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Biotransformation of 2,6-Diaminopurine Nucleosides by Immobilized *Geobacillus stearothermophilus*

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An efficient and green bioprocess to obtain 2,6-diaminopurine nucleosides using thermophilic bacteria is herein reported. Geobacillus stearothermophilus CECT 43 showed a conversion rate of 90 and 83% at 2 h to obtain 2,6-diaminopurine-2′-deoxyriboside and 2,6-diaminopurine riboside, respectively. The selected biocatalyst was successfully stabilized in an agarose matrix and used to produce up to 23.4 g of 2,6-diaminopurine-2'-deoxyriboside in 240 h of process. These nucleoside analogues can be used as prodrug precursors or in antisense oligonucleotide synthesis. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.,* 28: 1251–1256, 2012
Keywords: thermophilic microorganisms, nucleoside phosphorylase, whole cell immobilization, prodrug precursors, transglycosylation

Introduction

At present, there is a marked tendency towards the novel biotransformation of nucleoside analogues with pharmacological activity.[1] 2,6-Diaminopurine-2′-deoxyriboside (DAPurdRib) is used as precursor of 2′-deoxyguanosine analogues by deamination due to low solubility of guanine as starting substrate.[2] Furthermore, antisense oligonucleotides with 2,6-diaminopurine nucleosides exhibit enhanced thermal stability and are used in cancer therapies.[3] Besides, these compounds can be used to synthesize mRNA cap analogs for studies of *in vitro* genetic expression.[4]

Recently, Arico *et al*. have reported improvements in DAPurdRib synthesis by chemical methods.[5] However, biocatalysis is frequently recognized as an alternative to obtain nucleoside analogues by transglycosylation,[6] owing to its high catalytic efficiency[7] and stereoselectivity, which simplify the downstream processing steps.[8]

Transglycosylation reaction can be catalyzed by two groups of enzymes: nucleoside phosphorylases (NPs, 2.4.2.1, 2.4.2.2, 2.4.2.3, or 2.4.2.4) and 2′-*N*-deoxyribosyltransferases (NDTs, 2.4.2.6). Biotransformation of nucleoside analogues using NPs[9] or NDTs[10] was previously reported, but expensive and complex methods are required. Therefore, whole cells provide one-pot reactions in a natural environment for enzymes and regenerate cofactors efficiently.[11] Yokozeki and Tsuji have reported the use of free microorganisms for obtaining 2,6-diaminopurine nucleosides.[12]

Particularly, thermophilic microorganisms are very useful in industrial bioprocesses due to the high stability of their enzymes.[13] To date, only NPs were reported and characterized in *Geobacillus stearothermophilus*. These enzymes catalyze reversible phosphorolytic cleavage of *N*-glycosidic bonds of nucleosides in the presence of phosphate to form a free base and its respective activated pentose moiety, which is then coupled to the desired modified base to give a nucleoside analogue (only β-anomer).[14]

Entrapment is the most useful technique for microorganism immobilization.[15] This strategy simplifies product recovery, improves biocatalyst reusability[16] and bioprocess scale-up feasibility. The aim of this study was to obtain 2,6-diaminopurine-2′-deoxyriboside (DAPurdRib) and 2,6-diaminopurine riboside (DAPurRib) by immobilized *G. stearothermophilus* CECT 43 as biocatalyst using an inexpensive and environmentally friendly methodology.

Materials and Methods

Reagents and microorganisms

Nucleosides and bases were purchased from Sigma Chem (Brazil). Culture media compounds were obtained from Britania S.A. (Argentina). Chemical reagents were purchased from Sigma Chem (Brazil). HPLC grade solvents from Sintorgan S.A. (Argentina) were used. TLC aluminum sheets (Silica gel 60 F254) were from Merck (Germany). The microorganisms were kindly supplied by the 'Colección Española de Cultivos Tipo (CECT),' University of Valencia (Spain).

Growth conditions

Microorganisms were grown at 55°C and 200 rpm, harvested by centrifugation at 17500*g* for 10 min, washed with 30 mM phosphate buffer (pH 7) and stored at 4°C until use. *G. stearothermophilus* media contained 10 g/L meat peptone, 5 g/L yeast extract, 5 g/L NaCl, and 4 g/L glucose (pH 7).

