14 Enzymatic Synthesis of Modified Nucleosides

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CONTENTS

14.1 Introduction .............................................................................................................403
14.2 Enzymatic Synthesis of Modified Nucleosides.........................................................404
  14.2.1 Enzymes that Modify the Base ....................................................................404
  14.2.2 Enzymes that Modify the Sugar...................................................................407
  14.2.3 Enzymatic Oxidation of CH2OH in Nucleoside Analogs ............................409
  14.2.4 Enzymes that Catalyze the Synthesis of Nucleosides by Transglycosylation ..................................................................................410
    14.2.4.1 1-Characterization of the Enzymes Involved in the Biotransformation of N-2'-deoxyribosyl nucleosides ...................412
  14.2.5 Immobilization of Whole Cells ....................................................................413
  14.2.6 Synthesis of Different Nucleoside Analogs..................................................416
14.3 Conclusions..............................................................................................................420
References ..........................................................................................................................422

14.1 INTRODUCTION

Nucleosides are involved in many biochemical processes, notably the storage and transfer of genetic information. As a consequence, nucleoside analogs have been used in the treatment of anti-immunodeficiency syndrome (AIDS), and other viral infections, such as those caused by herpes viruses, and influenza A and B viruses [1]. Most of the approved antiviral drugs, such as 3'-azido-2',3'-dideoxythymidine (AZT, 1) or 2',3'-dideoxyinosine (ddI, 2), shown in Figure 14.1, are naturally occurring nucleoside analogs that act by interfering with the synthesis of viral nucleic acids.

In addition to the classical antiviral activity of nucleosides, there are some other therapeutical effects described for these nucleoside analogs, such as:

1. Nucleoside transport inhibitors: this strategy is used for cardio-protection and brain protection in ischemic heart disease and stroke, respectively, and in some human leukemia [2]
2. Treatment of inflammatory processes [3]
3. Antisense oligonucleotides, useful for preparing triple ADN helix, as potential and selective inhibitors of gene expression [4]
4. Antimicrobial drugs, such as oxetanocine (AXT-A) or oxanosine [5]
Therefore, much effort has been expended on the synthesis of nucleoside analogs. In this sense, the chemical syntheses of these compounds are often multistage processes, including several protection and deprotection steps in order to obtain the specific modification of certain groups of these polyfunctional molecules [6–11]. On the other hand, the enzymatic synthesis of nucleoside analogs offers several advantages over chemical methods:

1. Protecting groups are not usually required.
2. The enzymatic steps are highly stereospecific and/or regioselective.
3. Enzyme-catalyzed reactions are usually so efficient that the synthesized nucleosides can be used in further processes without purification. Some reviews about this topic have been performed [12,13].

Four different types of enzymes can be used to carry out the chemoenzymatic synthesis of nucleoside analogs:

1. Hydrolytic enzymes capable of modifying functional groups of the base; adenosine deaminase is the most interesting enzyme
2. Hydrolytic enzymes that can modify the functional groups of the sugar residue, such as penicillin acylase, lipases, proteases, and esterases
3. Oxygenases that can oxidize the CH$_2$OH group of the pentose ring to CO$_2$H
4. Enzymes that catalyze the transfer of glycosyl residues from a nucleoside donor to an acceptor base. There are two main subclasses of these enzymes:
   - Nucleoside phosphorylases
   - N-2'-deoxyribosyl transferases

14.2 ENZYMATIC SYNTHESIS OF MODIFIED NUCLEOSIDES

14.2.1 Enzymes That Modify the Base

Intracellular adenosine deaminase (ADA) is widely used in the preparation of antiviral structures by deamination of 6-aminopurines [14,15]. ADA specifically catalyzes the hydrolytic deamination of 6-aminopurine nucleoside, leaving the amino group of the 2’-amino sugar moiety intact (Figure 14.2).

In addition, this enzyme displays broad substrate specificity and its use can be extended to carbocyclonucleosides or acyclonucleosides [15]. Traditionally, it has been used in the synthesis of guanosine or inosine nucleoside analogs with moderated to high yields (40 to 80%)
[16], allowing the scalable synthesis of guanosine derivatives. ADA has also been used in the deamination of oxetanocin A (OXT-A, 11) to the corresponding hypoxanthine (OXT-H, 12) analog in quantitative yields, or in the specific transformation of 2-amino-OXT-A (13) in OXT-G (14) (Figure 14.2) [5].

