (abacavir) and 2',3'-dideoxycytidine (zalcitabine), which are applied to the treatment of infections caused by different virus like HBV, HCMV, HSV, HPV, VZV, HCV, RSV, and HIV (Scheme **1A**).

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Cancer therapies have been also benefited by the use of modified nucleosides based drugs [4], being some relevant examples: 2-fluor-9-β-D-arabinofuranosyladenine (fludarabine), 2-chloro-2'-deoxy-2'-fluoro-9-β-D-arabinofuranosyladenine (clofarabine); 1-β-D-arabinofuranosylcytosine (cytarabine); 6-methoxy-9-β-D-arabinofuranosylguanine (nelarabine), 2-chloro-2'-deoxyadenosine (cladribine), and 2',2'-difluorodeoxycytidine (gemcitabine) (Scheme **1B**).

In addition to their use as therapeutic agents, nucleoside analogues are employed in other fields. For example, IMP and GMP are excellent flavour enhancers and are also used as beef extract substitutes in the food industry [5]. Tuberculosinyladenosine, a diterpene nucleoside present in *Mycobacterium tuberculosis*, is explored as a new diagnostic test for tuberculosis [6]. AZT-prolinamide analogues were described as new organocatalysts for the enantioselective aldol condensation of aldehydes with ketones [7].

Therefore, new structures are constantly developed and their preparation in large scale and low cost is mandatory. Regardless of the several advances achieved in the large scale preparation of NAs, still nowadays the high cost of these compounds is a concern [8]. In this field as in many others, biocatalyzed strategies have shown many advantages over chemical synthesis due to their unique benefits, such as very mild reaction conditions, high stereo and regioselectivity, and a friendly environmental technology [9-11]. This biotechnological approach replaces multistep chemical processes, and has been widely applied in combination to chemical methods in the chemoenzymatic synthesis of nucleosides.

Different biocatalysts have been developed such as soluble and immobilized enzymes [12-14] or whole cells microorganisms con-

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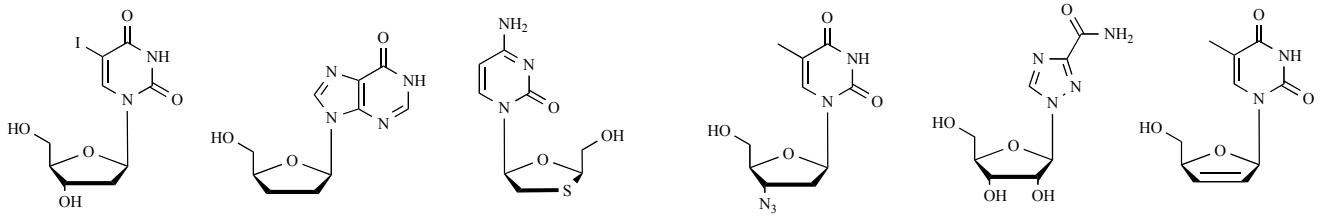
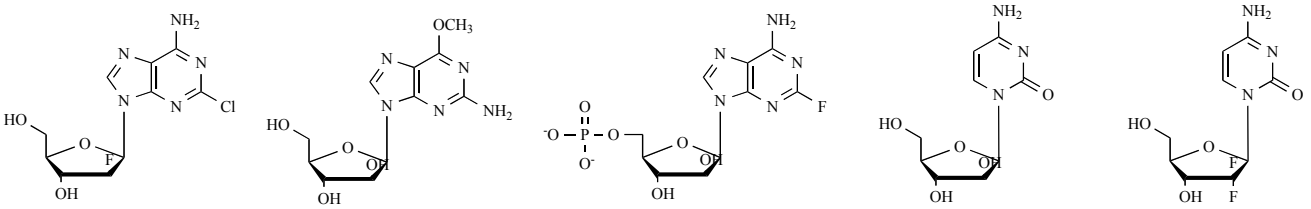
A. Antivirals



Idoxuridine **Didanosine** **Lamivudine** **Zidovudine** **Ribavirine** **Stavudine**

B. Antitumor agents



Clofarabine **Nelarabine** **Fludarabine phosphate** **Cytarabine** **Gemcitabine**

Scheme 1.

taining the required enzymatic system [15-17]. Since the choice of commercially available enzymes is limited, the screening of particular activities present in microorganisms offer an attractive alternative [18]. In addition, the direct uses of microbial whole-cells as biocatalysts avoids the cumbersome procedures involved in enzyme isolation and protects enzyme activity by keeping them in their natural environment, providing a potential cheaper route for industrial applications [19]. An additional advantage furnished by the use of whole cell systems is the ease of increasing production by genetic manipulation [20]. However, the use of whole cell systems as biocatalysts involves microorganism cultivation, the presence of side reactions and by-products from cell lysis, making in some cases isolation procedures more complex. Handling and recycling can be improved by immobilization methodologies, but sometimes this approach generates even more expensive routes [21].

Despite the fact that many processes have taken advantage of whole-cell biocatalysis, like biodiesel [22] and other high-value compounds poduction [23], this approach has attracted less attention compared with isolated enzymes in both research and applications [24]. While several reviews dealing with the biocatalyzed synthesis of nucleosides have been previously published [25-28], this work focuses on the whole cell biocatalyzed preparation of nucleoside analogues and their analogues. The subjects involved in this review deal with the biocatalyzed whole cell synthesis of nucleosides mediated by phosphorylases, key biocatalyzed steps involved in other chemoenzymatic routes and the transformation of the corresponding analogues in more useful analogues.

2. NUCLEOSIDE PREPARATION

Since the last decades of the past century, biocatalyzed synthesis of nucleosides has, in some cases, replaced chemical synthesis. Traditional approaches often require time-consuming multistep processes, glycosyl activation and the stereo- and regiochemistry control of glycoside formation [2, 8]. Therefore, biocatalysis offers attractive alternative routes in view of its well known characteristics, allowing a more efficient and cleaner process [29].

Glycosyltransferases (GT), enzymes that transfer glycosyl residues to acceptor bases, are the most widely spread biocatalysts for the synthesis of natural and nonnatural nucleosides *via* base exchange between specific combinations of purine and pyrimidine nucleosides. Whole cells containing nucleoside 2'-deoxyribosyltransferases (NdRTs) or nucleoside phosphorylases (NPs) have been employed in *one-pot*, *one-enzyme, one-step* or *one-pot, two-enzymes, two-steps* as biocatalytic systems for NAs synthesis, respectively. Reviews dealing with the biocatalyzed synthesis of nucleosides using these enzymes have been reported [9, 10, 25, 26, 30]. Since NdRTs will be the subject of another review in this issue, only an update of the significant progresses carried out in NAs synthesis using microbial NPs as biocatalysts will be herein reported. In addition, some examples of modified nucleosides obtained by combining microbial NPs with other whole cell biotransformations, as well as some whole cell biocatalyzed key steps for the chemoenzymatic synthesis of nonconventional NAs will be addressed.

2.1. Nucleoside Synthesis Mediated by Microbial Nucleoside Phosphorylases

Nucleoside phosphorylases are essential enzymes for the salvage and catabolism pathways of nucleotides. NPs are transferases that can reversibly catalyze the cleavage of the glycosidic bond of ribo- or deoxyribonucleoside to produce a base and ribose- or deoxyribose-1-phosphate in the presence of inorganic phosphate [9], existing both pyrimidine (PyNP) and purine (PNP) nucleoside phosphorylases. PNPs recognize 9-(β-D-pentofuranosyl)purines as substrates, being mammalian PNPs specific for 6-oxopurines while bacterial ones show broad specificity, accepting both 6-oxo- and 6-aminopurines. Thymidine phosphorylase (TP) reversibly catalyzes the phosphorolysis of 1-(2'-deoxy-β-D-ribofuranosyl)-pyrimidines whereas uridine phosphorylase (UP) accepts many 1-(β-D-pentofuranosyl)-pyrimidines as substrates. Several organisms contain a single PyNP with broad specificity at the 2'-position of the ribose. Cytosine and its nucleosides are not substrates for any PyNP.

These enzymes have been successfully employed for the synthesis of a high variety of natural and non-natural NAs allowing the access to efficient antiviral and antitumor drugs [3]. New sources of NPs are continually being discovered in order to expand the range of accepted substrates and improve the efficiency and productivity of these biocatalysts. At first, the biotransformations have been carried out employing isolated enzymes but the use of whole cells has risen due to some advantages, such as avoidance of isolation and purification of the enzymatic system and improved activity, stability and cost [20]. In addition, microorganisms supply a large diversity of biocatalysts with different substrate specificities. Nowadays, whole bacterial cells overexpressing recombinant nucleoside phosphorylases are replacing wild type strains, providing shorter reaction times and requiring less biomass [31].

The thermodynamic equilibrium for PNP, but not for PyNP, is shifted towards nucleoside synthesis, therefore, the combination of both enzymes makes possible the generation of purine nucleosides from pyrimidine ones in two sequential steps. The so-called transglycosylation, is a one-pot cascade reaction that consists of a first conversion of a pyrimidine nucleoside to α-ribose-1-phosphate (R1P), catalyzed by a PyNP and a second step where a PNP catalyzes the transfer reaction between R1P and a purine base affording the corresponding purine nucleoside (Scheme **2**).

Futhermore, limitations of this approach are the need of following the sequential order PyNP-PNP and the availability of the corresponding pyrimidine analogues. Additionally, some pyrimidine nucleoside analogues are not appropriate substrates for PyNPs and therefore, they cannot be used as pentose donors. Then, an alternative strategy (here named as glycosylation), which consists of using only one NP and α pentose-1-phosphate as substrates has been also applied to nucleoside synthesis.

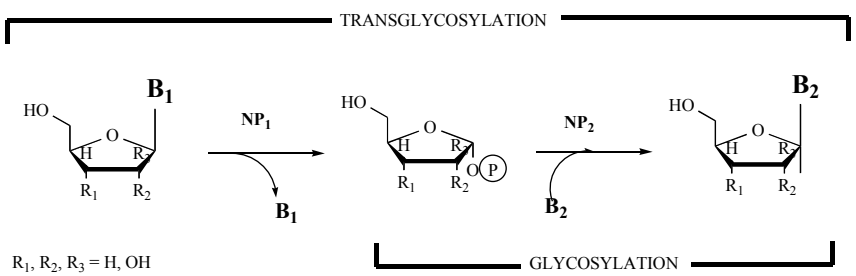
2.1.1. Transglycosylation Reaction

Microbial transglycosylation proved to be an efficient methodology for the synthesis of a high varietty of nucleoside analogues with biological and pharmaceutical importance [28, 32], by using natural or modified pyrimidine nucleoside as pentofuranose donors as well as natural or artificial heterocyclic bases as acceptors (Table **1**).

Ribavirin is a guanosine analogue that displays a broad spectrum activity against a variety of DNA and RNA viruses [33]. The mechanism of action involves the inhibition of inosine monophosphate dehydrogenase, an essential enzyme for viral replication that catalyzes the *de novo* guanosine synthesis [34]. More recently, its anticancer activity for the treatment of leukemia and breast cancer was also demonstrated [35]. We reported an efficient ribavirin synthesis procedure using *Aeromonas hydrophila* CECT4226 [36]. This wild type strain afforded a conversion of 77% starting from uridine and 1,2,4-triazole-3-carboxamide (TCA), carrying out the biotransformation at 60°C for 26 h. Temperature is an important parameter that needs to be set for each transglycosylation depending on the substrate and the involved biocatalysts. It is well known that in mesophilic microorganisms, UP is usually active up to 80°C, while TP is only active below 50°C. Besides, PNP activity drops above 65°C. Moreover, high temperatures help to increase the substrate solubility in aqueous solution and also produce an enhancement in the reaction rate [37]. Moreover, when whole cells are used as biocatalysts, deleterious enzymatic activities can be inhibited by increasing the reaction temperature. Typically, transglycosylations are performed at 60ºC, since most of the NPs retain over 70% of their activity and the action of enzymes such as deaminases can be minimized [9]. However, reactions involving TP are usually carried out at 45ºC. Then, in order to avoid deaminase interferences, 2'-deoxyuridine (dU) instead of thymidine can be used as an alternative source of the deoxyribosyl moiety. Despite these considerations, ribavirin bioconversion was reported to reach up to 86% using whole cells of *Escherichia coli* ATCC 12407 as biocatalyst [38], when the reaction was performed at 30ºC, representing an increase of more than 40% of the conversion obtained at 60°C with the same system.

Further characterization of previously mentioned *A. hydrophila* CECT4226 showed that, unusually, its NPs exhibit similar behaviour to the corresponding mammalian enzymes, since it only accepted natural occurring pyrimidine nucleosides and did not recognize 6-aminopurines. In contrast, different *A. hydrophila* strains (*A. hydrophila* CECT 4221) showed a distinct NP behaviour [36], since both adenine and hypoxanthine were accepted as substrates while using non-natural bases poor yields were achieved. Moreover, this strain was the best producer of dideoxynucleosides. The differential performance of *A. hydrophila* species reported in our paper suggests that a careful screening must be always carried out in the search of appropriate biocatalysts, since different strains of the same genus and species may provide diverse results. Didanosine (2',3'-dideoxyinosine) is an effective HBV and HIV inhibitor [39] that acts, after phosphorylation, as a chain terminator during reverse transcription [40]. This compound was prepared in 65% yield from dideoxyuridine and hypoxanthine using intact cells of the previously mentioned strain, *A. hydrophila* CECT 4221. The reaction temperature was set at 45°C according to the thermal stability of the pyrimidine phosphorylase involved in the biotransformation.

An alternative to overcome the temperature limitations of mesophilic NPs is to employ thermophilic microorganisms. In this sense, *Geobacillus stearothermophilus* [41] and *Thermus thermophilus* [42] have been applied to the synthesis of NAs. Almendros *et al*. [43] identified several strains of *T. thermophilus* capable of synthesizing purine nucleosides using pyrimidine ones as the sugar-donor and adenine or hypoxanthine as bases. High yields in short times and negligible adenosine deaminase (ADA) degradation of the products were obtained by carrying out the biotransformation at 65°C. Through a deep analysis of the biotransformation performance, the authors observed that adenine is better substrate than hypoxanthine; higher yields were obtained with 2'-deoxyribose nucleosides than with ribose ones without preference between thymidine and 2'-deoxyuridine as sugar donors; and productivity values were increased when Tris/HCl buffer was used instead of sodium phosphate buffer. As a consequence, they first postulated



Scheme 2.

Table 1. Whole cell biocatalyzed nucleoside synthesis by transglycosylation.

Product	Subtrates		Biocatalyst	T (°C)	Yield (%)	References
	Nucleoside	Base				
Ribavirin	Uridine	1,2,4-triazole-3-carboxamide	*Aeromonas hydrophila*	60	77	[36]
			Escherichia coli	30	86	[38]
Didanosine	Dideoxyuridine	hypoxanthine	*Aeromonas hydrophila*	45	65	[36]
6-Chloropurine-2'-deoxyriboside	2'-Deoxyuridine	6-Chloropurine	*Geobacillus stearothermophilus*	30	90	[48]
6-Methylpurine-2'-deoxyriboside	Thymidine	6-Methylpurine	recombinant *E. coli* DH5α	55	95	[65]
Vidarabine	Uridine arabinoside	Adenine	*Enterobacter aerogenes*	60	83	[55]
			Citrobacter koseri	60	71	[23]
Guanine arabinoside	Uridine arabinoside	2,6-Diaminopurine	*Enterobacter gergoviae* + ADA	60	46	[16]
2,6-Diaminopurine riboside	Uridine		*Geobacillus stearothermophilus*	30	83	[41]
			Aeromonas salmonicida	60	95	[51]
			recombinant *E. coli*	50	90	[63]
2,6-Diaminopurine-2'-deoxyriboside	2'-Deoxyuridine		*Geobacillus stearothermophilus*	30	90	[41]
			Enterobacter aerogenes	60	85	[51]
	Thymidine		*Proteus vulgaris*	45	66	[51]
			recombinant *E. coli*	50	90	[63]

that *T. thermophilus* strains present an active NdRT, rather than two NPs, however, after cloning and isolation experiments, three NPs activities with complementary substrate specificities were identified [44]. Two purine phosphorylases, *Tt*PNPI and *Tt*PNPII and one pyrimidine phosphorylase, *Tt*PyNP, were characterized. *Tt*PNPI showed high-molecular-mass and specificity for 6-oxopurines (guanine and hypoxanthine) while *Tt*PNPII displayed low-molecular mass and 6-aminopurines (adenine) specificity, which is the opposite of what happens in most organisms [45]. *Tt*PyNP uses thymine as well as uracil but not cytosine as substrates. As consequence of the tolerance at position 5, 5-Iodouridine was also tolerated by the enzyme. Interestingly, the three NPs phosphorolyse 2'-deoxyribosyl nucleosides more efficiently than the corresponding ribonucleosides. Same results were observed by Tomoike *et al.* [46] and, after an exhaustive structural study, they concluded that this enzymatic behaviour is conserved in most thermophilic species belonging to the *Deinococcus-Thermusphylum* phylum and other thermophilic organisms such as *G. stearothermophilus*.

Regarding *G. stearothermophilus*, the strain CECT43 was used as whole cell biocatalyst to prepare 6-halo- and 2,6-diaminopurine nucleosides [41]. 6-Halogenated purine nucleosides and their analogues play an important role in antiviral therapies, in particular, against hepatitis C virus (HCV) and SARS coronavirus (SARS Co-V) [47]. As expected, both ribo- and 2'-deoxyribosides of 6-halopurines were obtained with a conversion that reached 90% for the synthesis of 6-chloropurine-2'-deoxyriboside [48].