Selection of thermophilic bacteria

Different strains of *G. stearothermophilus* (CECT 43, 47 y 49) were tested as biocatalyst for thymidine hydrolysis.

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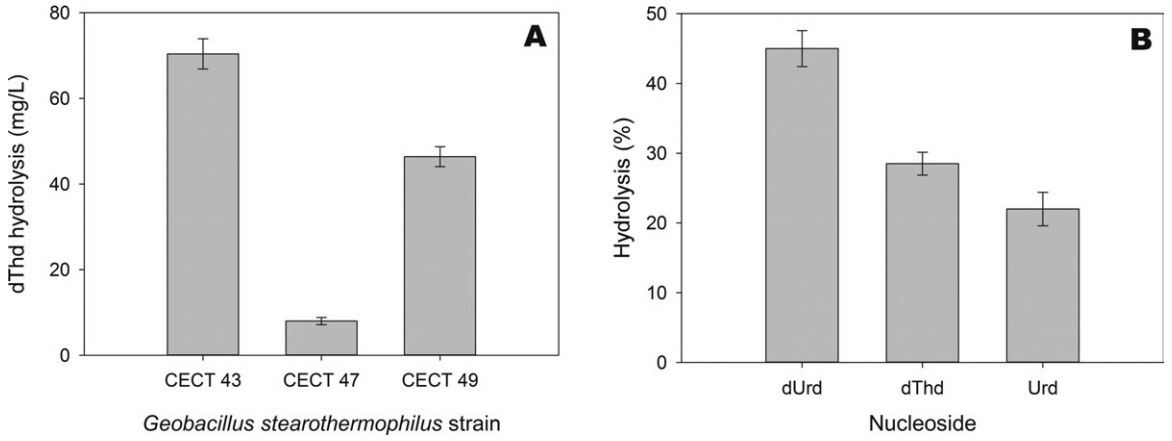


Figure 1. Biocatalyst and substrate selection.

A: Selection of thermophilic microorganisms. B: Hydrolysis of different sugar donors by *G. stearothermophilus* CECT 43.

Reactions were conducted with 5 mM thymidine, 3 x 10^9 CFU (Colony Forming Unit) in 0.5 mL of 30 mM phosphate buffer (pH 7) at 55°C during 2 h.

Sugar donor selection

Nucleosides such as uridine (Urd), thymidine (dThd), 2′-deoxyuridine (dUrd), uracil 1-β-D-arabinofuranoside (araUra) and 2′,3′-dideoxyuridine (ddUrd) were assayed as sugar donors. Reactions were made with 5 mM nucleoside, 3 x 10^9 CFU in 0.5 mL of 30 mM phosphate buffer (pH 7) at 55°C for 2 h.

Bioprocess parameters

Different reaction parameters such as microbial growth phase, microorganisms amount (1 x 10^7, 1 x 10^8, 1 x 10^9 and 1 x 10^{10} CFU), pH (5, 6, 7 and 8), buffer concentration (0, 10, 20, 30, 40, 50 and 60 mM), stirring speed (100, 200, 300 and 400 rpm), temperature (30, 45 and 60°C), cation effect (10–50 mM Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+} or Cu^{2+}) and 2,6-diaminopurine:2′-deoxyuridine ratio (6:2; 2:2 and 2:6 mM) were studied for DAPurdRib biotransformation in 0.5 mL of reaction.

Whole cell immobilization

G. stearothermophilus CECT 43 (1 x 10^{10} CFU) was stabilized by the entrapment techniques in agarose 2, 3, and 4% (w/v) and polyacrylamide 15, 20, and 25% (w/v) as previously described.[16] These immobilized biocatalysts were tested for bacterial release by analyzing with a spectrophotometer set at a wavelength of 600 nm (Shimadzu UV-1603) and also by plating in the corresponding medium.

Reusability of the immobilized biocatalyst was evaluated through successive DAPurdRib biotransformations and the reuse number that remained active was determined. Reaction conditions were 2 mM dUrd, 6 mM 2,6-diaminopurine (DAPur) in 3 mL of 30 mM phosphate buffer (pH 7) at 30°C and 200 rpm for 2 h. Storage stability was established by keeping the immobilized derivative at 4°C and determining its activity at different times as previously described for reusability assays.