Adenosine deaminase has also been used in the resolution of racemic nucleoside analogs derived from 6-aminopurines. Thus, Hertel et al. [17] reported the preparation of β-1-(2-amino-6-oxo-1H,9H-purin-9-yl)-2',3'-dideoxy-2',2'-difluororibose (β-15) from a racemic mixture of 1-(2,6-diamino-9H-purin-9-yl)-2',3'-dideoxy-2',2'-difluororibose (rac-15) (Figure 14.3). With the same strategy, the synthesis of both antipodes of the anti-HIV drug carbovir [(−)-17 and (+)-17] has been described [18], starting from racemic cis-[3-(2,6)-diamino-9H-purin-9-yl]cyclopentyl carbinol (rac-16) (Figure 14.3).

Margolin et al. [19] and Santaniello et al. [15] have used adenylic acid deaminase (AMPDA) from Aspergillus niger in the synthesis of 6-oxopurine nucleoside analogs with moderated to good yields (40 to 70%). This enzyme shows much broader substrate specificity than ADA, as depicted in Figure 14.4, and can lead to deamination of adenosine derivatives including phosphorylated cyclic (18), carbocyclic (20), and acyclic analogs [15]. In addition,
the enzyme can catalyze demethylation (22) and dechlorination (24) of purine ribonucleosides (Figure 14.4).

**14.2.2 Enzymes that Modify the Sugar**

The direct protection–deprotection of hydroxyl groups of nucleosides is a key step in the synthesis of nucleoside analogs. Although several chemical methods are available for regioselective acylation of the nucleoside sugar moiety, enzymatic methods offer advantages considering yield, regioselectivity, and overall number of synthetic steps that have to be carried out. Gotor et al. [20–22] described oxime esters and oxime carbonates as suitable acyl donors in the lipase-catalyzed regioselective acylation of different –OH residues of sugars (Figure 14.5) with good yields. More concretely, *Pseudomonas cepacia* lipase regioselectively acylates in 3'-position when using the oxime carbonate (27), while *Candida antarctica* lipase B leads to the acylation exclusively in the 5'-position (29) (Figure 14.5). The regioselectivity is only associated to the enzyme rather than to the acyl-donor structure, as it was proved by the same research group [21] using vinyl benzoate and lipase B from *C. antarctica*. 

Indeed, 2'-deoxyuridine, thymidine, 2'-deoxyadenosine, and 2'-deoxyguanosine were benzoylated in 5'-position with moderated yields (13 to 76%), although larger reaction times were necessary to achieve the maximum yields (71 to 80 h).

Subtilisin leads to the same regioselectivity as *P. cepacia* lipase with similar yields, though a higher amount (fivefold) of enzyme is required for this purpose. The lipase from *P. fluorescens* catalyzes the acylation of nucleosides, using anhydrides in dimethylformamide (DMF) or dimethyl sulfoxide (DMSO), but poor regioselectivity is observed [23–25]. Following an opposite hydrolytic strategy, regioselective deacylations can be achieved either with *P. fluorescens* lipase (3'-position) or with subtilisin and/or an alkaline protease from *Bacillus subtillis* (5'-position) [23,24], as shown in Figure 14.6.

The highly regiospecific acylation of the OH group in C-2' remains unsolved. Only lipase from *Mucor javanicus* shows a moderated regioselectivity for this position (42% yield), using *n*-octanoic anhydride as the acyl donor [12]. Another plausible alternative is the deprotection of peracetylated nucleosides using two enzymatic steps [13]. Recently, wheat germ lipase (WGL) has been used to deacylate in one step C-5' and C-3' positions, but only moderated yields (26–29%) are reported. [13].

Finally, Wang et al.[26] have described a highly diastereoselective thermophilic lipase ESL-001-02 that afforded in phosphate buffer at 60°C α-1-talofuranosyluronamide uracil (34) with 100% diastereomeric excess (de) from 5'-O-acetyl-β-n-allofuranosyl-uronamide uracil (33) (Figure 14.7).
14.2.3 Enzymatic Oxidation of CH$_2$OH in Nucleoside Analogs

The enzymatic oxidation of these polyfunctional and labile compounds is more advantageous than the chemical process, because it is environmentally cleaner, and the avoiding of protection–deprotection steps of other groups makes it more attractive from an economical point of view. In fact, the preparation of carboxylic nucleoside derivatives in C-5\textsuperscript{'} is a powerful tool for obtaining a new family of anti-inflammatory drugs [3,12]. In this sense, nucleoside oxidase from _Stenotrophomonas maltophilia_ FERM BP-2252 has been used for this purpose [12,27]. This enzyme shows high tolerance for different functionalities in the base, especially in C-2 position, as shown in Figure 14.8. Furthermore, the N-1 can be modified either to N-oxide or N-methylated groups.