2,6-Diaminopurine nucleosides (DAP-Ns) are used as pharmaceutical drugs or prodrugs in cancer and antiviral therapies [49, 50]. As prodrugs, DAP-Ns are converted *in vivo* into guanosine analogues by the deamination performed by endogenous ADA [39] (Scheme **3**). De Benedetti *et al*. [41] carried out the synthesis of DAP-2'-deoxyriboside and DAP-riboside at 30°C in 90% and 83% conversion respectively, using *G stearothermophilus* CECT43, a thermophilic strain. Similar conversions for the same products were previously reported [51] but employing 60°C and in addition, DAP-2',3'-dideoxyriboside (DAPddR) and DAP-arabinoside (DAPA) were also obtained. The synthesis of this range of DAP analogues was possible by using different mesophilic bacterial whole cells, which unlike thermophilic ones, are able to accept 2',3'-dideoxyuridine and uracil arabinoside (AraU) as substrates.

Other examples of pharmaceutical drugs that can be prepared by whole cell biocatalysis are adenine arabinoside (9-β-D-arabinofuranosyladenine, vidarabine), a broad spectrum antiviral agent used in the treatment of human diseases caused by HSV, CMV and HBV [52]; and fludarabine, a fluoro analogue prescribed for chronic lymphocytic leukaemia therapies [53]. Moreover, guanine arabinoside (AraG) as well as their prodrugs, such as the above mentioned DAPA and the commercially available nelarabine, are also powerful anti leukemic agents [54].

Whole cells of *Enterobacter aerogenes* HXY2222 were reported as biocatalyst for vidarabine synthesis starting from AraU and adenine [55]. The transglycosylation was carried out at 60°C, in phosphate buffer, achieving 83% yield after 30 h. In this regard, we also reported the production of different arabinonucleosides from AraU and the corresponding bases by transglycosylation reactions biocatalyzed by *Citrobacter koseri* whole cells [23]. Among other nucleosides, three pharmacologically active arabinonucleosides, fludarabine (58% yield in 14 h), vidarabine (71% yield in 26 h) and DAPA (77% yield in 24 h), were prepared.

Transglycosylation using guanine as base donor usually fails due to its poor solubility. To overcome this drawback, some alternative strategies have been reported including: the use of more

soluble guanine derivatives like glyoxal-guanine or guanosine [56], the increase of reaction temperature employing thermostable NPs and the use of basic pHs. Multienzymatic systems were also described, where transglycosylation is coupled to other biocatalyzed steps either to use more suitable reagents for NPs or to shift the reaction equilibrium [16]. As mentioned above, whole cells may display deleterious activities that can catalyze the transformation of transglycosylation substrates and/or products into undesirable compounds. This effect, that may produce a decrease in the biotransformation productivity, can be used however [in a profitable way]. For example, AraG can be obtained *in vitro* by a coupled system based on a combination of NPs and deaminases. After obtaining DAPA by transglycosylation, deamination can be achieved using commercially available ADA from mammalian sources [57] or isolated adenylate deaminase from *Aspergillus oryzae* DAW-01 [58]. A one pot alternative, based on the action of two whole cells to perform the simultaneous transglycosylation and deamination reactions was applied by us to synthesize AraG [16]. The deamination step was carried out by *Arthrobacter oxydans* [59], whose ADA has the peculiarity of remaining active at 60°C (Scheme **3**). The broad specificity of *A. oxydans* made possible to obtain other pharmacologically active compounds such as didanosine. The presence of adenine and guanine deaminases in *A. oxydans* was also demonstrated. The combined activity of NPs and ADA s*et al*so the basis for the *in vivo* action of guanosine prodrugs. 6-Substituted guanosine analogues can be synthesized by transglycosylation and after administration, human ADA hydrolyzes them to the parent active guanosine drug [60].

Another interesting strategy for the multienzymatic synthesis of guanosine analogues consists of converting the base released during transglycosylation, into a compound unable to be accepted by NPs in order to shift the reaction equilibrium towards the product. When hypoxanthine nucleosides are used as starting materials for the transglycosylation reaction, xanthine oxidase is added to the reaction medium, in order to convert the released base into uric acid [61]. Horinouchi *et a.* [62] described the first detailed oxidative pyrimidine metabolism in *Rhodococcus erythropolis* showing that pyrimidine bases are initially oxidized to barbituric acid analogues by uracil/thymine dehydrogenase, and then the barbituric acid analogues are further hydrolyzed to ureidomalonic acid as result of the barbiturase action; finally, an ureidomalonase, catalyzes the amidohydrolysis to urea and malonic acid. Therefore, *R. erythropolis* JCM 3132 whole cells can be used to carry out the removal of the pyrimidine base released during the transglycosylation reaction, increasing the productivity of guanosine analogues synthesis (Scheme **4**).

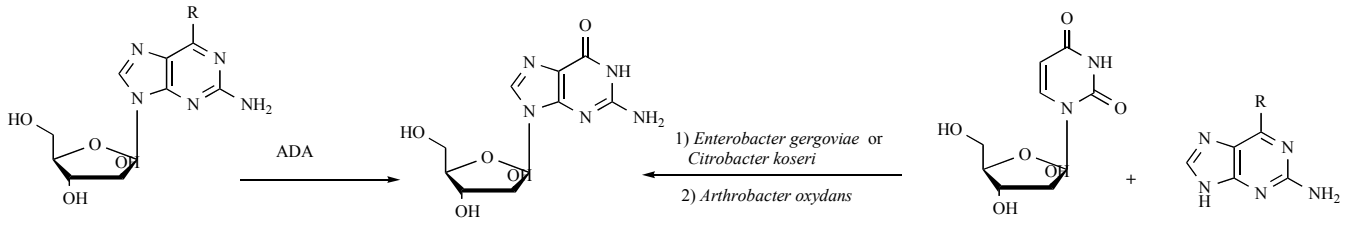
In addition, considerable progress has been achieved in the production of recombinant enzymes during the last decade, making available a wide set of biocatalysts for broader applications. Genetically engineered bacteria that express high levels of recombinant NPs have been successfully used to improve the production of NAs. These reactions require less amount of biomass and shorter reaction times. NAs such as 2,6-diaminopurine-2'-deoxyriboside [63] and 2-chloro-2'-deoxyadenosine [64] have been successfully synthesized using recombinant strains.

Liang *et al*. [65] cloned and overexpressed NPs genes from *E. coli* BL21 in *E. coli* DH5α and the recombinant bacteria were used for synthesizing purine deoxynucleosides such as 2'-deoxyadenosine (dA) and 6-methylpurine-2'-deoxyriboside (MePdR). Although we obtained a similar yield of dA (95%) using *E. coli* BL21 wild type [66], the results reported by of Liang *et al*. are significantly superior since they used fifty times less biomass and five times more substrate concentration to obtain the product in half the time. In addition, ADA deleterious activity was not detected probably, because of the low amount of cells and the short time employed for the reaction.

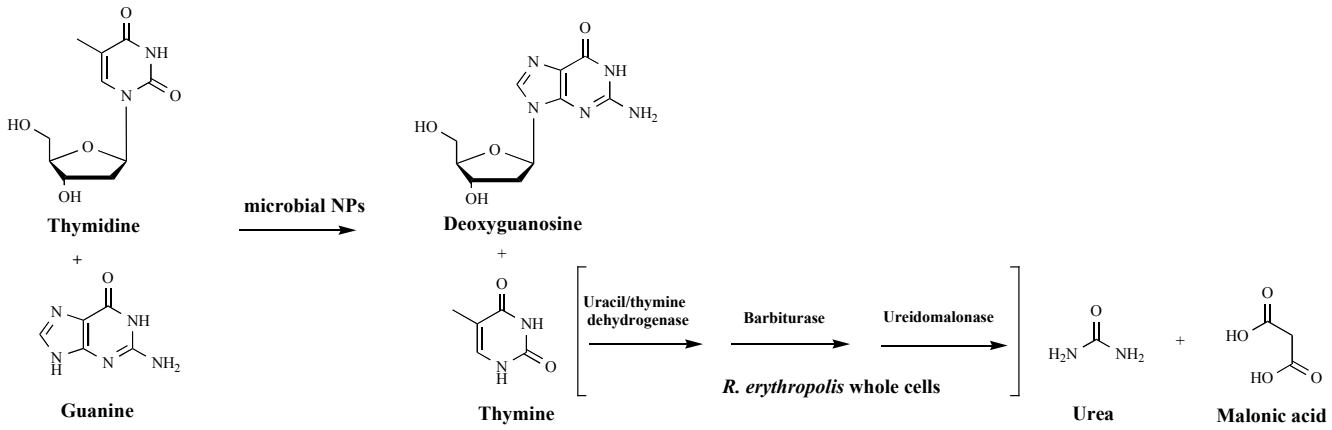
E. coli PNP, but not human PNP, converts MePdR into the cytotoxic drug 6-methylpurine (MeP). Therefore, an anti-tumour therapy system (*E. coli* PNP/MePdR) was developed to make use of MePdR as a tissue specific prodrug strategy [67]. In this system, *E. coli* PNP is either expressed or targeted by fusion to a tumour specific antibody or antibody fragment in order to efficiently generate the tumour cell killer MeP. The prodrug MePdR, could be produced in 95% yield in one hour using as biocatalysts the recombinant strains *E. coli* DH5α/pBV220-PNP and DH5α/pBV220-TP from thymidine and MeP [65].

Although expression systems that require induction with IPTG are widely used, the high cost, the usual inhibition of bacterial growth, and the complex cell harvesting have limited the industrial application of recombinant biocatalysts. In 2005, Studier developed an auto-induction method for culturing expression strains to saturation, with little or no exogenous induction [68]. Based on this work, Xiong *et al*. [69] used the auto-induction ZYM-Fe-5052 medium which contains 100 μM $FeCl_3$, 2 mM $MgSO_4$, 25 mM Na_2HPO_4, 25m MKH_2PO_4, 5 mM Na_2SO_4, 0.05% glucose, 0.5% glycerol and lactose (0.2%), for the culture of recombinant strains further used as biocatalysts for preparing dA and 5-methyluridine (5-MU). Genes encoding PNP, UP and TP from *E. coli* K12 were cloned and overexpressed into *E. coli* BL21(DE3), generating three recombinant strain BL21/pET28a-PNP, BL21/pET28a-UP and BL21/pET28a-TP called BP, BU and BT, respectively. ZYM-Fe-5052 medium promoted the generation of a high density culture of *E. coli* BL21(DE3). BT and BP strains were used to catalyze the synthesis of dA from thymidine and adenine, reaching 96% conversion in 1 h. In order to prepare 5-MU, the two PyNP strains BT and BU were used affording 56% yield in 1 h. Uridine was selected as the donor nucleoside and an excess of thymine was added to shift TD equilibrium towards nucleoside cleavage.

Two years later, the same group [70] reported the co-expression of UP and TP from *E. coli* K12 into *E. coli* BL21(DE3). Co-expression of different enzymes in a single strain provides an effi-



Scheme 3.



Scheme 4.

cient method to simplify both biocatalyst preparation and biotransformation process, especially in large-scale industrial production [71]. BTU strain, co-expressing BL21/pET28a-TP, pET21a-UP, was cultivated in ZYM-Fe-5052 medium for 10 h and used as catalyst in the synthesis of dU. This compound is a crucial intermediate in the preparation of deoxynucleoside analogues such as idoxuridine and 5-trifluoromethyl-2'-deoxyuridine, which are effective anti-herpes virus drugs [30]. Starting from thymidine and uracil, 61.6% yield of dU was obtained at 50°C and 1 h reaction time employing only 0.25% dry wt/v of recombinant whole cell biocatalyst, increasing dU productivity by 8-9 fold when compared to wild type *E. Coli* K12 and *E. coli* BL21(DE3) strains.

In order to obtain DAP-nucleosides, Ge *et al*. [71] designed two recombinant *E. coli* BL21(DE3) strains harbouring double recombinant plasmids, using genes encoding PNP, TP and UP, also from *E. coli* K12: PNP-UP (DUD) and PNP-TP (DAD) and two other strains containing tandem recombinant plasmids (TDU and TDA). After IPTG induction, these new biocatalysts were assayed in transglycosylation reactions. DAP-2'-deoxyriboside could be obtained from DAP and thymidine, reaching yields of 40.2% and 51.8% when the biocatalysts employed were DAD and TDA respectively. TDU and DUD showed higher activity, transforming uridine to DAP-riboside in 88.2% and 58.0% yield, respectively. Although in our work [51] the obtained yields using wild type whole cell biocatalysts were higher, with the use of recombinant strains reaction time was shortened and smaller amount of wet cells were used.

In a later work [63], lactose was used instead of IPTG for inducing the expression of the same four recombinant strains and after optimization of the reaction conditions, DAP-nucleosides production was improved. TDU was able to catalyze the transformation of 90% of uridine and DAP into DAP-riboside while using DUD 83% conversion was achieved. In the case of DAP-2'-deoxyriboside, similar conversions (>70%) were obtained starting from thymidine and DAP when TDA or DAD were employed as biocatalysts. In addition, some other ribo- and 2'-deoxyribonucleosides were synthesized with TDA, TDU, DUD, or DAD, such as 5-MU, 5-fluorouridine, uridine, adenosine, azauridine, ribavirin, dA, dI, and 2'-deoxyribavirin in yields ranging from 50 to 85%.

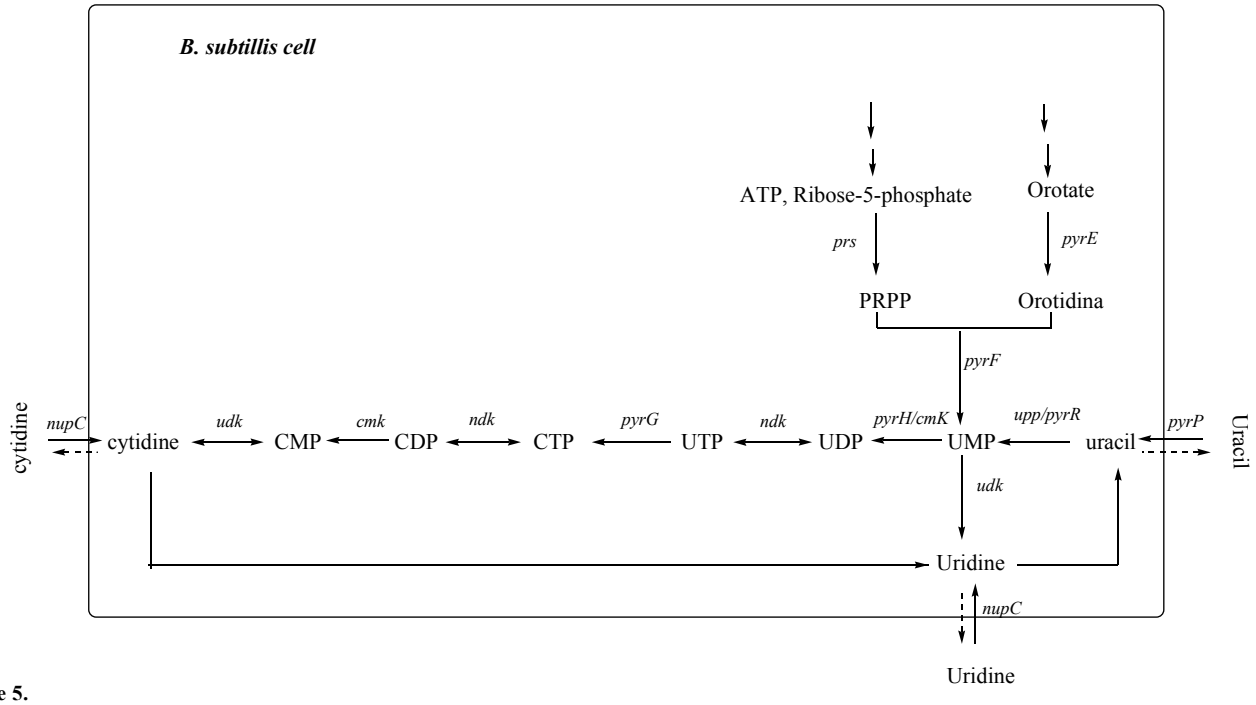
In recent years, there has been a great interest in biotechnology and synthetic biology research related to the design and construction of factory cells [72]. The removal of nonessential regions from a bacterial chromosome facilitates the optimization of metabolic pathways and energy utilization, tailoring cell functions to a particular need [73].

Bacillus subtilis and related bacteria are widely used as hosts for industrial production of enzymes and for the synthesis of small-molecule chemicals such as nucleosides, riboflavin, D-ribose, 2,3-butanediol and acetoin [74]. In particular, *B. subtilis* is commercially employed to produce cytidine and uridine. Zhu *et al*. [75] modified some key genes and operons related to the pyrimidine nucleotide biosynthesis in *B. subtilis* 168 and investigated the influence of these modifications on the production of pyrimidine nucleosides. In order to increase productivity, they constructed a series of recombinant strains by deregulation of the *pyr* operon and the overexpression of the *prs* gen, allowing the accumulation of uridine 5'-monophosphate (UMP) to be further converted to terminal metabolites (cytidine, uridine and uracil) (Scheme **5**). In addition, in these recombinant strains, *pyrG* and *pyrH* genes were overexpressed to improve the ratio of cytidine to other pyrimidine nucleoside products and the *nupC-pdp* gene was deleted to avoid uridine conversion to uracil. A key factor for the accumulation of cytidine instead of uridine is cytidine deaminase activity; therefore, *cdd* gene is activated or inactivated depending on the target of the recombinant cell. Through this methodology the production of uridine and cytidine could be enhanced to 1684.6 mg/L and 1423 mg/L, respectively. More recently [76], following a similar strategy, the authors constructed a guanosine and thymidine producing recombinant *B. subtilis* strains BSK814 and BSK756, with the genome size reduced by up to 814.4 kb. Recombinant strains produced 115.2 mg/L of guanosine and 151.2 mg/L of thymidine, which represented a 4.4 and 5.2-fold increase compared to full-genome control strains, respectively.