Analytical methods

Nucleoside analogue biotransformations were qualitatively evaluated by TLC in 80:20 (v/v) chloroform/methanol as mobile phase. The quantitative analysis was performed by HPLC (Pharmacia LKB) at 254 nm using a Nucleodure 100 C-18 column (5 μm, 125 mm × 5 mm). The isocratic mobile phase used was 90:10 (v/v) water/methanol at flow of 1.0 mL/min. Product identification was performed by MS-HPLC in LCQ-DECAXP4 Thermo Spectrometer using the Electron Spray Ionization method.[17] A Phenomenex C18 column (5 μm, 100 mm × 2 mm) and Xcalibur 1.3 software (Thermo-Finnigan, USA) were used. The mobile phase used for DAPurRib (M^+: 283.1) and DAPurdRib (M^+: 267.0) biotransformations was 85:15 (v/v) methanol/water + 0.1% acetic acid and flow rate of 0.2 mL/min.

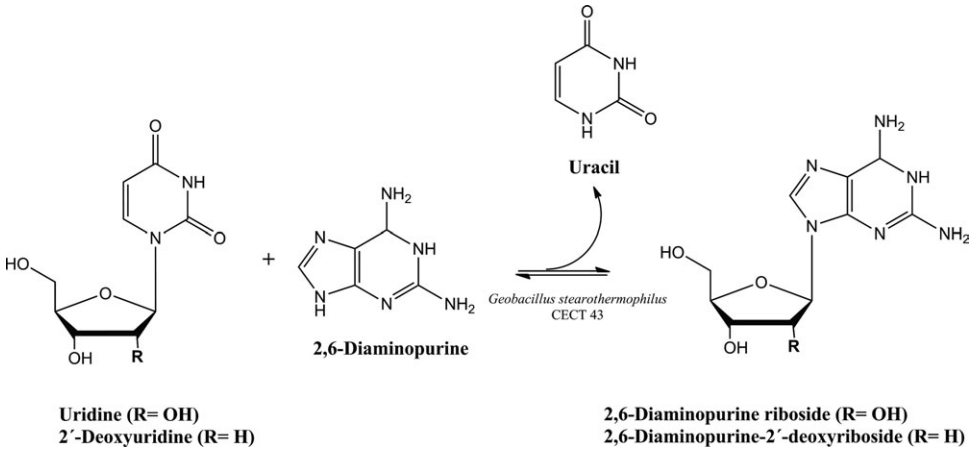
Results and Discussion

Selection of thermophilic bacteria

dThd hydrolysis was assayed to select the thermophilic strain.[18] Screening was carried out with three *G. stearothermophilus* strains (CECT 43, 47 y 49) (Figure 1A). All strains tested showed catalytic activity for dThd hydrolysis, whereas *G. stearothermophilus* CECT 43 was selected for subsequent reactions since it exhibited the highest conversion values at tested times. This differential behavior within the same genus could be due to different enzyme specificity. There are some reports which have postulated that it is not possible to establish a relationship between a given sequence or structure and its nucleoside specificity.[9]

Sugar donor selection

Urd, dThd, dUrd, ddUrd, and araUra were tested as starting nucleosides (Figure 1B). *G. stearothermophilus* CECT 43 preferentially hydrolyzed dUrd, dThd and Urd with conversions values of 45, 28, and 21% at 2 h, respectively. In contrast, no significant hydrolytic activity was detected when ddUrd and araUra were evaluated at short reaction times. There are previous reports which showed a hydrolysis increase (about 50%) of dUrd over Urd, when pyrimidine nucleoside phosphorylase activity was evaluated.[19] In view of these results, dUrd and Urd were used as starting



Scheme 1. Biotransformation of 2,6-diaminopurine nucleosides by *G. stearothermophilus* CECT 43.

nucleosides for DAPurdRib and DAPurRib biotransformations, respectively (Scheme 1).