![Figure 14.6](image)


![Figure 14.7](image)

14.2.4 Enzymes that Catalyze the Synthesis of Nucleosides by Transglycosylation

Nucleoside analogs can be prepared by base interchange using two different kinds of intracellular enzymes: nucleoside phosphorylases (NP) and N-2'-deoxyribosyl transferases.

NPs catalyze the reversible phosphorolysis of nucleosides and the transferase reaction involving purine or pyrimidine bases. Purine (E.C.2.4.2.2) and pyrimidine (E.C.2.4.2.1) nucleoside phosphorylases have been isolated from a large number of bacteria [28]. These enzymes display fairly broad substrate specificity. Contrarily, N-2'-deoxyribosyl transferases (EC.2.4.2.6.) specifically catalyze the exchange of the base from a 2'-deoxyribosyl nucleoside with a free purine or pyrimidine [29], and especially those from Lactobacilli are well documented [30–33]. Both types of enzymes display a high regio-(N-1 glycosylation in pyrimidine and N-9 in purine) and stereoselectivity (β-anomers are exclusively formed). The formal synthetic process could be pictured as shown in Figure 14.9.

In order to find new active strains that are able to catalyze this process, a taxonomic screening of 147 microorganisms was performed in our laboratories, using strains belonging to different microbial groups: Bacillaceae, Enterobacteraceae, Lactobacillaceae, Photobacteraceae, Pseudomonaceae, Psychrobacter, and Vibrionaceae. Two reaction tests (Figure 14.10) were used in the screening: reaction test I was the synthesis of adenosine from uridine, and reaction test II was the synthesis of 2'-deoxyadenosine from 2'-deoxyuridine.

The screening resulted in 41 microorganisms (28%) active in reaction test I, while 53 (36%) microorganisms gave positive results in reaction test II, and 35 strains (24%) were positive in both reactions. Only six microorganisms were specific for the synthesis of ribonucleosides.

The most interesting microorganisms were selected according to four main criteria:

1. Low production of hypoxanthine (low adenosine deaminase activity)
2. Nonpathogenic and easy to cultivate in standard culture media

![Figure 14.8](image-url)


![Figure 14.9](image-url)

**FIGURE 14.9** Synthetic activity of nucleoside phosphorilases (NPs).
3. Ability to catalyze the reactions at low temperature
4. High productivity in adenosine or 2'-deoxyadenosine

Using these criteria, the microorganisms shown in Table 14.1 were selected as the most interesting strains for the synthesis of ribonucleosides, while those in Table 14.2 show the best strains for the synthesis of 2'-deoxyribosyl nucleosides. The assays were performed in duplicate experiments. In all cases, the reproducibility of the results was good, as we show in the case of Xanthomonas translucens (57°C, Table 14.1), and for B. psychrosaccharolyticus (57°C) and Psychrobacter immobilis (70°C) in Table 14.2. These two strains, and both Photobacterium are described in this chapter for the first time as nucleoside phosphorylase producers.

![Test reactions used in the screening.](image)