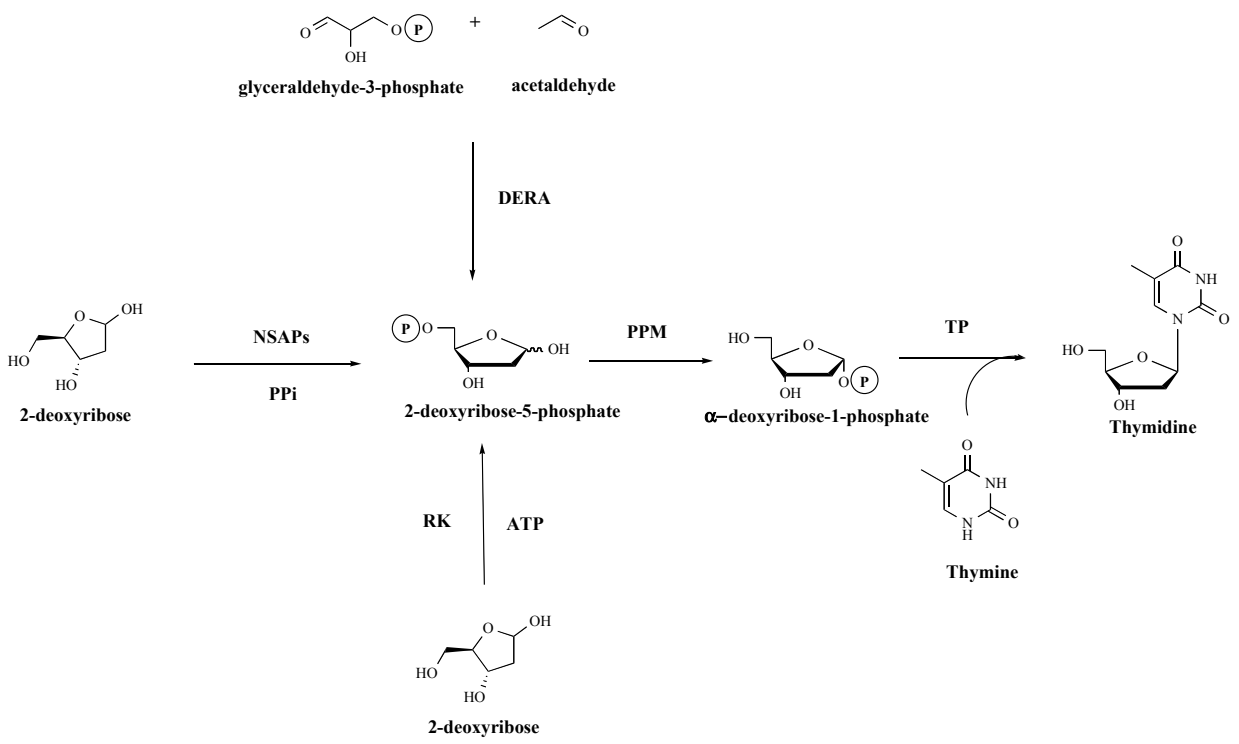
2.1.2. Glycosylation

As discussed previously, biocatalyzed transglycosylation using whole cells that contain nucleoside phosphorylases (NPs) is a one-pot cascade reaction that proceeds through the formation of the corresponding α-furanose 1-phosphate and requires pyrimidine nucleosides and inorganic phosphate as starting materials. An alternative approach is to employ furanose 1-phosphate intermediates as starting materials, being this strategy more suitable than transglycosylation for the preparation of pyrimidine nucleosides.

The synthesis of some interesting arabinonucleosides was described by Mikhailopulo group [77] using α-D-arabinofuranose 1-phosphate and recombinant *E. coli* PNP as the biocatalyst. However, the availability of furanose 1-phosphates is limited due to their intrinsic instability and their cumbersome synthesis [78, 79]. On the other hand, furanose 1-phosphates can be enzymatically obtained from furanose 5-phosphates employing phosphopentomutase (PPM). This enzyme takes part of the pentose pathway in bacteria and in mammal tissues, catalyzing the transfer of a phosphate group



Scheme 5.



Scheme 6.

between the hydroxyls of positions 5 and 1 of ribose and 2-deoxyribose [11] (Scheme **6**).

We reported a chemoenzymatic synthesis of furanose 5-phosphates [80] through the lipase biocatalyzed regioselective protection and deprotection of the corresponding sugars. The obtained furanose 5-phosphates were further employed, for the synthesis of a set of natural and modified nucleosides using an overexpressed PPM from *E. coli* and commercial PNP or TP as biocatalysts.

Additionally, the 5-*O*-phosphorylation of furanoses can be carried out by ribokinase (RK) [81] using ATP as a phosphate donor in presence of Mg^{2+}. *E. coli* RK was expressed in *E. coli* ER2566 by Chuvikovsky *et al.* [82] and used as the biocatalyst for the first step of the cascade transformation of different pentoses into nucleosides. Notably, cladribine, a standard drug for the treatment of hairy cell leukaemia was prepared from 2'-deoxyribose and 2-chloroadenine in 90% yield [83].

Sugars can also be regioselectively phosphorylated using bacterial nonspecific acid phosphatases (NSAPs) and disodium acid pyrophosphate (PPi) as the phosphate source [84]. NSAPs are a group of enzymes that are able to hydrolyze a broad range of organic phosphoesters, displaying optimal activity in acidic or neutral pH. In addition to their intrinsic phosphatase activity, some enzymes also exhibit phosphotransferase activity. In this sense, we described that intact cells of recombinant *E. coli* BL21(DE3) strains carrying *Enterobacter aerogenes* and *Raoultella planticola* acid phosphatases [85] proved to be efficient catalysts for the synthesis of ribose-, 2'-deoxyribose- and arabinose 5-phosphates.

There is a growing demand for 2'-deoxyribonucleoside (dNs) since they are key supplies for PCR techniques and constitute also essential starting materials for the synthesis of medicinal drugs. This is the reason why there is a constant search for the development of efficient dNs production processes starting from inexpensive materials.

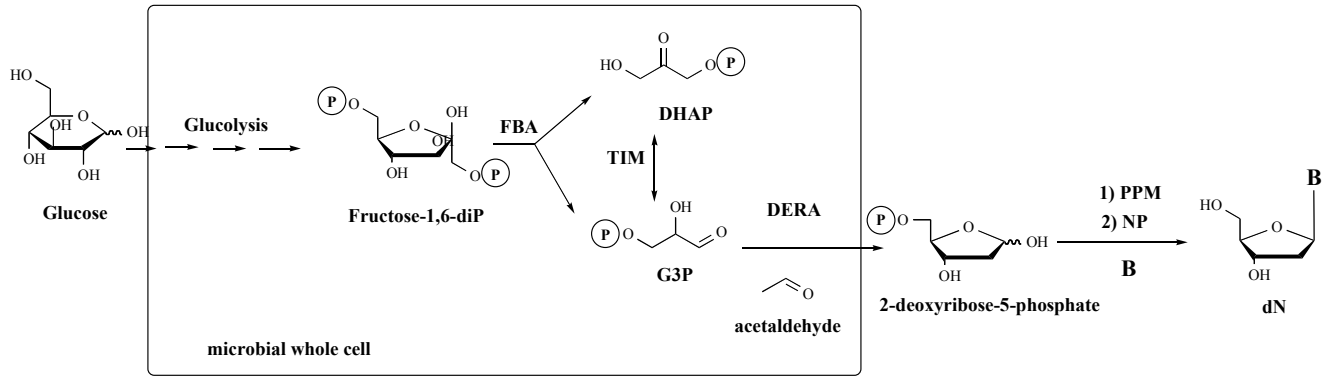
Shimizu's group [86] developed a very attractive multienzymatic process to prepare dNs that involved glycolytic enzymes. In this route, D-glyceraldehyde 3-phosphate (G3P) was prepared from glucose and further converted to 2-deoxyribose 5-phosphate (DR5P) through the condensation with acetaldehyde catalyzed by 2-deoxy-D-ribose-5-phosphate aldolase (DERA). DR5P was transformed into 2-deoxyribose 1-phosphate (DR1P) by the action of PPM, and finally a NP catalyzed the formation of dNs in the presence of a nucleobase (Scheme **7**).

Aldolases are enzymes that catalyze the aldol condensation between two carbonylic compounds by a stereoselective and reversible reaction, constituting an attractive tool in the synthesis of chiral bioactive compounds, such as carbohydrates, amino acids, and their analogues [87]. Whereas aldolases can typically use a broad range of aldehydes as acceptors, the donor compound is often structurally invariable [88]. DERA is the only known member of the acetaldehyde-dependent aldolase family and is also the only known aldolase that catalyzes the aldol reaction between two aldehydes [89]. In the past few decades, much attention has been paid to the isolation and characterization of DERA variants from different microorganisms like *Paenibacillus sp*. EA001 [90], *Yersinia sp*. EA015 [91], *Pyrobaculum aerophilum, Thermotoga maritime* [92], *Thermus thermophilus* HB8 [93], *Streptococcus mutans* GS-5 [94], *Haemophilus influenza* [95], and *Streptococcus suis* [96] and also from some extremophilic organisms [97]. However, the practical application of DERA is limited due to its low affinity towards acetaldehyde and low stability at high concentrations of this reagent. Apart from interfering with surface lysine residues (thereby destabilizing the enzyme tertiary structure), acetaldehyde can also exert a detrimental effect on enzyme activity when the catalytic lysine residue in the active site is inactivated [98]. To increase its tolerance to high aldehyde concentrations [99] and also to improve its affinity to non-phosphorylated substrates [100, 101], DERA has been mutated *via* several random mutagenesis strategies, such as error-prone PCR and DNA shuffling and/or targeted mutagenesis. Alternatively, screening of environmental DNA libraries [102] has also proven to be successful for this purpose. In view of industrial applications, a DERA overexpressing *E. coli* fermentation broth has been employed as a whole-cell DERA biocatalyst [103].

In earlier works, Shimizu's group reported for the first time the synthesis of 2'-deoxyinosine (dI) [104] from glucose [105] or fructose 1,6-diphosphate (FBP), produced by yeast glucose fermentation [106], acetaldehyde, and adenine through three microbial steps without expensive energy sources such as ATP. This route comprised: a recombinant phosphatase-negative *E. coli* 10B5 cells expressing DERA from *Klebsiella pneumoniae*, *E. coli* BL21 cells overexpressing PPM from *E. coli* and a commercial PNP. It is noteworthy that dI was produced from adenine due to the ADA activity displayed by the *E. coli* transforming strains. Unfortunately, PPM from *E. coli* is markedly inhibited by phosphorylated compounds, such as FBP and G3P and also by inorganic phosphate. Therefore, after optimization of the reaction conditions in order to achieve low concentration of phosphate to prevent PPM inhibition, the authors were able to successfully produce dI in a one-pot process [107]. The yield of dI afforded in this work (9.9 mM, 80.2% yield based on DR5P and 17.7% on glucose) was similar to the one obtained in the *de novo* fermentative production of dI [108] and is better than the previously reported yield obtained through hydrolysis of the DNA generated by fermentation with glucose [109]; for this reason, they suggest that this methodology could result in an attractive industrial process and encouraged further improvements.

In order to select new DERA sources with both high synthetic activity and tolerance to elevated acetaldehyde concentration, we [110] described a hierarchical screening procedure. In this way, a non-pathogenic wild type microorganism, *Erwinia carotovora* ATCC 33260, was identified as a new DERA-containing bacterial whole cell biocatalyst. Starting from glucose and acetaldehyde, and the addition of non-ionic detergents to enhance cells permeability [111], 14.1 mM DR5P were obtained. To synthesize thymidine, this reaction was coupled to the action of a recombinant PPM isolated from *E. coli* and commercial TP. After DR5P formation, the final reaction medium was lyophilized to completely remove acetaldehyde, and the residue re-dissolved to fit PPM and TP requirements. After 2 h of the addition of thymine, thymidine was obtained in 85% conversion relative to DR5P [112]. This strategy was also applied to the synthesis of 5-bromo-2'-deoxyuridine achieving 100% conversion after 45 min reaction [80].

As previously mentioned, the one pot strategy for dNs synthesis faces the limitation imposed by the inhibition of *E. coli* PPM activ-



Scheme 7.

ity by phosphorylated compounds. To overcome this drawback, Horinouchi *et al*. [113] screened microorganisms isolated from soil and stock of bacterial and fungal cultures, selecting *Bacillus sphaericus* AKU229 as a source of both acetaldehyde and phosphorylated compounds tolerant PPM. This enzyme was cloned and expressed in *E. coli* BL21 and tested as whole cell biocatalyst for dI synthesis using as DR5P source the solution coming from the reaction of glucose and acetaldehyde using baker's yeast and DERA-expressing *E. coli* 10B5/pTS8 cells. Recombinant *B. sphaericus* PPM resulted 2 times more active than the *E. coli* PPM under the same conditions.

Later, these authors [114] constructed an *E. coli* BL21 co-expressing *K. pneumoniae* DERA-*E. coli* PPM that showed specific activities 9.8- and 7.0-fold higher than the host strain, respectively. They developed an efficient one pot production method for dNs consisting of the coupled reactions of the already mentioned recombinant strain, a commercial NP and acetone-dried baker's yeast. 75 mM of dI, the deaminated product of dA, was produced in high yield (83% relative to adenine).

Current advances in recombinant DNA techniques allow the construction of artificial bio-synthetic pathways for the production of value-added compounds. In particular, the overproduction of thermophilic enzymes in mesophilic organisms have additional advantages such as the elimination of undesired side reactions by thermal denaturation of host cell proteins, as well as the reduction of diffusion restrictions by disruption of the cell membrane barrier of mesophilic hosts by heating [115]. Honda *et al.* [116] provided an interesting alternative for the production of DR5P from fructose using six recombinant *E. coli* strains producing thermophilic enzymes from *Thermus thermophilus* (Scheme **8**). The biotransformation consists of 2 steps where the first one is the phosphorylation of fructose to FBP carried out at 70°C for 4 h by *E. coli* producing fructokinase (FK), 6-phosphofructokinase (PFK), and polyphosphate kinase (PPK) [117] from *T. thermophilus* HB27. After cell removal by centrifugation, the supernatant was directly used for DR5P production employing three recombinant *E. coli* strains, previously pre-heated at 70°C for 30 min, expressing FBP aldolase (FBA), triosephosphate isomerase (TIM) and DERA from *T. thermophilus* HB8 respectively. The reaction was incubated with acetaldehyde at 30°C affording a molar yield of 55% after 1 h.

2.2. Whole-cell Biotransformations as Key Step for NAs Chemoenzymatic Synthesis

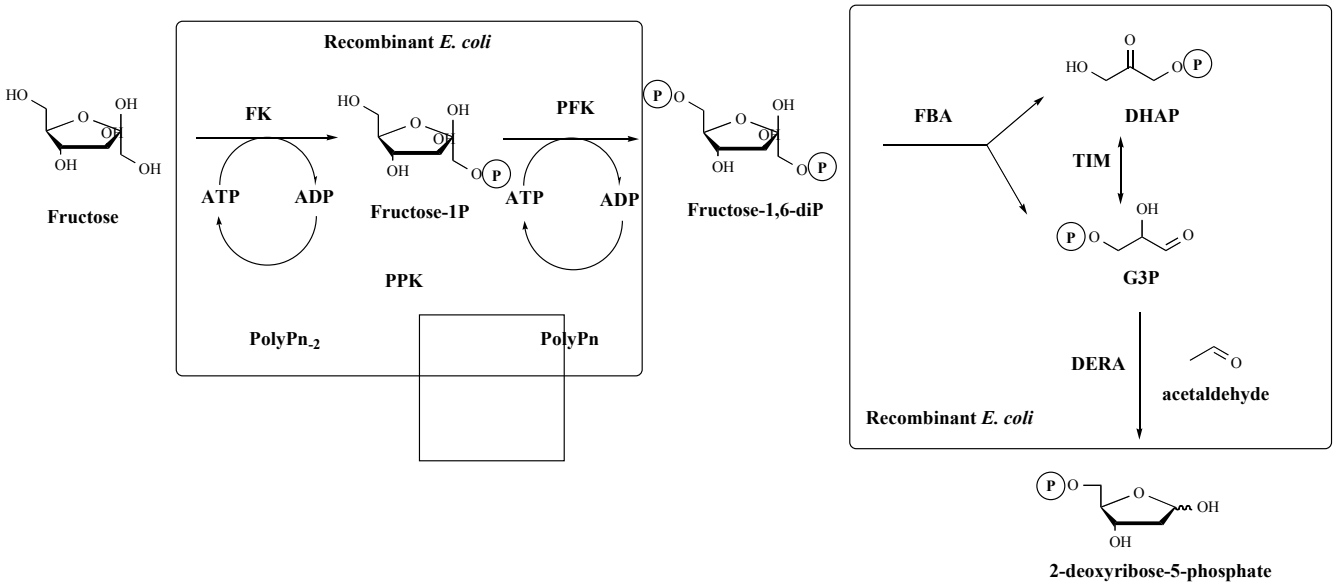
The continuous appearance of emerging diseases as well as the resistance developed to already available drugs make mandatory the constant generation of new active compounds. Although NPs show broad substrate specificity, there are many structural modifications of the heterocyclic bases and/or of the sugar moiety present in biological active nucleoside analogues that are not accepted by these enzymes. Among them are worth mentioning inversion of hydroxyl groups configuration, their elimination, their substitution or functionalization by a variety of functional groups, the replacement of the endocyclic oxygen and the presence of open sugars [3]. In addition, *C*-, homo- and reversed nucleosides whose structures are also not recognized by NPs, have emerged as particularly interesting drug candidates [118]. Scheme **9** shows different types of NAs.

Therefore, this section intends the revision of the new bibliography referring to particular whole cell biocatalyzed reactions involved in key steps of multistep synthetic routes of NAs that cannot be obtained by NP mediated glycosylations. For a better understanding, the discussion is structured by class of compounds.