**FIGURE 14.10** Test reactions used in the screening.

<table>
<thead>
<tr>
<th>Strain</th>
<th>T (°C)</th>
<th>Test I</th>
<th>Test II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas salmonicida ssp. achromogenes CECT 895</td>
<td>57</td>
<td>10.0</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus cereus CECT 131</td>
<td>57</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>1.72</td>
<td>6.17</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>B. subtilis CECT 4524</td>
<td>70</td>
<td>1.1</td>
<td>4.5</td>
</tr>
<tr>
<td>B. subtilis ssp niger CECT 4071</td>
<td>57</td>
<td>6.7</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter amnigenus CECT 4078</td>
<td>70</td>
<td>9.0</td>
<td>1.25</td>
</tr>
<tr>
<td>E. sakazakii CECT 858</td>
<td>70</td>
<td>9.0</td>
<td>2.25</td>
</tr>
<tr>
<td>E. gergoviae CECT 857</td>
<td>70</td>
<td>18.0</td>
<td>12.25</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>15.5</td>
<td>20.5</td>
</tr>
<tr>
<td>Erwinia amylovora CECT 222</td>
<td>70</td>
<td>20.8</td>
<td>0</td>
</tr>
<tr>
<td>Photobacterium leiognathi CECT 4191</td>
<td>57</td>
<td>12.1</td>
<td>2.2 (70°C)</td>
</tr>
<tr>
<td>P. phosphoreum CECT 4192</td>
<td>57</td>
<td>11.6</td>
<td>0</td>
</tr>
<tr>
<td>Serratia marcenses CECT 977</td>
<td>57</td>
<td>7.6</td>
<td>4.01</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>6.6</td>
<td>0</td>
</tr>
<tr>
<td>S. rubidea CECT 868</td>
<td>70</td>
<td>19.7</td>
<td>0</td>
</tr>
<tr>
<td>Xanthomonas translucens CECT 4643</td>
<td>57</td>
<td>15.5</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>18.8</td>
<td>13.4</td>
</tr>
</tbody>
</table>
Regarding the synthesis of ribonucleosides, some strains yielded adenosine without detectable production of hypoxanthine at 57°C: *B. cereus*, *B. subtilis*, *B. atrophaeus*, *Serratia marcescens*, and *X. translucens* (Table 14.1). This temperature is lower than that usually described for other strains in the synthesis of this nucleoside, which is normally carried out at temperatures higher than 60°C in order to reduce the production of hypoxanthine [33,34]. The productivity values for 2'-deoxyadenosine (Table 14.1) are generally lower than those obtained for adenosine in the case of nonspecific strains (Table 14.1). Nevertheless, *B. cereus* (70°C), *B. subtilis* (70°C), *Enterobacter gergoviae*, *S. marcescens*, and *X. translucens* showed good productivity in both reactions. It has been previously reported [35–37] that *B. subtilis*, *S. marcescens*, several *Enterobacter* strains and *X. campestris* AJ 2797 are nucleoside phosphorylase producers.

From the results presented in Table 14.2 we can deduce that the most interesting strains (considering the productivity obtained) are *B. coagulans*, *B. psychrosaccharolyticus*, and *P. immobilis*. These strains displayed the best productivity values and the lowest hypoxanthine levels in reactions at 57°C. It must be considered that the presence of 2'-deoxyribosyl transferases in *Lactobacillus* spp. [30,31] is well documented, but that is not the case for psychrotrophic strains [38].

14.2.4.1 1-Characterization of the Enzymes Involved in the Biotransformation of N-2'-deoxyribosyl nucleosides

As indicated in Table 14.2, six strains were selected as active and specific for modification of 2'-deoxyribosyl nucleosides. Two different enzymes can catalyze the synthesis of 2'-deoxyribonucleosides by means of base interchange. The mechanism proposed for 2'-deoxyribosyl transferases involves a covalent catalysis, by analogy with the glycoside hydrolases that use

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**TABLE 14.2**
Productivity Obtained in the Synthesis of 2'-Deoxyadenosine from 2'-Deoxyuridine. Specific Strains in the Synthesis of 2'-Deoxyribosyl Nucleosides (Adenine = 2'-Deoxyuridine = 5 mM, Reaction Time = 1 h; Reaction Volume = 50 mL)

<table>
<thead>
<tr>
<th>Strain</th>
<th>T (°C)</th>
<th>Yield (%)</th>
<th>[Cells] (10^6 cells/ml)^a</th>
<th>Productivity [mM/(h × 10^6 cells)] × 10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus coagulans</em> CECT 12</td>
<td>57</td>
<td>14</td>
<td>514</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>24</td>
<td>1,204</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>13</td>
<td>1,404</td>
<td>2.3</td>
</tr>
<tr>
<td><em>B. psychrosaccharolyticus</em> CECT 4074</td>
<td>57</td>
<td>71</td>
<td>554</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>56</td>
<td>590</td>
<td>27</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em> CECT 684^b</td>
<td>70</td>
<td>42</td>
<td>686</td>
<td>15</td>
</tr>
<tr>
<td><em>Lactobacillus alimentarius</em> CECT 570^b</td>
<td>57</td>
<td>19</td>
<td>1,677</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>6.5</td>
<td>750</td>
<td>2.2</td>
</tr>
<tr>
<td><em>L. pampilonensis</em> CECT 4219^b</td>
<td>57</td>
<td>42</td>
<td>2,068</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>7.7</td>
<td>1,623</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>8.8</td>
<td>2,223</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Psychrobacter immobilis</em> CECT 4492</td>
<td>57</td>
<td>66</td>
<td>690</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>53</td>
<td>713</td>
<td>18</td>
</tr>
</tbody>
</table>

^aCell cultured at 28°C.

^bHypoxanthine producer (yield >10% referred to adenine).
either a glutamyl or an aspartyl residue for catalytic activity. Indeed, several researchers [29,39,40] have demonstrated the presence of a glutamyl residue involved in the rate-controlling step in several N-2'-deoxyribosyl transferases, as depicted in Figure 14.11.

Another possibility involves two enzymes: thymidine nucleoside phosphorylase (TNP) and purine nucleoside phosphorylase [41,42]. TNP (EC 2.4.2.4) is specific for thymidine and the reaction takes place in two steps, through a 2'-deoxyribose-1-phosphate intermediate, as shown in Figure 14.12. TNP recognizes thymidine better than 2'-deoxyuridine [39,40].

The difference between both mechanisms is the presence of the ribose 1-α-phosphate intermediate in the case of TNP but not in the case of N-2'-deoxyribosyl transferases. Therefore, two parallel experiments were performed at the same time and with the same microbial culture. One reaction was performed using 30 mM NaH₂PO₄/Na₂HPO₄ buffer (pH = 7) and the other using 25 mM Tris/HCl buffer (pH = 7). When the microorganisms expressed a TNP, the yield and productivity of the reaction were higher in phosphate buffer that in the presence of Tris/HCl buffer. Contrarily, if the microorganisms expressed a nucleoside 2'-deoxyribosyl transferase, the yield were similar in both reactions.

From the results in Table 14.3 we can deduce that B. subtilis (nonspecific strain, Table 14.1) cannot give the test II in the presence of Tirs buffer. E. aerogenes clearly express a TNP because the reaction does not take place in Tris/HCl buffer. On the contrary, B. psychrosaccharolyticus, B. coagulans, P. immobilis, and Lactobacillus pampilonensis gave similar yields in Tris/HCl buffer as in phosphate buffer. This confirms the presence of nucleoside 2'-deoxyribosyl transferases in these strains.

### 14.2.5 Immobilization of Whole Cells

Nucleoside phosphorylases are extracellular and multimeric enzymes [43,44]. A synthesis with these enzymes at semi-industrial scale would demand a co-immobilization step after the overexpression of the enzymes in genetically modified microorganisms and disruption of cells by consecutive passage of biomass in a high-pressure homogenizer [45–47]. Therefore, the immobilization–stabilization of these enzymes is a rather complex, but not impossible, process. In fact, nucleoside phosphorylases from B. subtilis have been expressed in Escherichia
coli, purified, and immobilized in sepabeads with polyethyleneamine, yielding an enzymatic derivative that is very active at pH $= 10$ and $45^\circ C$ [46,47]. A cheap alternative is the immobilization of the whole cells [48,49]. With these biocatalysts, efficient synthesis of some purine nucleosides have been reported using *Erwinia herbicola* [34], *E. coli* BL21 [35,50], *B. stearothermophilus* [28], and *B. cereus T.* [51]. In order to scale up the synthetic process using whole cells, two main conditions are necessary:

1. Absence of secondary reactions such as deamination of adenine in adenosine ring
2. Possibility of reusing the biocatalyst [34,52,53]

Thus, the most widely used technique for cell immobilization is the cell entrapment [54], in which the living cells are enclosed in a polymeric porous matrix that allows the diffusion of substrates and products. The main advantages of this methodology are the high operational stability, the ease of cell handling and separation, and finally the feasibility of the scaling up [55–61].

Therefore, the best microorganisms as deduced by their performance shown in Table 14.1 and Table 14.2 were immobilized in different supports, in order to select the best biocatalyst according to the maximum yield reached and the maximum number of reuse cycles. The immobilized derivatives in agars (with different methoxylation degree) or in agaroses provided the best results. Different shapes of the biocatalysts — small beads, microbeads, or small parallelepipeds — were prepared in our laboratories, while the immobilization in microbeads was rejected because the biocatalysts were not interesting from the reuse and scale-up aspects, as we show in Figure 14.13 compared to Figure 14.14 (immobilization in small plates).