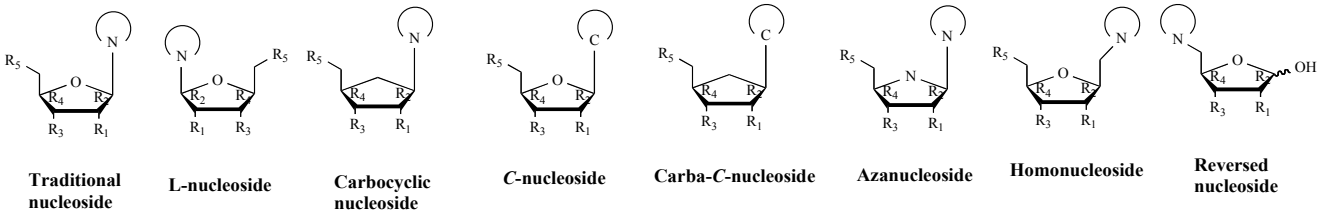
2.2.1. L-nucleosides

Much impetus was given to the use of L-nucleosides as drugs since the discovery, at the beginning of the 90's, of lamivudine, the first L-nucleoside approved against HIV and HBV. Since then, a large number of L-NAs have been synthesized, playing an effective role in the treatment of HBV, HCV, Epstein–Barr virus, and some other diseases [119]. L-Nucleosides are potentially attractive drug candidates since they are less cytotoxic and many of them show higher activity than the corresponding D-enantiomers.

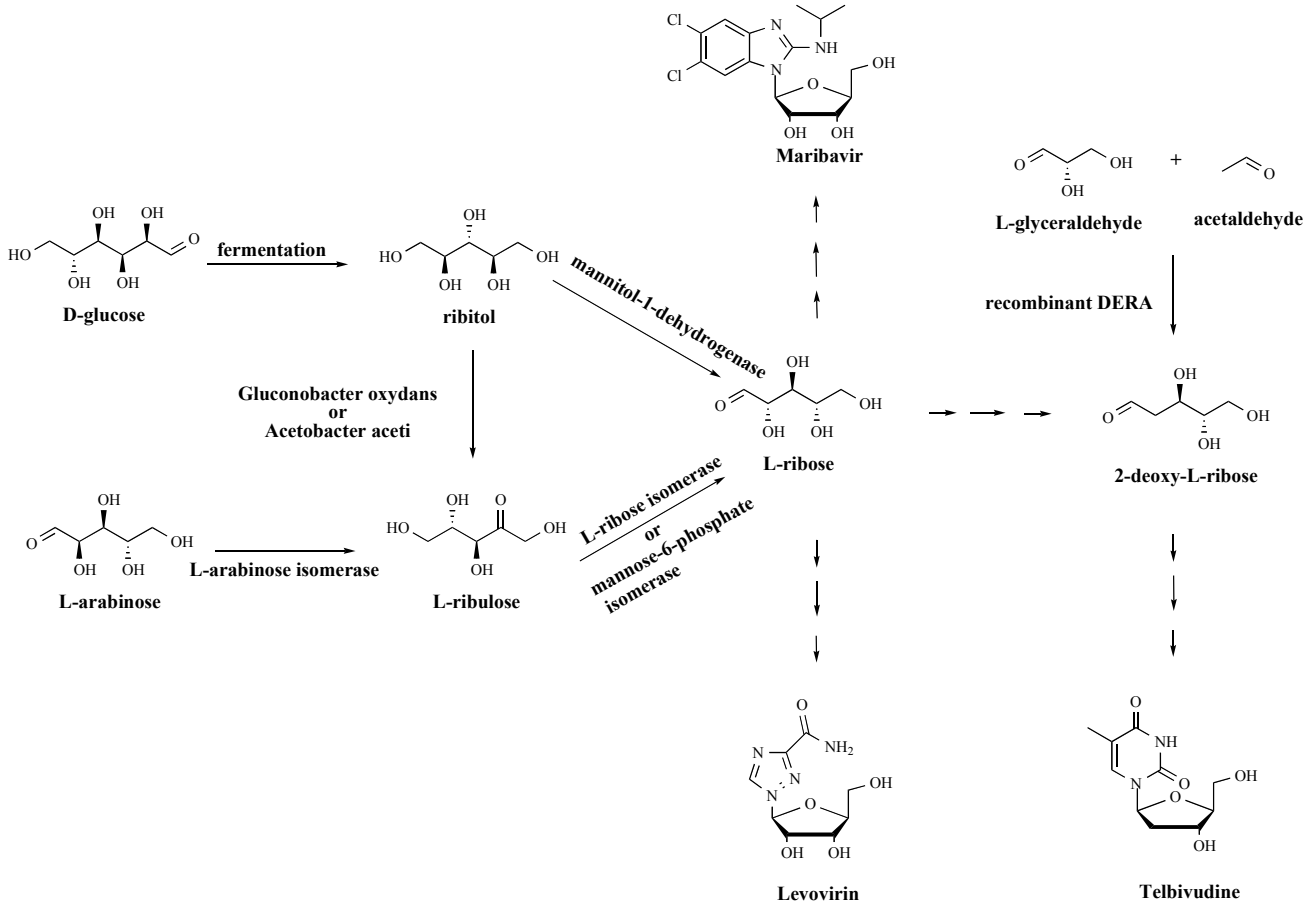
Not only NAs but also their oligomers, the oligonucleotides, have received much attention as potential therapeutic agents [120]. These functional oligonucelotides show different structures and ways of action like antisense, ribozymes, DNAzymes, siRNA, aptamers, among others. They find applications in the treatment of different diseases and also in fields such as molecular biology, diagnosis and development of new sensors. However, these molecules require chemical modifications in order to show proper stability in biological media. In this sense, L-oligonucleotides have been largely studied and in particular as proof of their wide range of



Scheme 8.



Scheme 9.



Scheme 10.

application, we have recently applied this kind of oligonucleotides as catalyst in the oxidation of diverse compounds [121].

L-Ribose is a non-natural sugar that can be used as intermediate for the synthesis of L-NAs. Since its use as starting material is hampered because of its high price, the development of an efficient synthesis is highly desirable. Several good synthetic routes to L-ribose from D or L-carbohydrates and non-sugar compounds have been reviewed [122, 123]. In contrast to chemical production, biocatalyzed methods can produce L-ribose from cheap raw materials under environmentally friendly conditions [124]. Although some revisions about the enzymatic synthesis of L-ribose [125-127] are available in literature, herein only the relevant works related to whole cell biocatalyzed processes published in the last years will be discussed.

Traditionally, to prepare L-ribose two multienzymatic strategies have been developed starting from L-arabinose or from glucose (Scheme **10**). Both routes proceed *via* a common intermediate, L-ribulose, which can be converted into L-ribose by the catalytic action of L-ribose isomerase (L-RI) or mannose-6-phosphate isomerase (MPI).

L-ribulose was obtained from D-glucose through a first fermentation step by *Trichosporonoides oedocephalis* or *Trichosporonoides megachillensis* followed by a second step where the formed ribitol was oxidized using washed cells of *Acetobacter aceti* [128] or *Gluconobacter oxydans* [129]. Since the overall yield was low and the purification of ribitol was difficult, this procedure was largely overcome by the process that starts from L-arabinose. L-arabinose is naturally abundant as a common component of polymers of lignocellulosic materials in plants and can be easily isolated. Furthermore, in the next future it will become more readily and cheaply available with the development of the biorefining industry [125]. L-Ribulose is an intermediate in the pathway for L-arabinose metabolism in many bacteria, being L-arabinose isomerase (L-AI) the enzyme responsible for the transformation of L-arabinose into L-ribulose. Since the next metabolic path consists

in its phosphorylation to L-ribulose-5-phosphate, Helanto *et al*. [130] designed a ribulokinase-deficient *Lactobacillus plantarum* strain. They applied resting cells of this mutant to the efficient production of L-ribulose by the action of its endogenous L-AI. The main drawback of this approach lies in the unfavourable reaction equilibrium towards the formation of L-ribulose. To solve this problem borate was added to the reaction medium, which forms a complex with ketoses resulting in a shift of chemical equilibrium and an enhanced final yield. In this way, they were able to achieve a productivity of 14.8 g/ L.h, much higher than that obtained from ribitol.

L-AI gene from *Bacillus licheniformis* was cloned and expressed in *E. coli*. Compared to other L-AIs, *B. licheniformis* L-AI has a wider pH range, a higher substrate specificity, and better catalytic efficiency for L-arabinose conversion [131]. Zhang *et al.* [132] reported the optimization of the process conditions to prepare L-ribulose in the presence of borate, employing whole cells of this recombinant *E. coli,* which led to a very high productivity, about 375 g/L.h. In addition, L-ribulose–borate complex could be easily separated from L-arabinose, making the method suitable for application on an industrial scale.

In the final step for L-ribose production, isomerization of L-ribulose was achieved both by L-RI and by MPI [133]. Since L-RI was rarely found in microorganisms, few sources are available. This enzyme presents lower k_{cat} values and poorer thermal stability than MPI, not existing directed evolution or site-directed mutation studies to overcome these drawbacks. In contrast, all MPIs used as biocatalysts were modified to increase their selectivity towards pentoses respect to hexoses, since natural isomerization activity is specific for all aldose substrates possessing hydroxyl groups oriented in the same direction at the C-2 and C-3 positions. A possible concern for MPI applications could be the required high reaction temperature, 70–75°C, which might cause degradation of L-ribulose during the biotransformation [127].

L-RI is an aldose-ketose isomerase that was first found in *Acinetobacter sp*. DL-28 by Kagawa *et al*. [134]. Enzymes from different organisms showed broad substrate specificity, an optimal temperature in the range of 30–40°C and optimal pH between 7.0 and 9.0. New sources of L-RI were explored with the aim of improving thermostability and productivity. Among them, a thermostable *Paenibacillus* RI-39 L-RI was reported by Pyun *et al.* [135], an L-RI from *Raoultella ornithinolytica* MB426 was described by Izumori *et al*. [136] and a thermoactive and thermostable L-RI from *Cellulomonas parahominis* MB426, a strain isolated from soil, was further expressed in *E. coli* [137].

In order of develop a new and efficient process for L-ribose production from L-arabinose, *Helanto et al*. [138] introduced an L-RI into an L-ribulokinase-deficient mutant of *L. plantarum*, and resting cells of this recombinant strain and L-arabinose were incubated at 39°C in MOPS buffer pH 8. Conversions achieved were around 20%, which were higher than those obtained using L-AI and L-RI as isolated enzymes [139].

MPI catalyzes the reversible isomerization of β-D-mannose-6-phosphate to D-fructose-6-phosphate [140]. In eukaryotes and prokaryotes, MPI is involved in the synthesis of mannosylated proteins which are essential for the biosynthesis of yeast cell wall. This function makes MPI an interesting target for inhibition of fungal infections. After Yeam *et al*. reported MPI ability to convert L-ribulose into L-ribose [141], different MPIs were discovered and genetically engineered to enhance isomerase activity and thermostability, including those from *T. thermophilus* [142], *G. thermodenitrificans* [143], and *B. subtilis* [141]. In particular, Kim *et al.* [144] developed a triple-site variant MPI from *G. thermodenitrificans* that showed a catalytic efficiency 7.1-fold higher than that of the wild-type MPI [145], being this mutant further co-expressed together with L-AI, from the same origin, in *E. coli* ER2566 (NEB) cells. This system was applied to the conversion of L-arabinose to L-ribose, being the first report about MPI used as whole cell biocatalyst. The productivity of this reaction was 33 g/L.h, constituting the highest conversion reported among processes using L-arabinose as the substrate.

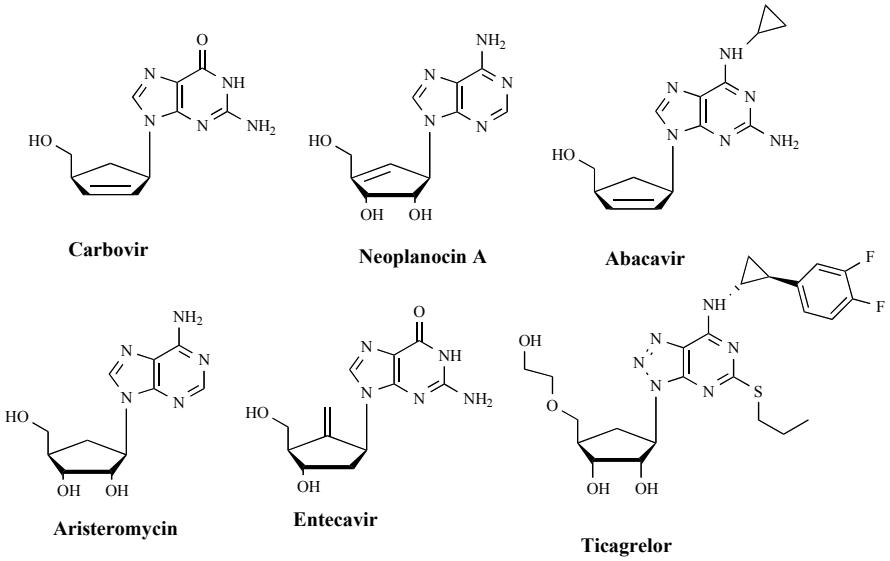
An original strategy was described by Woodyer *et al*. [146] for the preparation of L-ribose in one enzymatic step *via* regioselective oxidation of the terminal carbon of ribitol using an NAD-dependent mannitol-1-dehydrogenase (MDH) from *Apium graveolens* (garden celery). This enzyme, with notable 2*R* stereoselectivity, was cloned and overexpressed in *E. coli* and used as a whole-cell catalyst, yielding a volumetric productivity of 17.4 g/L.day. Mutants generated by directed evolution were selected for enhanced thermostability and conversion to L-ribose, affording an overall 19.2-fold improvement [147]. Although it is remarkable that the reaction consists of a single step, once more the problem of starting from ribitol limits its use for large-scale production.

Additionally, it is well-known that aldolases have been employed to prepare rare sugars [148]. As discussed in the previous section, DERA catalyzes the condensation of acetaldehyde with both, G3P to generate DR5P, and other nonphosphorylated aldehydes to synthesize many 2-deoxysugars. Recently, the above mentioned recombinant DERA from *K. pneumonia* [107] was subjected to multi-site-directed mutagenesis strategy and the generated $KDERA^{K12}$ (S238D/F200I/ΔY259) mutant exhibited a 3.15-fold improvement in enzyme activity and a 1.54-fold increase in substrate tolerance to polyhydroxy aldehydes compared with the wild type enzyme [100]. As confirmed by activity analysis of single mutants, S238D substitution increased the D-glyceraldehyde-binding capacity, F200I substitution enhanced the enzyme activity towards D-glyceraldehyde and deletion of Y259 improved substrate tolerance. Moreover, performing the aldol addition using resting cells, further increase in product yield and substrate tolerance was observed. Therefore, the ability of the *E. coli* BL21 ($pKDERA^{K12}$) strain to produce other 2-deoxysugars from non-phosphorylated aldehydes was tested. When using L-glyceraldehyde, not only the natural product 2-deoxy-L-xylose (3*R*, 4*S*) but also the non-natural product 2-deoxy-L-ribose (3*S*, 4*S*), were produced. This loss of stereochemical control of DERA was not a surprising behaviour since the same authors observed similar results when using other aldolases [149]. In this way, they showed the potential for using DERA in the *de novo* synthesis of many kinds of 2-deoxysugars.

2.2.2. Carbocyclic Nucleosides

Carbocyclic nucleosides (CNs) are NAs in which a methylene group replaces the oxygen atom of the furanosidic moiety, having some of them applications as antiviral, antitumor or antibiotic drugs [150]. They can be found in Nature like aristeromycin [151] and neplanocin A [152], or be the result of synthetic routes such as entecavir [153], carbovir [154], abacavir [155] and ticagrelor [156] (Scheme **11**). The structural modification present in CNs results in an increase in the chemical stability of the *N*-glycosidic bond and confers metabolic resistance to the action of several enzymes such as NPs.

Chiral Vince lactam (γ-lactam) 2-azabicyclo [2.2.1] hept-5-en-3-one, which is efficiently synthesized from an aza-Diels–Alder cycloaddition reaction, has been used as one of the most successful intermediates for the synthesis of carbocyclic nucleosides for more than 30 years. Vince and Singh [157] were the first to realize that this intermediary has the distinctive characteristic of possessing the required *cis* orientation between the hydroxymethyl and the nucleobase functions. Both enantiomers of the γ-lactam are key chiral precursors of many CN analogues and other compounds with important pharmaceutical relevance [158]. In particular, (−)-γ-lactam is a common precursor for the synthesis of carbovir, abacavir and peramivir. Abacavir is a carbocyclic guanosine analogue that is used in the treatment of human HIV and HBV, acting as a prodrug



Scheme 11.

of carbovir with improved toxicity profile and higher oral bioavailability [159]. Peramivir has emerged as a promising long-acting neuraminidase inhibitor for the treatment and prophylaxis of human influenza virus infection [160].

Enzymatic resolution of γ-lactam has been widely studied, and there are several enzymes that provide excellent enantioselectivity. Among them, γ-lactamase, a type of amidase, is the preferred one for obtaining optically pure γ-lactam, and both (+)-γ- and (-)-γ-lactamases exist, although their *in vivo* activity is unknown [161]. The resolution of γ-lactam to both optical forms has been performed by Taylor *et al*. [162] in very high optical purity using whole cell catalysts for the first time (Scheme **12**). Two strains, ENZA-1 (*Rhodococcus equi* NCIB 40213) and ENZA-20 (*Pseudomonas solanacearum* NCIB 40249), were isolated from the environment as organisms capable of growing in presence of *N*-acyl compounds as the sole source of carbon and energy. However, wild-type strains often do not me*et al*l the requirements to be robust biocatalysts and therefore, the heterologous expression of γ-lactamases is a favoured approach [163]. To improve stability and activity, mutant strains of ENZA-1 and ENZA-20 have been constructed and used as whole cell biocatalysts. Using 100 g/L of the racemic substrate, (+)- γ-lactam was recovered after 3 h reaction employing ENZA-1 as biocatalyst (>98% ee, 45% yield) while ENZA-20 produced the (-)-γ-lactam with the same performance. After isolation of the lactam from the reaction medium, the desired (1*R*,4*S*)-(-)-enantiomer was further used to synthesize abacavir.