The qualitative behavior of other microorganisms immobilized in these matrixes was similar. This finding shows that all thermogels (obtained at their optimum percentage and with optimum shaking speed) do not exert dramatic restrictions on the diffusion of either

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**TABLE 14.3**

| Productivity Obtained in the Synthesis of 2'-Deoxyadenosine from 2'-Deoxyuridine + Adenine in Tris/HCl or in Phosphate Buffer ($T = 57^\circ C$, Reaction Volume = 4 mL, 2'-Deoxyuridine = adenine = 5 mM, Reaction Time = 20 min) |
|---|---|---|
| Strain | Buffer | Yield$^b$ (%) | Productivity [mM/(h x $10^6$ cells)] $\times 10^3$
| Bacillus coagulans | Tris/HCl | 15 (8) | 3.7 |
| CECT 12 | Phosphate | 14 (9) | 3.8 |
| B. subtilis | Tris/HCl | 0 | 0 |
| CECT 4524 | Phosphate | 35 (7) | 31.5 |
| B. psychroactarolyticus | Tris/HCl | 48 | 31 |
| CECT 4074 | Phosphate | 62 | 35 |
| Enterobacter aerogenes$^a$ | Tris/HCl | 0 | 0 |
| CECT 4074 | Phosphate | 14 (6) | 17 |
| Lactobacillus pampilonensis | Tris/HCl | 26 (8) | 3.1 |
| CECT 4219 | Phosphate | 23 (11) | 2.7 |
| Psychrobacter immobilis | Tris/HCl | 27 | 13 |
| CECT 4492 | Phosphate | 24 | 10 |

$^a$Reaction time when it is not 1 h.

$^b$Yield in 2'-deoxyadenosine and (yield in hypoxanthine).
reagents or products for obtaining an optimum yield in adenosine. In Figure 14.15 we observe that the bacteria are immobilized inside small cavities where only a few cells are entrapped, linked to the matrix through small linkers that probably could be mucopolysaccharides secreted by the cell. These microphotographs justify that in the washing of the biocatalyst particles for reuse, no free bacteria were observed in the reaction medium with a conventional microscope.

The stability of the biocatalysts in standard storage conditions (4°C) was also tested. After 60 to 75 days all the immobilized biocatalysts retained the same activity in the synthesis of adenosine starting from uridine and adenine [38].

In Table 14.4 we compare the catalytic activity of several strains immobilized in agar or agarose microplates using resting cell conditions. We can observe that *E. ammigenus* and *E. gergoviae* gave similar yields in all cases, while *S. marcenses* showed a moderated diminution in the yield in adenosine after immobilization. So, these biocatalysts can be used for a further scaling up.

**FIGURE 14.13** Synthesis of adenosine from uridine/adenine (30/10) mM using immobilized *Serratia marcenses* CECT 977 whole cells. Microbeads of highly methoxylated agar A28/03. Temperature = 57°C. Stirring speed = 250 r.p.m. Reaction time = 120 h. 40 g of wet biocatalyst.

**FIGURE 14.14** Synthesis of adenosine from uridine/adenine (30/10) mM using immobilized *Serratia marcenses* CECT 977 whole cells. Microplates (1 cm × 1 cm × 0.2 cm) of highly methoxylated agar A28/03. Temperature = 57°C. Stirring speed = 250 r.p.m. Reaction time = 120 h. 40 g of wet biocatalyst.
In the case of the synthesis of 2'-deoxyribonucleosides, the immobilized whole cells in agar (A28/03) were as active in agar as in agarose, as we show for a nonspecific strain such as *E. gergoviae* (Table 14.5).

Even though better yield was obtained using the immobilized biocatalysts compared to free resting cells, the productivity was lower (Table 14.5), due to the longer reaction times demanded for the immobilized cells. Finally the best biocatalyst — *E. gergoviae* immobilized in small plates of highly methoxylated agar — was reused several times, as shown in Figure 14.16. This biocatalyst could be reused at least seven times without diminution of the yield. The immobilized biocatalysts in agarose gave good yield in the first cycle (Table 14.5), but only 60% yield was achieved in the second cycle. The yields obtained with immobilized *E. gergoviae* were similar to those described by Yokozeki and Tsuji [53], using free cells of *E. aerogenes* AJ-11125, or better than those described by Prasad et al. [62].

### 14.2.6 Synthesis of Different Nucleoside Analogs

The syntheses of several nucleoside analogs have been described in the literature using whole cells as biocatalysts [36,49,63–66].

Kulikowska et al. [64] showed that 7-methylguanosine and 7-methylinosine are substrates of purine nucleoside phosphorylases. Taking into account this finding, Hennen and Wong

**TABLE 14.4**

Catalytic Activity of the Most Interesting Strains in the Synthesis of Adenosine from Uridine. Uridine/Adenine (30 mL/10 mM). Resting Cells and Immobilized in Agar A28/03 and in Agarose D5 (T = 70°C, Reaction Time = 30 min (resting cells) or 1 h (immobilized cells), Reaction Volume = 15 ml, Wet Catalyst Weight = 20 g)

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Adenosine (%)</th>
<th>Adenosine (%)</th>
<th>Adenosine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting Cells</td>
<td>Immobilized Agar</td>
<td>Immobilized Agarose</td>
</tr>
<tr>
<td><em>Enterobacter amnigenus</em> CECT 4078</td>
<td>98.5</td>
<td>88</td>
<td>94</td>
</tr>
<tr>
<td><em>E. gergoviae</em> CECT 857</td>
<td>100</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td><em>E. sakazakii</em> CECT 858</td>
<td>91</td>
<td>5.2</td>
<td>48</td>
</tr>
<tr>
<td><em>Serratia rubidea</em> CECT 868</td>
<td>90</td>
<td>11</td>
<td>7.5</td>
</tr>
<tr>
<td><em>S. marcenses</em> CECT 977</td>
<td>100</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>
proposed the use of 7-methylguanosinium or 7-methylinosinium as water-soluble glycosyl donor to obtain analogs of nucleosides in an irreversible and quantitative process, caused by the low solubility of guanine and inosine at pH $\approx 7.0$ to 7.4.

Shirae et al. [67] described the use of different \textit{E. coli} strains (resting cells) for the preparation of different nucleoside analogs, such as 2',3'-dideoxyadenosine (39) or 2',3'-dideoxyinosine (40) (Figure 14.17). After a taxonomic screening of 436 microorganisms belonging to 39 genera, they selected one strain (optimal pH was $\approx 6.5$, and optimal temperature was 50°C). These authors indicated that 2',3'-dideoxypyrimidine nucleosides (38 and 41) could also be obtained from 2',3'-dideoxyadenosine (39), but with moderated yields, as shown in Figure 14.17, so this result indicates the great versatility of the strain.

Recombinant \textit{E. coli} BL21 strain was used by Rogert et al. [50] to prepare some ara-nucleosides (43 and 44) from ara-uridine (42) using resting cells at 60°C and pH $= 7.0$ (Figure 14.17). On the other hand, Murakami et al. [68] described the use of \textit{E. coli} JA-300

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>2'-Desoxyadenosine (%)</th>
<th>Cell Concentration (10^6 cell/ml)</th>
<th>Catalyst Load (10^6 cell/g cat)</th>
<th>Productivity [mM/(h × 10^5 cells)] × 10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting Cells</td>
<td>42</td>
<td>1319</td>
<td>—</td>
<td>84</td>
</tr>
<tr>
<td>Immobilized Agar A28/03</td>
<td>90</td>
<td>—</td>
<td>1319</td>
<td>23</td>
</tr>
<tr>
<td>Immobilized Agarose</td>
<td>85</td>
<td>—</td>
<td>1110</td>
<td>25.5</td>
</tr>
<tr>
<td>Resting Cells</td>
<td>43</td>
<td>1319</td>
<td>—</td>
<td>86</td>
</tr>
</tbody>
</table>

[FIGURE 14.16 Recycling of immobilized biocatalysts: whole cells of \textit{Enterobacter gergoviae} CECT 857 immobilized in small plates of agar A28/03, $T = 57°C$.]
FIGURE 14.17 Synthesis of different nucleoside analogs using some *Escherichia coli* strains.
whole cells (50°C and pH = 6.5) in the synthesis of 6-halo-2',3'-dideoxypurine nucleosides (45 and 46), used as anti-HIV drugs, starting from 2',3'-dideoxyuridine (38) (Figure 14.17).

Finally, recombinant E. coli BM-11 cells have been used by Zinchenko et al. [69] to obtain 9-(β-D-arabinofuranosyl)guanine (47, Figure 14.18), active against viruses of herpes type and also for inhibiting T-lymphocyte proliferation. The synthesis was performed from 1-(β-D-arabinofuranosyl)uracil (42) and three different guanine donors: guanine (48), guanosine (4a) and 2'-deoxyguanosine (49), as shown in Figure 14.18.