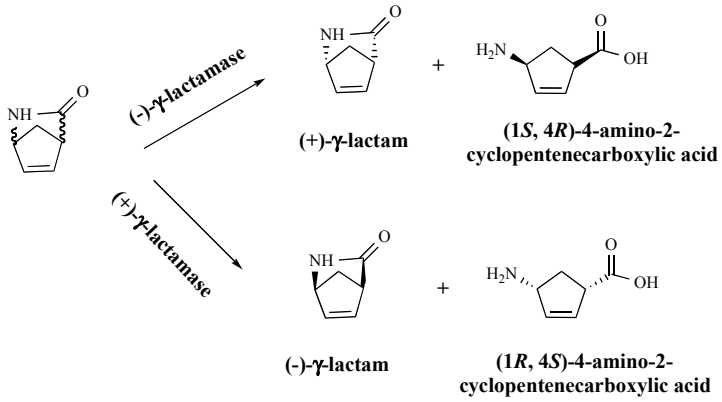
There are few microorganisms reported to date with (+)-γ-lactamase activity and only Microbacterium hydrocarbonoxydans, Sulfolobus solfataricus, Comamonas acidovorans, Pseudomonas cepacia, Pseudomonas solanacearum, Pseudomonas fluorescens, Delftia sp.CGMCC 5755 and Pseudomonas granadensis B6 have been used as whole-cell biocatalysts [158, 164-169]. Among them, the last one, a non-thermophilic bacterial strain recently discovered, is expected to be suitable for large-scale (−)-γ-lactam production since it does not require high reaction temperatures, can tolerate high substrate concentration (up to 300 g/L) and the ee could reach 99.9%. The other wild type microorganisms resolved lower lactam concentration and/or afforded lower ee.

In order to obtain more active and low cost biocatalysts, the gene coding for (+)-γ-lactamase from *Delftia* sp. CGMCC 5755 was overexpressed in *B. subtilis*168, since a previous attempt in *E. coli* BL21(DE3) failed because most of the proteins were located in inclusion bodies. Recombinant *B. subtilis* whole cells were used, for the first time for the stereoselective preparation of (−)-γ-lactam, resulting highly efficient; starting from 100 g/L *rac* γ-lactam, 53.1% conversion rate to (−)-γ-lactam with 99.9% ee was produced [170].

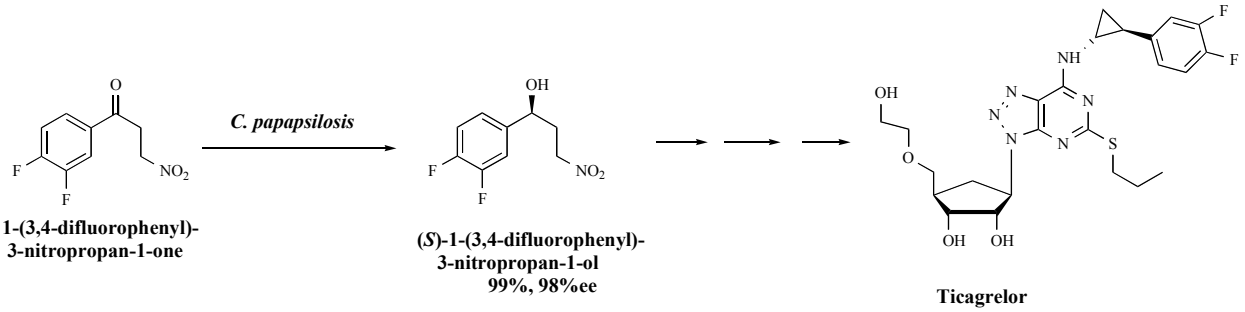
Zhu *et al*. [171] described the first identification of a (+)-γ-lactamase from *B. japonicum* USDA6 based on sequence-structure guided genome mining methodology, unlike all the other lactamases that were isolated from microorganisms selected through traditional screening methodologies [172]. The novel (+)-γ-lactamase was expressed in *E. coli* and both *B. japonicum* USDA6 wild type and recombinant whole cells were tested for the production of chirally pure (−)-γ-lactam. While recombinant *E. coli* hydrolyzed the (+)-γ-lactam specifically with a yield of 49% and an enantiomeric excess of 99%, the stereoselectivity of the *B. japonicum* USDA6 wild type was poor. Based on these evidences, the authors proposed, and later verified, the existence of two totally different enantioselective lactamases: a (+)-γ-lactamase belonging to the amidase family, with 504 amino acids, and a (-)-γ-lactamase from the hydrolase family containing 274 amino acids [173]. Both enzymes were able to be easily overexpressed in a different host to prepare both optically pure lactam enantiomers.

At the same time, Wang *et al*. [174] also reported the existence of two complementary γ-lactamases in *Microbacterium hydrocarbonoxydans*. However, contrasting what happened in *B. japonicum,* both, the quantity and the activity of the (+)-γ-lactamase were higher than those of the (−)-γ-lactamase, being the reason why whole cells presented (+)-γ-lactamase activity.

Ticagrelor is a P_2Y_{12} receptor antagonist that functions by blocking adenosine diphosphate-mediated platelet aggregation and is recently approved by FDA for the prevention of thrombotic events such as stroke or heart attack. There are many published chemical syntheses of this CN and its derivatives [156] that involve the key chiral intermediate difluorophenyl cyclopropylamine. Zhang *et al*. [175] developed a convergent synthesis where this intermediate was formed from (*S*)-1-(3,4-difluorophenyl)-3-nitropropan-1-ol prepared by stereoselective reduction of the corresponding prochiral phenyl ketone (Scheme **13**). However, this chiral chemical catalyzed procedure afforded low yield and enantioselectivity, being in addition the employed catalyst expensive. As an alternative, Singh *et al*. [176] proposed to carry out ketone reduction *via* a whole cell biocatalytic process. Sixteen different mi-



Scheme 12.



Scheme 13.

croorganisms were screened and tested both, in growing or resting, for the bioreduction of 1-(3,4-difluorophenyl)-3-nitropropan-1-one to the corresponding alcohol. *Candida* species demonstrated superior activity and selectivity in comparison to other strains and among them, *Candida parapsilosis* was chosen. After optimization of fermentation and reaction media, *C. parapsilosis* exhibited high conversion (>99%) and enantioselectivity (98%) for the *S*-enantiomer at 4 g/L substrate concentration with the advantage of not requiring external cofactors or cofactor recycling systems.

2.2.3. C-nucleosides

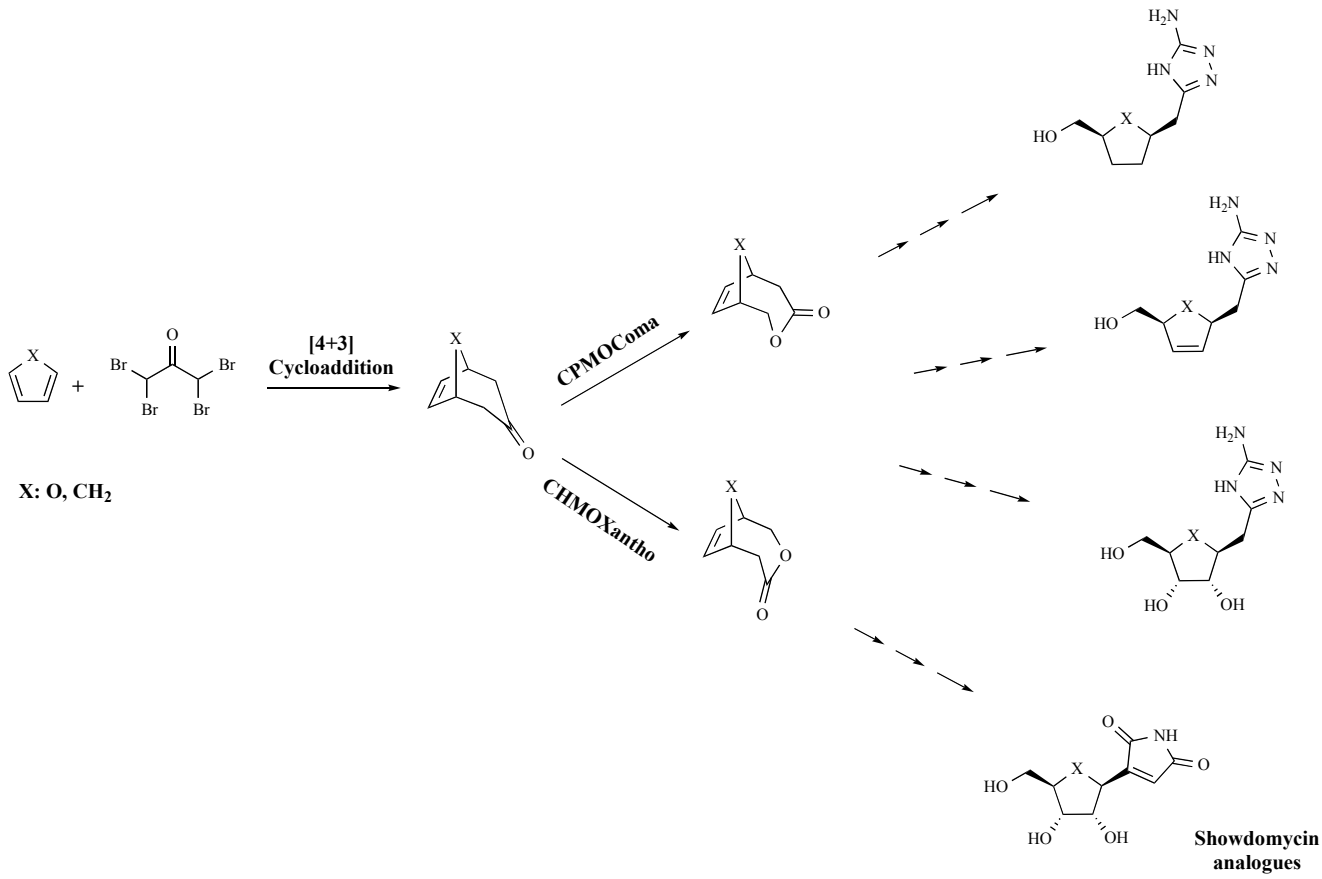
NAs in which C-N bond between sugar and base is replaced by a C-C bond are called *C*-nucleosides (Scheme **9**). This structural characteristic confers increased hydrolytic and enzymatic stability, showing these compounds, in addition, significant therapeutic properties such as antiviral, antitumor and antibiotic activities [177]. Since the biological activity of natural *C*-nucleosides, such as showdomycin, pseudouridine, and formycin was discovered, several synthetic analogues have been synthesized [178].

On the other hand, carba-*C*-nucleosides, which are *C*-NAs in which the tetrahydrofuran ring is substituted by a cyclopentane, are only sporadically documented in the literature. It is expected that carba *C*-nucleosides become a class of potential bio-active compounds by combining the common features of classical *C*-nucleosides and a noncarbohydrate core [179].

Classical *C*-nucleosides synthesis usually starts from pentoses. In contrast, their carba analogues often require the development of stereoselective routes, attracting the interest of medicinal chemists in the last time [180]. Seven-membered bicyclic ketones were achieved by [4+3] cycloaddition, starting from furan or cyclopentadiene depending on if tetrahydrofuran- or cyclopentane-based compounds are desired [181]. Using these prochiral ketones as precursors, Mihovilovic's group recently carried out the chemoenzymatic synthesis of some *C*-nucleosides such as showdomycin [182] and their corresponding carba analogues. The key stereoselective step was an enantiodivergent biooxidation [183] mediated by two different Baeyer–Villiger monooxygenases (BVMOs): cyclopentanone monooxygenase (CPMOComa) [184] from *Comamonas* sp. NCIMB9872 and cyclohexanone monooxygenase from *Xanthobacter* sp. ZL5 (CHMOXantho) [185]. Both enzymes were overexpressed in *E. coli* [186], in order to avoid both isolation of the unstable enzymes and cofactor recycling required for NADPH-dependent BVMOs [187]. The recombinant whole-cells were applied as biocatalysts to prepare both antipodal chiral lactones with high chiral purity [188], which were considered as potential building blocks for the synthesis of *C*-nucleosides, homonucleosides -in which a methylene linker is placed between the ring and the nucleobase- and other analogues (Scheme **14**).

By analogy with *C*- and carba-*C*-nucleosides, aza-*C*-nucleosides are NAs in which the nucleobase is linked to a pyrrolidine unit through a C–C bond. Azanucleosides have attracted much attention owing to their anticancer and antiviral activity, and increased resistance towards nucleases [189]. A very promising class of aza-*C*-nucleosides are immucillins [190], which act as transition state analogue enzyme inhibitors of several nucleosidases and NPs [191]. Some immucillins also exhibit potent antiviral activity; among them, an adenosine analogue is currently being developed as a potential treatment for Ebola virus infection [192].

The traditional synthetic strategy requires multiple steps and harsh conditions, thereby limiting the structural and functional diversity of these products. A typical approach involves the direct addition of the heterocyclic base to a pyrrolidine ring containing an electrophilic functionality. In this sense, pyrrolidine-2,5-dicarboxamide, easily available as both racemic and meso form, proved to be a versatile synthetic intermediate to achieve enantiomerically pure



Scheme 14.

disubstituted pyrrolidines. By kinetic resolution mediated by amidase containing *Rhodococcus erythropolis* AJ270 whole cells, racemic *trans*-pyrrolidine-2,5-carboxamide was resolved in (2*S*,5*S*)-pyrrolidine-2,5-dicarboxamide and (2*R*,5*R*)-5-carbamoylpyrrolidine-2-carboxylic acid in high yields and excellent enantioselectivity [193]. Similarly, biocatalytic desymmetrization of meso *cis*-pyrrolidinedicarboxamide afforded enantiomerically pure (2*R*,5*S*)-5-carbamoylpyrrolidine-2-carboxylic acid in almost quantitative yield [194]. In both, kinetic resolution and desymmetrization, the amidase always exhibited excellent 2*R*-enantioselectivity giving products with >99.5% ee. *R. erythropolis* AJ270 whole cells displayed higher enzymatic activity for *trans*-configured dicarboxamides than for the *cis*-isomers, being this result attributed by the authors to the steric effect involved in the substrate−enzyme interaction. The synthetic potential of the biotransformation was demonstrated by the scalable preparation of the chiral intermediates and their conversions to aza-nucleoside analogues by substitution of the anomeric position with tetrazole, affording compounds of pharmaceutical interest (Scheme **15**).

3. NUCLEOSIDE DERIVATIVES

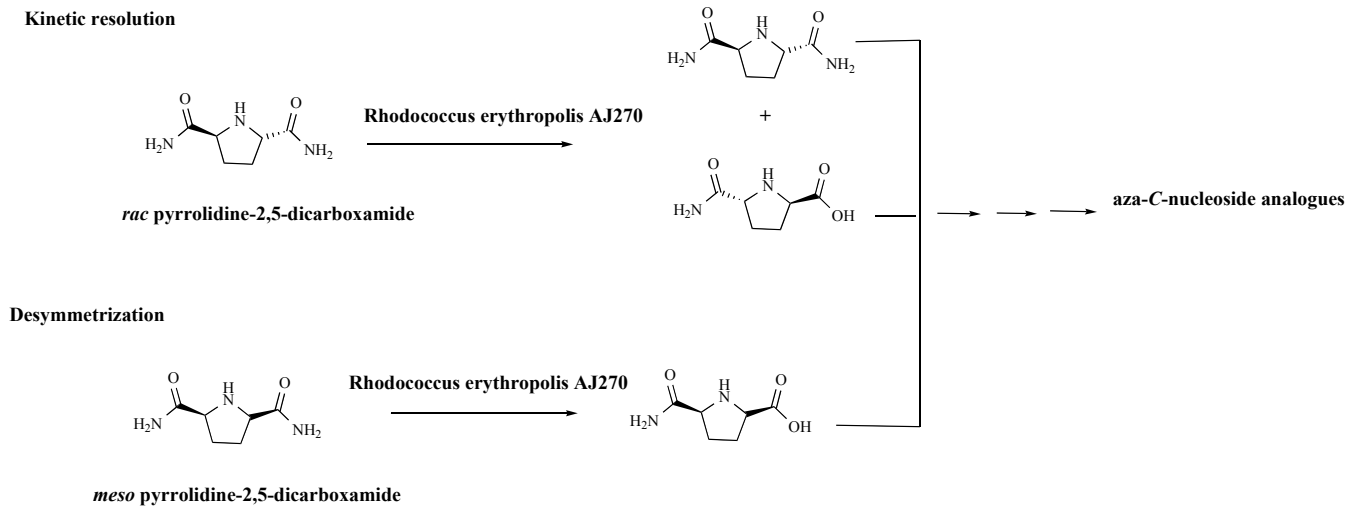
The attachment of other moieties to nucleosides and their analogues may confer upon them particular properties. Well-known examples of nucleoside derivatives are provided by prodrugs. These compounds are conjugates of active drugs with either hydrophilic functionalities (e.g., phosphate) that increase their water solubility, or lipophilic moieties (e.g., ester) that improve their passive permeability. In addition, other type of nucleoside derivatives that expand the diversity of this family of compounds are sugar conjugates of nucleoside phosphates. These analogues are the essential glycosyl donors involved in many glycosylation reactions.

Considering the above presented context, the aim of the following section is to summarize the available methodology for the biocatalyzed preparation of some representative nucleoside derivatives that make use of whole cell systems.

3.1 Acylnucleosides

Nucleosides that do not exhibit proper physicochemical, pharmacokinetic or pharmacodynamic properties can be transformed to the corresponding prodrugs. In particular, acylated nucleosides show several advantages as prodrugs such as increased chemical or *in vivo* stability, improved uptake, better absorption by oral delivery and enhanced circulation times. Cytarabine is a clear example of this kind of strategies. This compound has been extensively employed in the chemotherapy of acute leukaemia, as antiviral and also as immunosuppressive agent [195]. However, cytarabine is rapidly inactivated *in vivo* by the action of cytosine deaminase and is also ineffective against solid tumours due to its high hydrophilicity. Acylated cytarabine analogues provide a solution to these drawbacks [196].

In this sense, a biocatalytic acetylation of cytarabine using whole cell of *Aspergillus oryzae* was reported by Li *et al*. [197]. This whole cell biocatalyst showed regioselectivity toward the 3'-hydroxyl group. Since cell-bound lipases are considered inducible enzymes [198, 199], their production is expected to be influenced by growth conditions. Therefore, the authors tested 11 different lipids as carbon sources, which acted not only as nutrients, but also as inducers, thereby affecting the microorganism growth and the



Scheme 15.

cell-bond lipase production. They observed, as previously reported [195, 200], that a wide range of fatty acyl esters (olive oil, Tweens and Spans) strongly triggered cell-bond lipase production. In addition, an improvement in lipase activity was noticed with the increase of fatty acid carbon chains of Tweens and Spans. It is remarkable that when Spans were used higher 3'-regioselectivity was achieved, suggesting that more than one type of lipases might be synthesized by *A. oryzae* and Span 80 might favour the production of the enzyme with 3'-OH preference (Scheme **16**).

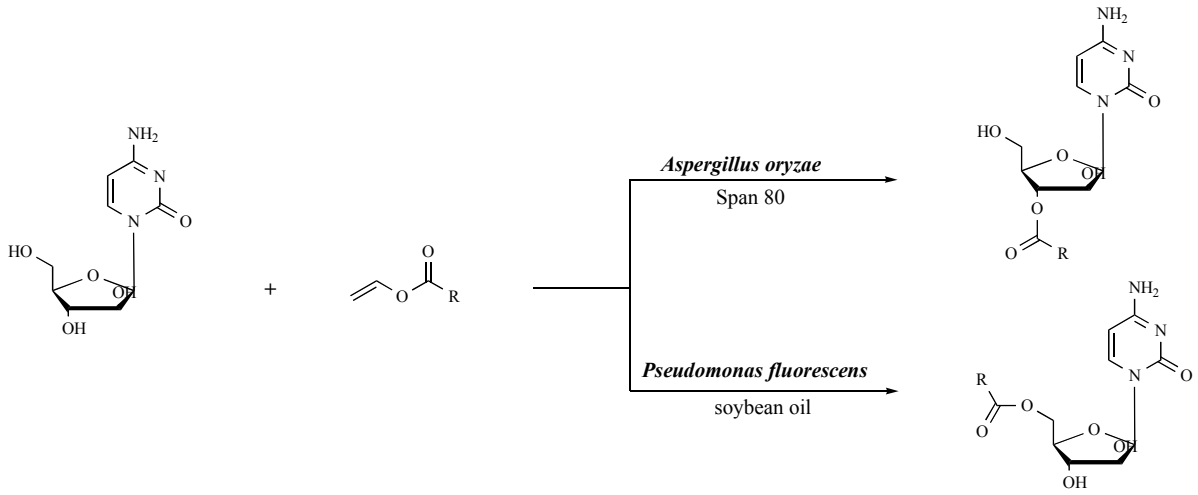
The same group also analyzed the dependence of growth conditions on the catalytic performance of freeze-dried *Pseudomonas fluorescens*, used as whole cell biocatalyst for cytarabine acetylation [201]. The genus *Pseudomonas* is widespread in nature and suitable for biotechnological applications due to the diversity of lipases that expresses, with unique substrate specificity and toxic solvent tolerance [10, 202]. The shown results indicate that the catalytic activity of the freeze-dried *P. fluorescens* whole cells was significantly improved by cultivation with mixed carbon sources containing yeast extract and additional lipidic substrates, especially soybean oil. In contrast to the above commented work, this biocatalyst exhibited 5'–regioselectivity. Previous reports have shown that extracellular lipases from *Pseudomonas* sp. favour the 3'-acylation of various nucleosides [198]. The difference in regioselectivity might be attributed to the different enzyme structures between the known extracellular lipases and the lipases located in the whole-cells [198, 203].

The best results with the *P. fluorescens* whole cell biocatalyst were obtained using 0.5% (w/v) soybean oil, 0.1% (w/v) yeast extract and 48 h culture time, under which the yield and 5'-regioselectivity of the reaction reached 75.4% and 96.8%, respectively. These results suggest that freeze-dried *P. fluorescens* whole cell is a green and economic alternative to enzymes for cytarabine regioselective acylation in non-aqueous media.

Whole cell biocatalyzed acylation of polar substrates such as nucleosides in organic media has been hampered by the poor solubility of the hydrophilic substrates in organic solvents with low cytotoxicity [204]. Therefore, an extension of the previously discussed work was carried out by Li *et al.* [205], who analyzed the influence of organic solvents in the regioselective synthesis of monoacylated cytarabine. The acylation reaction catalyzed by lyophilized *P. fluorescens* cells exhibited a clear solvent dependence, showing an improvement of the catalytic activity with the increase of the organic solvent polarity, with the exception of the acetonitrile–pyridine mixture. Among all the tested solvents, pure and binary mixtures, the best results were obtained with isopropyl ether–pyridine, in which the cells also showed good operational and thermal stabilities. The optimal isopropyl ether concentration, water content, acyl donor/ cytarabine ratio, biocatalyst amount and reaction temperature were 30% (v/v), 4%, 45, 50 mg/ml and 30ºC, respectively. Using these conditions the observed initial rate was 2.93 mM/h, affording the 5'-*O*-propionyl cytarabine in 77% yield with 97.3% 5'-*O*-regioselectivity. The bacterial cells and immobilized *Candida antarctica* lipase B (Novozyme 435), exhibited comparable regioselectivity, suggesting environmental and cost advantages of the whole cell biocatalyst.

Another study involving the synthesis in non aqueous media of 3'-*O*-propionyl cytarabine using dehydrated whole cells of *A. oryzae* was reported by Yang *et al.* [206]. Six different isopropyl ether (IPE)/pyridine ratio were tested and other variables were optimized. When varying the IPE content from 10% (v/v) up to 30% (v/v), the initial rate, yield and 3'-regioselectivity of the reaction improved significantly. However, higher concentrations of the hydrophobic IPE caused visible insolubility of cytarabine in the reaction media. Thus, an increase from 30% (v/v) to 40% (v/v) in IPE content produced a decrease in both the yield and 3'-regioselectivity of the reaction. The results obtained in this work showed that the best reaction medium was IPE-pyridine, being IPE concentration 30% (v/v), the vinyl propionate/cytarabine ratio 90, the reaction temperature 30°C and the shaking speed 140-180 rpm. Under these conditions the desired 3'-*O*-propionyl cytarabine analogue was obtained in 88% yield and 70% regioselectivity. The whole cell biocatalyst also exhibited good thermal stability in both IPE-pyridine and hexane-pyridine mixtures, showing only a slight loss of catalytic activity after incubation in IPE-pyridine 30% (v/v) at 30-35°C. Further increase of the incubation temperature led to a deleterious effect on relative activities.

Acylation of nucleosides with longer acyl chains can produce nucleoside esters with higher bioactivity [207]. Having this in mind Yang *et al* developed a whole-cell based method for the 5'-regioselective synthesis of arabinocytosine laurate [208]. Due to the differential substrate recognition by microbial enzymes from different sources [209, 210], a screening became necessary in order to explore microbial cells with capability to catalyze the formation of long-chain nucleoside esters. Among the seven strains of bacteria tested, *P. fluorescens* gave the highest productivity and a regioselectivity higher than 99%. The influence of the solvent was also assessed. Compared with pure organic solvents, solvent mixtures greatly promoted better yield of the whole-cell catalyzed reaction,



Scheme 16.

showing little influence on the 5'-regioselectivity. The best result was found in isopropyl ether/pyridine followed by isopentanol/pyridine. However, the whole cells showed much lower thermostability in isopropyl ether/pyridine than in THF-pyridine. To understand more deeply the deleterious effects of the organic solvents, growing cells and whole cells were further examined. Significant effects of organic solvents on the biomass of the *P. fluorescens* cells were observed, which depended on the type of solvents used. SEM analysis showed clearly the changes in the surface morphology of whole and growing cells cultured in media containing different organic solvents, revealing differences in surface smoothness, bulges and modified cell sizes. These results established that organic solvents toxicity produce cell structure modifications that play an important role in whole cell mediated catalysis. However, for practical applications of this whole cell biocatalytic route, improvement of reaction efficiency still needs to be achieved.

Since organic solvents may suffer from drawbacks such as toxicity to the environment, volatileness and lower biocatalyst, ionic liquids (ILs) are promising alternative and environmental friendly [211] solvents for nonaqueous biocatalysis. Ionic liquids are liquids comprised of ions with melting point below 100°C and, preferentially, are liquid at room temperature. They show unique characteristics such as nonvolatility, nonflammability, and excellent chemical and thermal stability. There are several examples of biocatalyzed reaction using ILs [212] and some useful generalizations are available. For example, ILs composed of a chaotropic cation and a kosmotropic anion can positively influence on enzyme activity and stability [213, 214]. Hydrophilic ionic liquids with strong hydrogen bonding capacity tend to behave as water-mimicking liquids that can dissolve enzymes while maintaining their activity [215, 216]. Regarding catalytic processes using whole cells in ILs, a review that also involves this subject has been recently published [217].

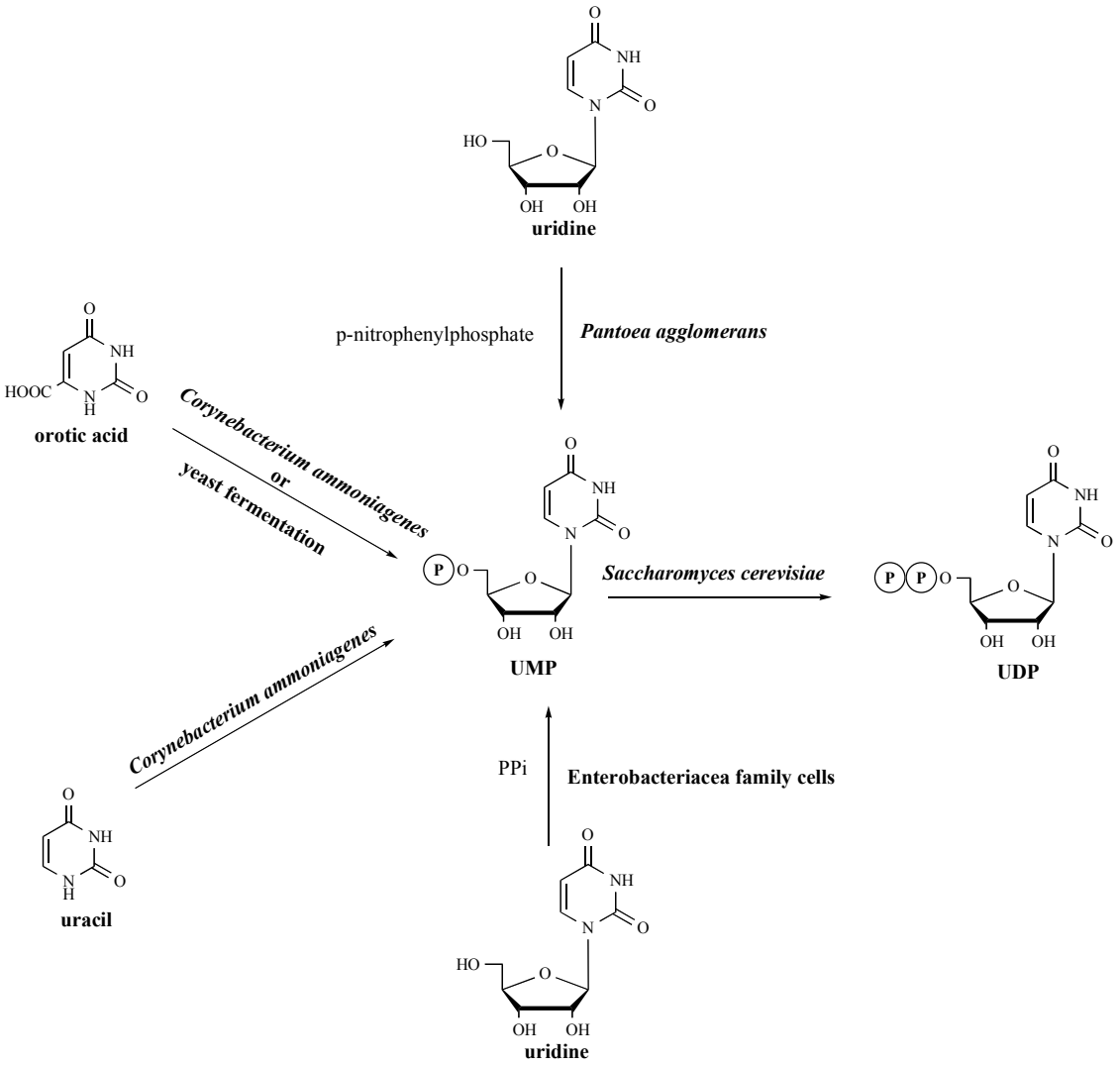
In the specific field of nucleoside biotransformation, Yang *et al.* reported the synthesis of long-chain nucleoside esters using ILs as solvents [218], based on their achievements in whole cell biotransformation. The synthesis of laurate ester of cytarabine was chosen as the model reaction using as biocatalyst *P. fluorescens* GIM1.209 whole cells. The influence of different IL types on the biocatalyzed reaction was analyzed and their effects on the cell morphology were evaluated in terms of the biomass and surface morphology. The obtained results showed that their effects were closely related with both the anions and cations of the ILs, being 10% [BMI][PF6]/THF the solvent that afforded the highest reaction efficiency. Under this condition the observed initial rate was 2.34 mmol/L.h, product yield reached 81% and 5'-regioselectivity was higher than 99%. In addition, SEM analysis revealed that ILs can modify the cell surface morphology, improving cell envelopes permeability and thus facilitating substrates mass transfer to the active sites of cell bound enzymes. This result highlights the potential of ILs as promising reaction medium for efficient and regioselective whole-cell catalysis in particular in the field of nucleoside chemistry.

3.2. Nucleoside Phosphates

Monophosphates provide many advantages in the formulation and development of poorly water-soluble compounds, due to their increased solubility respect to the parent drugs [219]. Nucleosides monophosphates (NMPs) can be obtained by the reaction of the corresponding nucleosides with phosphorylating agents, such as phosphorus oxychloride [220]. This corrosive and volatile reagent is toxic to humans due to its action on the central nervous system. Furthermore, the regioselectivity of the reaction is poor, producing also the corresponding 2'- and 3'-nucleotides. By these reasons, alternative synthetic routes for large scale preparation of NMPs are a challenging goal; therefore, biocatalyzed strategies provide an attractive option.

Phosphate esters of active nucleosides are rapidly hydrolyzed by endogenous phosphatases, liberating the pharmacologically active component and for this reason, they are well established prodrugs. The most representative case of a nucleoside phosphate prodrug is fludarabine monophosphate (FaraAMP), an analogue of the antiviral agent vidarabine. The presence of a fluorine in the purine base and a phosphate group in the arabinose moiety produces an improved water solubility and enhanced resistance to adenosine deaminases compared to vidarabine. FaraAMP is prescribed for the treatment of B-cell chronic lymphocytic leukaemia and in non-Hodgkin's lymphoma. After administration, FaraAMP, like other nucleoside phosphate prodrugs, is rapidly dephosphorylated by serum phosphatases and the parent drug is then transported into cells by nucleoside transport systems. Once inside the cell, it is re-phosphorylated by deoxycytidine kinase to regenerate FaraAMP and subsequently accumulated as its biologically active 5′-triphosphate analogue [221].

For most nucleosides, 5'-monophosphorylation is the rate-limiting step in their transformation to the corresponding 5'-triphosphates; therefore, the involved kinases have been subject to many functional and structural studies, constituting this information a powerful toolbox for the development of enzymatic strategies for the preparation of non-natural NMP. In addition to the use of kinases, other enzymes with phosphorylating activity have been applied to the synthesis of unnatural nucleotides such as phos-



Scheme 17.

photransferases, phosphoribosyltransferases and acid phosphatases. A previous review dealing with nucleoside prodrugs has also covered the biocatalyzed synthesis of NMPs and can also be consulted [27]. However, there is not available review, as far as we know, dealing exclusively with whole cell methodologies and therefore, this revision will include reports from earlier stages of this field. The different methodologies that are described below for the synthesis of NMPs have been summarized in Scheme **17**.

One of the papers that set the basis for posterior research in the use of whole cells for the preparation of NMPs was published by Zinchenko *et al*. [222]. They made use of the nucleoside phosphotransferase (NPT) activity present in *Pantoea agglomerans* 47/3 (previously *Erwinia herbicola* 47/3) whole cells and of p-nitrophenylphosphate as phosphate source for the synthesis of 5'-monophospho esters of ribonucleosides, 2'-deoxyribonucleosides, 3'-fluoro-3'-deoxyribonucleosides and 3'-fluoro-2',3'-dideoxyribonucleosides. The described results showed that nucleoside transformation to the corresponding 5'-monophosphate did not depend on the heterocyclic base or on the presence of hydroxyl groups at C-2'or C-3'. However, in the case of deoxynucleosides longer times are required to obtain maximum conversions to NMP.

Zhernosek *et al*. [223] applied the same methodology described above using *P. agglomerans* 47/3 whole cells for the phosphorylation of 2',3'-dideoxyuridine and 2',3'-dideocytidine. They also observed the selective formation of 5'-monophosphorylated analogues, although lower yields were informed. Due to the lack of proper information of protein sequences, further characterization of the enzyme responsible of the *P. agglomerans* 47/3 phosphorylating activity was not possible. However, the pH range of activity, cellular localization, and phosphohydrolase activity of this enzyme suggest a possible wrong classification and it may belong to a broader group of enzymes with phosphatase/phosphotransferase activity such as acid phosphatases (Class B) family.