The synthesis starting from guanine nucleosides leads to higher yields than using free base. The phenomenon is related to the poor solubility of guanine in the reaction conditions (~0.042 g/L) which leads to a low base concentration and thus the unfeasibility of an effective transglycosidation mediated by the cells. Contrarily, both nucleosides are used by the cells to render a high yield in ara-guanosine. As can be seen, the cross-linking with glutaraldehyde of whole cells increases both the stability of biocatalysts and the obtained yield.

Some other researchers have described the synthesis of nucleoside analogs using phosphorylases in combination with other enzymes. Thus, Pal and Nair [28] described how the addition of xanthine oxidase (xodase) to a synthesis of nucleosides catalyzed by whole cells of B. stearothermophilus ATCC 12980 leads to higher yields in the synthesis of thymidine (51) starting from 2'-deoxyinosine (50) than those described in the absence of xodase (Figure 14.19). This fact is explained because xodase shifts the equilibrium of the reversible transglycosylation reaction toward completion by the formation of uric acid (52, Figure 14.19), which is not recognized by phosphorylase.

Finally, Yokozeki and Tsuji [53] described the synthesis of 2'-deoxyguanosine (4a, Figure 14.20) with 100% yield at 25°C, using E. gergoviae AJ-11125 whole cells. This strain has a very active adenosine deaminase, so that the synthesis of 4a could be described using

2,6-diaminopurine (54) and 2′-deoxyuracil (53) as base acceptor, through 3a intermediate (Figure 14.20). This indirect methodology overcomes the direct synthesis of 4a from guanine and 53 due to the low water solubility of the base in the medium.

14.3 CONCLUSIONS

The enzymatic synthesis of nucleoside analogs using either free enzymes or whole cells can be advantageous over chemical synthesis due to the reduction in the number of steps and the high regio- and stereoselectivity observed. Different enzymes can be used in the synthesis of

![Diagram of nucleoside analog synthesis](image1)


![Diagram of nucleoside analog synthesis](image2)

these compounds in order to selectively modify either the base or the sugar functional groups. In addition, different expensive nucleoside analogs can be prepared starting from cheap nucleosides by base interchange using immobilized whole cells in processes that are easy to scale up. The main problem for this synthetic methodology is to design a “homogeneous reaction media” for both nucleosides (hydrophilic) and bases (hydrophobic). Therefore, the development of new solvents displaying low toxicity for cells or enzymes and possessing good solving properties is the main work in the future for scaling up these syntheses.

### TABLE 14.6

<table>
<thead>
<tr>
<th>Pyridine Nucleoside</th>
<th>W</th>
<th>Z</th>
<th>Z'</th>
<th>Microorganism</th>
<th>Base</th>
<th>Yield in Nucleoside (%)</th>
<th>Time (h)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>E. gergoviae CECT 857</td>
<td>6-Mercaptopurine</td>
<td>56</td>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td>2'-deoxyuridine</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>E. gergoviae CECT 857</td>
<td>6-Mercaptopurine</td>
<td>18</td>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td>Uridine</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>E. coli BMT-ID/1A</td>
<td>3-carboxyamido1,2,4-triazol</td>
<td>45</td>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td>Uridine</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>E. coli Free enzymes</td>
<td>Purine</td>
<td>80</td>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td>Ara-Uridine</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>E. coli BMT-ID/1A</td>
<td>Adenine</td>
<td>83</td>
<td>133</td>
<td>55</td>
</tr>
<tr>
<td>Uridine</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>E. coli Free enzymes</td>
<td>4-Chloro-Imidazo[4,5-c]pyrimidine</td>
<td>57</td>
<td>—</td>
<td>59</td>
</tr>
</tbody>
</table>
TABLE 14.7
Synthesis of Some Nucleoside Analogues Using 7-Methyl Guanidinium or 7-Methylinosinium Salts

<table>
<thead>
<tr>
<th>Initial Compound</th>
<th>Base</th>
<th>Yield in Nucleoside</th>
<th>Reaction Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>adenine</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Adenine</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Adenine</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>3-diaza adenine</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>3-diaza adenine</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1,2,4-triazol-3- carboxamide</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1,2,4-triazol-3- carboxamide</td>
<td>57</td>
<td>2</td>
</tr>
</tbody>
</table>

*100 mol.
*25 mol.

Enzymatic Synthesis of Modified Nucleosides


Enzymatic Synthesis of Modified Nucleosides


Author Query

AQ1: Table citation is missing in text for Table 14.6 and Table 14.7