The use of whole cells carrying nonspecific acid phosphatases (NSAPs) was first assessed by Asano *et al*. [224]. They studied the 5'-phosphorylation of inosine to produce inosine-5'-monophosphate (IMP) using several microorganisms from their culture collection and PPi as the phosphate donor. Although many of the screened microorganisms were able to phosphorylate inosine, phosphotransferase activity specific to the 5'-position was detected among the bacteria belonging to the Enterobacteriacea family and in particular, *Morganella morganii* NCIMB10466 was selected for further studies of IMP production. When wet cell past of *M. mor-*

ganii intact cells was employed, 6.02 mg/ml (11.4 mM) of IMP were synthesized from 10 mg/ml (37.3 mM) of inosine and 250 mg/ml (560 mM) of tetrasodium pyrophosphate decahydrate in 9 h.

Wang *et al*. successfully developed a two-step biocatalytic process for uridine 5'-monophosphate (UMP) production from orotic acid using *Corynebacterium ammoniagenes* ATCC 6872 [225]. They analyzed the effect of cultivation conditions on UMP production, finding that the cells exhibited the highest biocatalytic activity when cultivated in a medium containing corn steep liquor at pH 7.0 for 15 h in the exponential phase of growth. The author explored "one-factor-at-a-time" and statistical methods for reaction optimization, reporting orotic acid, glucose, phosphate ion (equimolar KH_2PO_4 and K_2HPO_4), $MgCl_2$, Triton X-100 as the optimum components. They also concluded that α-D-5-phosphoribosyl-1-pyrophosphate (PRPP) synthesis was the rate-limiting step for pyrimidine nucleotides production. After optimization, yield was improved in three folds, observing accumulation of 38.5 mM UMP in 24 h.

In view of the high cell concentration (40 g dry cell weight/L) used in the above mentioned paper, a whole cell biocatalytic process for UMP production starting also from orotic acid but using *S. cerevisiae* as biocatalyst was developed by Chen *et al*. [226]. UMP was synthesized by means of yeast catalyzed fermentation or biotransformation, but this last methodology was chosen for large-scale UMP production in view of its accessibility. The enzymes involved in this process are orotatephosphoribosyltransferase (OPRTase) and orotidine 5′-monophosphate decarboxylase (ODCase). By addition of 1 g/L of sodium citrate to the broth, UMP production was improved by 23%. Additional effects were observed such as elevation of the glucose-6-phosphate pool, a decrease of FBP and pyruvate concentration and reduction of organic acid pools. These results demonstrated that the control of citrate levels could be used as a novel tool to regulate the metabolic fluxes distribution among glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle.

We have also reported the phosphotransferase activity of NSAPs in the phosphorylation of nucleosides, using wild type and recombinant strains [85]. First, a screening of 26 bacteria belonging to the genera *Citrobacter, Escherichia, Erwinia, Proteus, Klebsiella* and *Serratia* were tested using PPi as phosphate source for the regioselective phosphorylation of the 5'-OH of different nucleosides. *R. planticola* and *E. aerogenes* strains showed the best activities and were further applied to the synthesis of natural and modified nucleosides. In general terms, *E. aerogenes* cells were more effective in the phosphorylation of purine nucleosides, whereas *R. Planticola* cells showed higher activity on 2'-deoxynucleosides and arabinosides. NSAPs from *E. aerogenes* and *R. planticola* were later heterologously expressed in acid phosphatase deficient *E. coli* BL21 cells and further employed as whole cell biocatalysts. The use of these recombinant strains afforded, in most of the cases, increased yields and significant reductions in the reaction times (from days to no more than 2.5 h). The observed higher yields could be the result of the circumvention of competitive side reactions that occurred inside the cells. In particular, 2.1 mM of FaraAMP was obtained in 50 min.

Within the group of nucleoside monophosphates, cyclic adenosine-3',5'-monophosphate (cAMP) is of high importance due to its biological relevance. It acts as second messenger in cellular signal transduction and takes part of many biological activities in both prokaryotes and eukaryotes [227]. cAMP has numerous applications in clinic, such as in the treatment of hepatopathy, hyperthyreosis and cardiovascular, respiratory and nervous system diseases. Therefore, an efficient production of cAMP is of crucial importance. Since no whole cell catalyzed synthesis of this nucleotide had been previously reported, Li *et al*. [228] addressed this objective making use of the adenylate cyclase activity. This enzyme catalyzes the synthesis of cAMP from adenosine 5'-triphosphate (ATP). Adenylate cyclase from *Arthrobacte*r was overexpressed in *E. coli* Rosetta by the induction of lactose. The recombinant *E. coli* Rosetta (pET28a-cya) was permeabilized using Triton-X 100 and directly employed as whole cell catalyst for the mentioned biotransformation. Parameters of the biocatalytic process such as pH, temperature, Mg^{2+}, Triton X-100 and pyruvate, were evaluated, being the most decisive ones pH and Mg^{2+}. Compared to fermentation, the biocatalyzed cAMP synthesis using recombinant whole cells provided enhanced productivity, from 0.17 g/h to 2.49 g/h, and significantly shortened cycle production, from 66 h to 6 h, setting the basis for a potential industrial production of cAMP.

Due to the complexity of the classical methodologies applied to the preparation of nucleoside 5'-diphosphates (NDP), we [112] assessed multistep enzymatic systems for the synthesis of pyrimidine NDP starting from readily available reagents. We combined different strategies to prepare uridine- and thymidine-5'-diphosphates as ribo- and deoxyribonucleoside models, respectively. Using a simple methodology that involved commercial yeast and biocatalytically *in situ* prepared UMP, UDP synthesis was carried out with conversions ranging from 38 to 66%. *Corynebacterium ammoniagenes* ATCC 19350 was used for the first time as biocatalyst to synthesize UMP using uracil and orotic acid as starting materials, while *R. planticola* was selected as the proper biocatalyst for UMP synthesis from uridine. Although the overall performances of all the assessed approaches were similar, the use of uracil leads to a more suited and cheaper process. For TDP synthesis two consecutive one pot multistep enzyme systems were assayed. In the first one, as mentioned above, DR5P was formed starting from glucose by *E. carotovora* whole cells; subsequent action of PPM and TP afforded thymidine. Finally, in the second one pot reaction, thymidine was converted to the corresponding 5'-diphosphate by the combined action of *E. coli* BL21 pET22b-phoRp and *S. cerevisiae*.

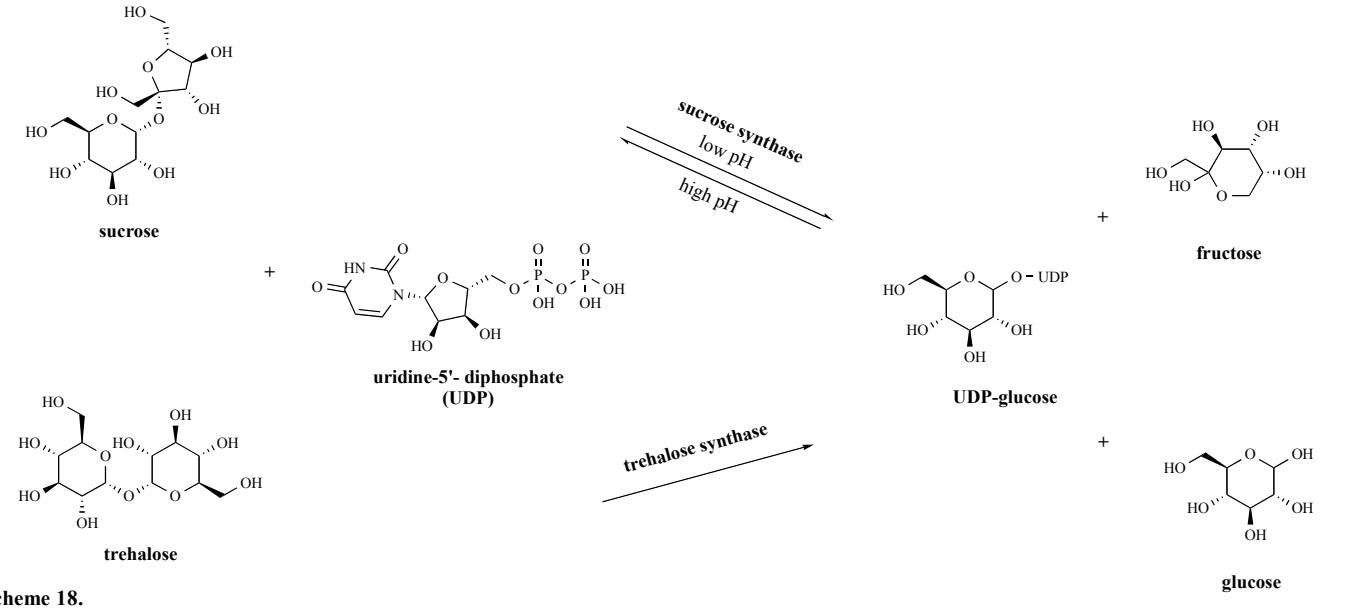
NDP analogues of sugars like glucose, galactose and mannose, among others, are the naturally activated substrates employed in cellular biosynthesis, involving nucleotide sugar-dependent ("Leloir") GTs [229]. These enzymes are key catalysts in the synthesis of many glycocompounds, such as oligo- and polysaccharides, glycosylated natural products, glycolipids and glycoproteins [230] and due to their particular ability to glycosylate unprotected substrates, constitute efficient biocatalytic tools for the production of fine chemical glycosides. Glycosides are attractive and highly demanded compounds since many of these molecules possess relevant characteristic respect to the corresponding aglycones.

Uridine diphosphate glucose (UDP-glucose) is the most employed glucosyltransferases substrate and also the precursor of other UDP-sugars, such as UDP-galactose, UDP-xylose and UDP-glucuronic acid (UDP-GA) as will be later discussed. Therefore, the synthesis of UDP-glucose has been the subject of several researches. Most of the published works deal with whole cell approaches since *in vitro* ones require the regeneration of several cofactors. De Bruyn *et al*. reported an excellent review [231] regarding the advances in the biotechnological glycosylation of small molecules that also addresses the synthesis of UDP-sugar analogues. This review should be consulted for extended information; in this work only the last reports about this subject will be discussed.

Several methods that were developed so far for the preparation of UDP-sugar analogues made use of synthases (Table **2**). This is an attractive strategy since the reversible reactions catalyzed by these enzymes generate UDP-sugars starting from a disaccharide, being sucrose and trehalose synthases the most studied biocatalysts. A major challenge when applying synthases as catalysts involves the improvement of their stability and kinetic parameters. For example, synthase from *Pyrococcus horikoshii* showed low K_m values for UDP and trehalose. Likewise, K_m of sucrose synthase for sucrose is below desired values but can considerably be increased,

Table 2. Different strategies for UDP-sugar analogues preparation

Compound	Biocatalyst	Yield	References
UDP-glucose	*E. coli* MC1061 expressing trehalose synthase	27 %	[233]
	E. coli expressing sucrose synthase from *Acidithiobacillus caldus*	73 %	[234]
	E. coli JW 0675-1SP strain	60 %	[238]
GDP-glucose	*E. coli* MC1061 expressing trehalose synthase	82 %	[233]
ADP-glucose	*E. coli* MC1061 expressing trehalose synthase	60 %	[233]
	E. coli expressing sucrose synthase from *Acidithiobacillus caldus*	86 %	[234]
UDP-glucuronic acid	*S. pombe* expressing human UGDH	100 %	[243]
GDP-L-fucose	*E. coli* BL21star(DE3) overexpressing manB, manC, gmd and wcaG genes	4.4-fold respect to the control strain	[245]
	E. coli overexpressing G6PDH	21-fold respect to the control strain	[247]



Scheme 18.

when recombinantly overexpressed in a prokaryotic host [232]. For this reason, growing wild type cells cannot accumulate the respective disaccharides intracellularly at the required concentrations, hampering the use of synthases in fermentation conditions for UDP-sugar formation (Scheme **18**).

With the aim of producing NDP-sugars, Ryu and Lee [233] explored the use of trehalose synthase by constructing recombinant *E. coli* MC1061 cells harbouring this enzyme. Using trehalose as a glucosyl donor and NDPs such as UDP, ADP, and GDP, this whole cell biocatalyst was capable of producing the corresponding NDP-glucoses in the extracellular medium. The authors suggested that the recombinant *E. coli* cells act as entrapment elements and that the immobilized overexpressed enzyme produced and excreted NDP-glucose in the extracellular reaction solution. This system produced ADP-glucose from ADP, affording a yield of 60% after 12 h. The recombinant cells also produced 16.5 GDP-glucose and 5.4 UDP-glucose from 20 mM GDP and UDP, in yields of 82 and 27%, respectively. In addition, the authors assessed the performance of a whole cell system having a UDP-galactose- 4-epimerase (pGALE) for the synthesis of UDP-galactose, reporting a yield of 26% based on the amount of employed UDP-glucose. They also carried out a comparison between the production of NDP-sugars using recombinant whole cells and enzyme-coupled reactions finding yields 1.5 times higher for the cell system. These results encourage the use of trehalose and recombinant whole cells harbouring trehalose synthase as an alternative and practical method for the production of NDP-sugars and α-galacto-oligosaccharides.

In contrast to the few reports found in the literature concerning the applications of trehalose synthase, the other member of this family of enzymes, sucrose synthase, has received much more attention by different research groups (Scheme **18**). Schmölzer *et al*. addressed the study of UDP-glucose production catalyzed by sucrose synthase, using an integrated whole cell process [234]. This work involved the coordinated development of biocatalyst production, biotransformation and downstream processing. The production of recombinant sucrose synthase from *Acidithiobacillus caldus* was shifted largely to the stationary phase by its constitutive expression in *E. coli*, enhancing the specific enzyme activity to a level suitable for whole cell biotransformation. This system provided excellent results: ca. 100 g product/L, 86% yield (based on UDP) and a total

turnover number of 103 g UDP-glucose/g cell dry weight at a space-time yield of 10 g/L.h. UDP-glucose could be isolated in a single batch with ca. 90% purity and in 73% isolated yield, potentially allowing the production of 0.7 kg of isolated product/L of *E. coli* bioreactor culture.

Gutmann and Nidetzky also proposed the use of sucrose synthase and showed in a recent study [235] that, to unlock the synthetic potential of the GT reaction, it was fundamental to combine a kinetic characteristics-based enzyme selection with a reaction design aligned to thermodynamic constraints. The equilibrium constant for the conversion of sucrose and UDP into the target product UDP-glucose and D-fructose decreased with increasing pH because of the deprotonation of the UDP-β-phosphate group above pKa~6.0. Therefore, pH was carefully controlled throughout the reaction. Comparing the sucrose synthases from *A. caldus* and *Glycine max* (soybean), it was concluded that substrate inhibition by UDP was more important selection criteria than catalytic efficiency, choosing the bacterial GT for use at high UDP concentrations. Reaction at the operational optimal pH (5.0), afforded 255 mM (144 g/L) of UDP-glucose in 85% yield from UDP. Using a low enzyme concentration (0.1 g/L), a space-time yield of 25 g/L.h was obtained. The mass-based turnover number (g product formed per g enzyme) reached a value of 1440 from a single batch conversion. This excellent performance for UDP-glucose synthesis demonstrates that reactions catalyzed by GTs can be enhanced to reach process efficiencies typically required for achieving fine chemicals production.

A novel metabolic engineering strategy for the *in vivo* glucosylation of small molecules was reported by De Bruyn *et al*. [236] using *E. coli* W. This fast-growing and safe strain has a good tolerance for acidic conditions and osmotic stress, being therefore, a good host for industrial fermentations [237]. This strategy involved the introduction of a different sucrose metabolism using sucrose phosphorylase for the direct and effective synthesis of glucose-1-phosphate (G1P) as the precursor for UDP-glucose generation and fructose, which can be further used as carbon source for growth. By targeted gene deletions, a split metabolism is created, resulting in the reduced or eliminated use of G1P for growth, saving it for UDP-glucose formation. Further, the production pathway was enhanced by increasing and preserving the intracellular UDP-glucose pool.

A multistep synthesis of UDP-glucose was reported by Weyler and Heinzle [238]. They built recombinant *E. coli* cells containing over expressed sucrose phosphorylase of *Leuconostoc mesenteroides*. The cells were later treated with proper amounts of surfactants, becoming permeable to low molecular weight compounds whereas enzymes and other large molecules remain within the cells. These ''enzyme bags'' can be used for multienzyme synthesis under defined conditions [239]. Choosing appropriate substrates, a restricted number of reactions can proceed, thus limiting by-product formation. Moreover, permeabilized cells can easily be separated from the reaction mixture via centrifugation or filtration. Using this methodology, sucrose phosphorylase of *L. mesenteroides* was overexpressed in *E. coli* and its *pgm* gene encoding for phosphoglucomutase was deleted, yielding the *E. coli* JW 0675-1SP strain. The cells were permeabilized by means of detergent Triton X-100, allowing the direct synthesis of UDP-glucose using UMP as starting material with simultaneous regeneration of ATP and G1P. This reaction was optimized with regard to pH, temperature, growth phase during cell harvest, cell density during the synthesis and different media components. A yield of 37% with respect to UMP was achieved at 33°C using sucrose, phosphate, UMP, and ATP as substrates and the following conditions: pH 7.8, 27 mg/ml cell dry weight, cell harvest during the early stationary phase of growth and Mn^{2+} as the cofactor. A further improvement was achieved by ATP regeneration through glucose feeding and the addition of catalytic amounts of ATP and NAD^+. Using this system UDP-glucose yield respect to UMP rose to 60%. With the same setup but without the addition of external ATP, the yield was 54%. The multienzyme biocatalyst system developed in this research showed to be a simple and efficient methodology for the one-pot synthesis of UDP-glucose. The procedure is straight forward, since only cell cultivation and harvest, permeabilization, washing, and incubation is needed for UDP-glucose synthesis. In addition, the biocatalyst can be easily separated by centrifugation or filtration from the reaction mixture and downstream processing is facilitated due to the much smaller number of metabolites present in the reaction mixture. In addition, the system also allows the combination with other enzymatic processes.

UDP-GA is a donor of glucuronic acid in detoxification processes and is also an intermediate in polysaccharide biosynthesis [240]. Moreover, the formation of glucuronide metabolites, with increased drug activity, is a potential toxicological problem to patients [241]. Therefore, the synthesis of glucuronated compounds is particularly attractive because it requires highly regiospecific oxidation reactions. In this line, a pioneer study [242] was carried out by Dragan *et al*. who expressed human UDP-glucose-6-dehydrogenase (UGDH) together with several human or rat UDP-glucuronosyltransferase (UGT) isoforms in the fission yeast *Schizosaccharomyces pombe*. As this fission yeast lacked an endogenous UGDH enzyme, a second set of strains was created that co-expressed the UGTs mentioned above together with human UGDH. Thus, a successful whole cell biotransformation using recombinant human UGTs expressed in a unicellular organism was established for the first time for the synthesis of glucuronosides. An important advantage of this system is its autonomy with respect to the expensive cofactor UDP-GA. In addition to up-scaling, scaling down could also provide a simple high-throughput screening method for UGT profiling or inhibition studies using living cells. Besides, a strategy was developed for the synthesis of ^{13}C6-labeled UDP-GA starting from ^{13}C6-glucose as a metabolic precursor using the UGDH expressing fission yeast strains.

Taking advantage of the previously mentioned benefits provided by the use of permeabilized cells, Weyler *et al*. explored the selective oxidation of UDP-glucose to UDP-GA [243]. They also used recombinant cells of *S. pombe* that expressed human UGDH. The cells were permeabilized with Triton X-100 and low molecular weight metabolites were subsequently washed away. The resulting permeabilized cells were directly used as whole cell biocatalyst. UDP-glucose and NAD^+ were used as substrates but since divalent cations promoted UDP-glucose hydrolysis, they were not added to the reaction medium. With this reaction system 5 mM UDP-glucose were converted into 5 mM UDP-GA within 3 h. Therefore, this well-defined system afforded UDP-GA in 100% yield without observable formation of by-products. Further improvements to this system could potentially include the introduction of a regeneration system for NAD+ and an optimized supply method for UDP-glucose, for example by employing a strain that overexpresses the endogenous UDP-glucose pyrophosphorylase fyu1 [244].

GDP-L-fucose plays an important role in many biological processes. This activated nucleotide sugar is a key starting material for the fucosylation of relevant metabolites like 2'-*O*-fucosyllactose (2-FL), which is a functional oligosaccharide present in human milk that provides protection against enteric pathogen infections. Lee *et al*. [245] explored the enhancement of GDP-L-fucose production by mannose supplementation, since this sugar may act as a better carbon source than glucose. They carried out the combinatorial overexpression of the genes involved in the biosynthesis of GDP-D-mannose, a precursor of GDP-L-fucose. They found that the use of a fed-batch fermentation of recombinant *E. coli* BL21star(DE3) overexpressing the *gmd* and *wcaG* genes, and the supply of mannose and glucose led to a 1.3-fold-increase in GDP-L-fucose concentration in comparison with the result obtained with the same system but providing only glucose as the sole carbon source. The authors observed a 4.4-fold enhancement in the final GDP-L-fucose

concentration when using a glucose-limited fed-batch fermentation of a recombinant *E. coli* BL21star(DE3) strain, overexpressing *manB*, *manC*, *gmd* and *wcaG* genes, respect to the control strain that overexpressed only *gmd* and *wcaG* genes. Further improvement of GDP-L-fucose production was not obtained by additional overexpression of the *manA* gene.

Since the biosynthesis of GDP-L-fucose requires NADPH as a reducing cofactor, Lee *et al*. [246] also studied the influence of the overexpression in recombinant *E. coli* of endogenous NADPH regenerating enzymes such as glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (Icd), and NADP(+)-dependent malate dehydrogenase (MaeB) on GDP-L-fucose production. The effects of overexpression of each of the previously mentioned enzymes were investigated in batch and fed-batch fermentations. In batch fermentations the overexpression of G6PDH afforded the highest GDP-L-fucose production, but in the glucose-limited fed-batch fermentation no effect was observed. Glucose feeding optimization for the fed-batch fermentation was assessed using a pH-stat feeding mode, achieving a 21% enhancement respect to the control strain. In view of these results the authors concluded that appropriate glucose supply and efficient NADPH regeneration are key factors for NADPH-dependent GDP-L-fucose production in recombinant *E. coli*.

Finally, taking into account that guanosine 5'-triphosphate (GTP) is an essential substrate for the biosynthesis of GDP-L-fucose, Lee *et al*. [247] explored the effect of the overexpression of the enzymes involved in the biosynthetic pathway for guanosine nucleotides. IMP-dehydrogenase, GMP-synthetase (GuaB and GuaA), GMP-reductase (GuaC) and guanosine–inosine kinase (Gsk) were overexpressed in a recombinant *E. coli* producing GDP-L-fucose. Among the enzymes evaluated, overexpression of Gsk led to a significant improvement of GDP-L-fucose production. Maximum concentration of this sugar nucleotide was achieved in a pH-stat fed-batch fermentation of recombinant *E. coli*-overexpressing Gsk, which corresponds to a 58% enhancement in the GDP-L-fucose production compared with the control strain.

As previously mentioned, 2-FL is a biologically relevant oligosaccharide employed as a prebiotic in various foods. It can be synthesized by the enzymatic fucosylation of lactose with GDP-L-fucose using α-1,2-fucosyltransferase (FucT2). Hence, Lee *et al*. [248] introduced the *FucT2* gene from *Helicobacter pylori* to the GDP-L-fucose producing recombinant *E. coli* BL21star(DE3) strain. But using a batch fermentation, only small amount of 2-FL was produced because the cells consumed the supplied lactose. Therefore, they employed *E. coli* JM109(DE3) as an alternative host, since this strain is not able to metabolize lactose. The results of batch fermentations using different concentrations of lactose showed that *E. coli* JM109(DE3) whole cells could synthesize 2-FL without supplementation of another auxiliary sugar for cell growth. The obtained yield (g 2-FL/g lactose) corresponded to 20% of the theoretical maximum yield calculated by the elementary flux mode (EFM) analysis.

Another NMP analogue that has been synthesized *via* whole cells biocatalysis is cytidine 5-monophosphate *N*-acetylneuraminic acid (CMP-NeuAc).

Unlike the other sugar nucleotides, CMP-NeuAc can be synthesized without the intervention of a sugar-1-P intermediate by N-acylneuraminate cytidylyltransferase (CMP-NeuAc synthetase) [249]. CMP-NeuAc is the essential, and very expensive, substrate of sialyltransferases, enzymes involved in the sialylation of many oligosaccharides and glycoconjugates. Sialic acids are a complex group of nine-carbon monosaccharides that are present at the end of many glycans and secreted proteins. They are involved in the modulation of several biological processes, being a determinant aspect in the design of glycoprotein therapeutics.

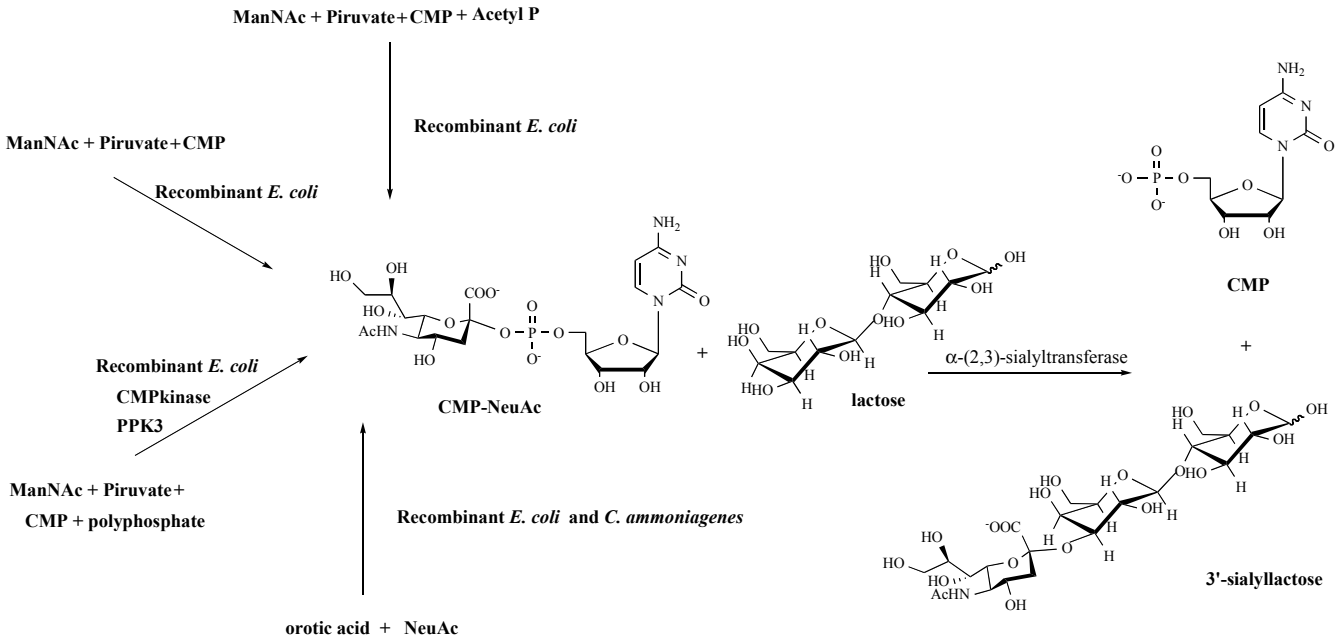
In order to avoid the long and complex synthetic route required for CMP-NeuAc preparation, Lee *et al*. [250] developed a whole cell biocatalyst based on an *E. coli* strain that expressed three recombinant enzymes: CMP-kinase, NeuAc-aldolase and CMP-NeuAc synthetase (Scheme **19**). This system catalyzed the production of CMP-NeuAc starting from CMP, N-acetylmannosamine, pyruvate, ATP, and acetylphosphate, in 90% conversion based on the initial CMP concentration. The endogenous acetate kinase was able to catalyze ATP regeneration, conversion of CMP to CDP and conversion of CDP to CTP. In contrast, endogenous pyruvate kinase and polyphosphate kinase could not efficiently regenerate ATP. This recombinant *E. coli* strain was also employed for *in situ* regeneration of CMP-NeuAc in a sialyltransferase catalyzed reaction. By this methodology, 2,3-sialyl-*N*-acetyllactosamine was obtained in 90% conversion yield starting from *N*-acetyllactosamine, CMP, *N*-acetylmannosamine, pyruvate, ATP, and acetyl phosphate.

Endo *et al*. [251] reported a system for CMP-NeuAc and sialyl-oligosaccharides large-scale production. They designed a whole cell reaction combining the activity of *E. coli* and *C. ammoniagenes* recombinant strains. In the case of CMP-NeuAc synthesis they used two recombinant *E. coli* strains that overexpressed the genes of CTP-synthetase and CMP-Neu5Ac synthetase. *C. ammoniagenes* catalyzed the transformation of orotic acid to UTP. Applying this procedure, CMP-NeuAc was accumulated at 27 mM (17 g/L) after 27 h reaction using as starting materials orotic acid and *N*-acetylneuraminic acid. When *E. coli* cells that overexpressed the α-(2,3)-sialyltransferase gene of *Neisseria gonorrhoeae* were added to the CMP-NeuAc production system, 3'-sialyllactose was accumulated at 52 mM (33 g/L) after 11 h reaction using orotic acid, *N*-acetylneuraminic acid, and lactose. The production of 3'-sialyllactose was up-scaled to a 5-l jar fermenter affording almost the same yield as that at a beaker scale, indicating the high potential of this methodology in industrial applications.

Song *et al*. [252] also reported a large-scale production system of CMP-NeuAc but employing a single bacterial strain. They assembled the genes of two enzymes: NeuAc-aldolase and CMP-NeuAc synthetase to generate an artificial gene cluster in a single plasmid. Later transformation of the plasmid into an *E.coli* strain afforded a simple whole cell catalyst for carbohydrate production, which could be up-scaled to large quantities. This methodology produced syalylated oligosaccharides starting from inexpensive materials, since the whole cell system only employed *N*-acetylmannosamine, excess of pyruvate and CTP to produce CMP-Neu5Ac.

To the same whole cell biocatalyst developed by Song *et al*., Nahálka and Pätoprstý coupled the activity of a novel polyphosphate kinase (PPK3), allowing the synthesis of CMP-NeuAc and 3'-sialyllactose using cheap polyphosphates [253]. Active inclusion bodies of PPK3 and CMP-kinase were combined with whole cells that co-expressed NeuAc-aldolase and CMP-NeuAc synthetase. This biocatalytic system was applied to the synthesis of CMP-NeuAc, which was further converted to 3'-sialyllactose by whole cells. The efficiency of CTP cofactor synthesis was high enough to overcome its degradation by the whole cell, producing the accumulation of 52 mM of CMP-NeuAc in the reaction mixture. In theory, up to 300 mg of CMP-NeuAc could be isolated from a 10 ml reaction volume. The author claimed that physiologically aggregated PPK3 offers an already immobilized enzyme with comparable activity to the acetate kinase/acetylphosphate system but with the advantage of using significantly lower cost substrates.

Antoine *et al*. used a metabolically engineered *E. coli* strain that overexpressed the bifunctional sialyltransferase *cstII* gene from *Campylobacter jejuni* to synthesize the carbohydrate portion of gangliosides GD3 and GT3. In this system the activated sialic acid donor (CMP-NeuAc) was generated from exogenous sialic acid, which was transported into the cells by the permease NanT [254]. Taking into account that sialic acid is an expensive compound the same research group developed a more economical process [255].



Scheme 19.

They used a genetically engineering *E. coli* K12 capable of generating CMP-NeuAc employing its own internal metabolism. Mutant strains, which lacked the NeuAc-aldolase and ManNAc-kinase, could efficiently produce 3′-sialyllactose by co-expressing the α-(2,3)-sialyltransferase gene from *Neisseria meningitidis* with the *neuC*, *neuB* and *neuA Campylobacter jejuni* genes encoding *N*-acetylglucosamine-6-phosphate-epimerase, NeuAc- synthase and CMP-NeuAc synthetase, respectively. A sialyllactose concentration of 25 g/L was obtained in long-term high cell density culture with a continuous lactose feed. This production is 10 times higher than the yield previously reported by the same group using a system based on the exogenous supply of NeuAc [256]. The high cost of sialic acid was a serious limitation for optimizing the microbial production of sialyllactose, and therefore this methodology could provide an attractive alternative. However, since the used heterologous genes come from pathogenic bacteria, the search of equivalent genes from non-pathogenic organisms could be more appealing for the food industry.

CONCLUSION

The literature covered in this work confirm that biocatalytic procedures that make use of whole cell systems can be successfully applied to obtain a wide variety of nucleoside analogues and their derivatives, providing alternative and complementary routes to traditional chemistry.

Although the information presented herein shows that these methodologies have reached a high degree of development, it is expected that future contributions of protein engineering and nucleoside metabolism knowledge, among other disciplines, will expand the already wide range of applications in nucleoside chemistry of whole cell biocatalysis.

LIST OF ABBREVIATIONS

AraG	=	Guanine arabinoside
AraU	=	Uracil arabinoside
AraA	=	Adenine arabinoside
ATP	=	Adenosine 5’-triphosphate
BVMO	=	Baeyer–Villiger monooxygenase
cAMP	=	Cyclic adenosine-3’,5’-monophosphate
CHMOXantho	=	Cyclohexanone monooxygenase from *Xanthobacter* sp. ZL5
CMP-NeuAc	=	Cytidine 5-monophosphate *N*-acetylneuraminic acid
CN	=	Carbocyclic nucleoside
CPMOComa	=	Cyclopentanone monooxygenase from *Comamonas* sp. NCIMB9872
dA	=	2’-deoxyadenosine
DAP	=	2,6-diaminopurine
DAPA	=	DAP-arabinoside
DAPddR	=	DAP-2’,3’-dideoxyriboside
DERA	=	2-deoxy-D-ribose-5-phosphate aldolase
DR1P	=	2-deoxyribose 1-phosphate
DR5P	=	2-deoxyribose 5-phosphate
FaraAM)	=	Fludarabine monophosphate
2-FL	=	2’-*O*-fucosyllactose
G1P	=	Glucose 1- phosphate
G3P	=	D-glyceraldehyde 3-phosphate
G6PDH	=	Glucose-6-phosphate dehydrogenase
GT	=	Glycosyltransferase
GTP	=	Guanosine 5’-triphosphate
Icd	=	Isocitrate dehydrogenase
IL	=	Ionic liquid
MaeB	=	NADP(+)-dependent malate dehydrogenase
MePdR	=	6-methylpurine-2’-deoxyriboside
MDH	=	NAD-dependent mannitol-1-dehydrogenase
MeP	=	(γ-lactam) 2-azabicyclo [2.2.1] hept-5-en-3-one, 6-methylpurine
NA	=	Nucleoside analogue
NDP	=	Nucleoside 5’-diphosphate

NdRT	=	Nucleoside 2'-deoxyribosyltransferase
NMPs	=	Nucleosides monophosphates
NP	=	Nucleoside phosphorylase
NPT	=	Nucleoside phosphotransferase
NSAP	=	Nonspecific acid phosphatase
ODCase	=	Orotidine 5′-monophosphate decarboxylase
OPRTase	=	Orotatephosphoribosyltransferase
pGALE	=	UDP-galactose- 4-epimerase
PNP	=	Purine nucleoside phosphorylase
PPM	=	Phosphopentomutase
PRPP	=	5-phosphoribosyl-1-pyrophosphate
PyNP	=	Pyrimidine nucleoside phosphorylase
R1P	=	α-ribose-1-phosphate
SARS Co-V	=	SARS coronavirus
TP	=	Thymidine phosphorylase
UDP-GA	=	UDP-glucuronic acid
UDP-glucose	=	Uridine diphosphate glucose
UGT	=	UDP-glucuronosyltransferase
UMP	=	Uridine 5'-monophosphate
UP	=	Uridine phosphorylase

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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