Bioprocess parameters

Microbial Growth Phase. dUrd hydrolysis was assayed using *G. stearothermophilus* CECT 43 at different growth phases, using 3 x 10^9 CFU and 5 mM dUrd in 30 mM phosphate buffer (pH 7) at 55 °C. The best results were obtained with microorganisms in stationary growth phase, which could be due to differential expression of NPs, enzymes involved in nucleoside salvage pathway during stationary growth phase.[14]

Amount of microorganisms. Reactions were performed with increasing amounts of *G. stearothermophilus* CECT 43, using 6 mM DAPur and 2 mM dUrd as substrates in 30 mM phosphate buffer (pH 7) at 55°C. DAPurdRib biotransformation was detectable with 1 x 10^9 CFU, reaching 286 mg/L of this product at 4 h (Figure 2A). When the amount of microorganisms was increased by 10-fold (1 x 10^{10} CFU), the conversion rise to 505 mg/L. Higher amounts of microorganisms were not evaluated due to operational difficulties.

pH, Buffer Concentration and Stirring Speed. No significant differences were observed when DAPurdRib biotransformation was carried out at pH 5, 6, 7 and 8. Similarly, no significant differences were detected when buffer concentration (20–60 mM) and stirring speed (100–400 rpm) were evaluated.[20]

Temperature. DAPurdRib biotransformation was performed at different temperatures (30, 45 and 60°C) using 6 mM DAPur and 2 mM dUrd as substrates (Figure 2B).

DAPurdRib productivity at 30°C was 0.65 mM/h. However, a significant decrease in DAPurdRib productivity was observed at higher temperatures, possibly due to adenine deaminase activity (ADE, EC 3.5.4.2).[17]

Deaminase activity was detected at 45 and 60°C using 6 mM DAPur with 1 x 10^{10} CFU at 2 h reaction. At 30°C, DAPurdRib biotransformation was 90% and residual deaminase activity was observed (Table 1). Therefore, the selected temperature for subsequent reactions was 30°C.

Cations. DAPurdRib biotransformations were carried out with cation addition. Relative activity (ra) was calculated with respect to the standard reaction (ra: 1) (Figure 2C).

Relative activities were considerably enhanced when Cu^{2+} and Zn^{2+} were added to the reaction medium, and slightly increased by the addition of Mn^{2+}. However, no significant effect was observed with alkaline earth cations (Mg^{2+} or Ca^{2+}). The cation effect was independent of their concentration, because 10–50 mM of each cation was assayed and the obtained results were not significantly different.

It is known that Zn^{2+} and Cu^{2+} interact with ABC transporter proteins, which results in increased transport activity.[21] Additionally, previous reports have demonstrated that these kinds of proteins are involved in nucleoside uptake to be used by intracellular bacterial NPs.[22] Moreover, some of these ABC proteins have been found in *G. stearothermophilus*.[23] Thus, activation of transport may account for the cation effect.

Initial Molar Ratio. It has been widely reported that transglycosylation reactions are reversible[19] and previous reports have demonstrated that using an excess of any substrate significantly improved conversion values.[24] For these reasons, the initial ratio of DAPur and dUrd was analyzed (Figure 2D). When 2:2 mM ratio was assayed, DAPurdRib conversion was 20%. However, using an excess of some substrate, the reaction yields were significantly increased. When DAPur/dUrd ratio was 6:2 mM DAPurdRib conversion reached 90% at 2 h. We postulate that the obtained results could be due to hydrolysis (first reaction step) evolved satisfactorily in short times of reaction (higher than 90% at 1 h), suggesting that it would not be the limiting step of reaction. Therefore, a base excess promotes the second reaction step (nucleoside biosynthesis) because the modified base could compete more efficiently with existing unmodified base within the cell (for example, adenine).

On the basis of these results, reaction parameters for DAPurdRib biotransformation were 6 mM DAPur, 2 mM dUrd, 10 mM Zn^{2+} (or Cu^{2+}), 30 mM phosphate buffer (pH 7) at 30°C and 200 rpm. Finally, this work improves yields and reduces reaction times significantly improving bioprocess productivity. This parameter is a major variable for subsequent scale-up. In addition, we have been able to carry out the reaction at low temperatures, reducing the energy cost.[12]

Entrapment immobilization

Matrix Selection. The selected thermophilic strain was immobilized by entrapment in agarose and polyacrylamide at different concentrations.[24] Agarose 2% (w/v) showed no adequate microorganism retention. However, when 3% (w/v)

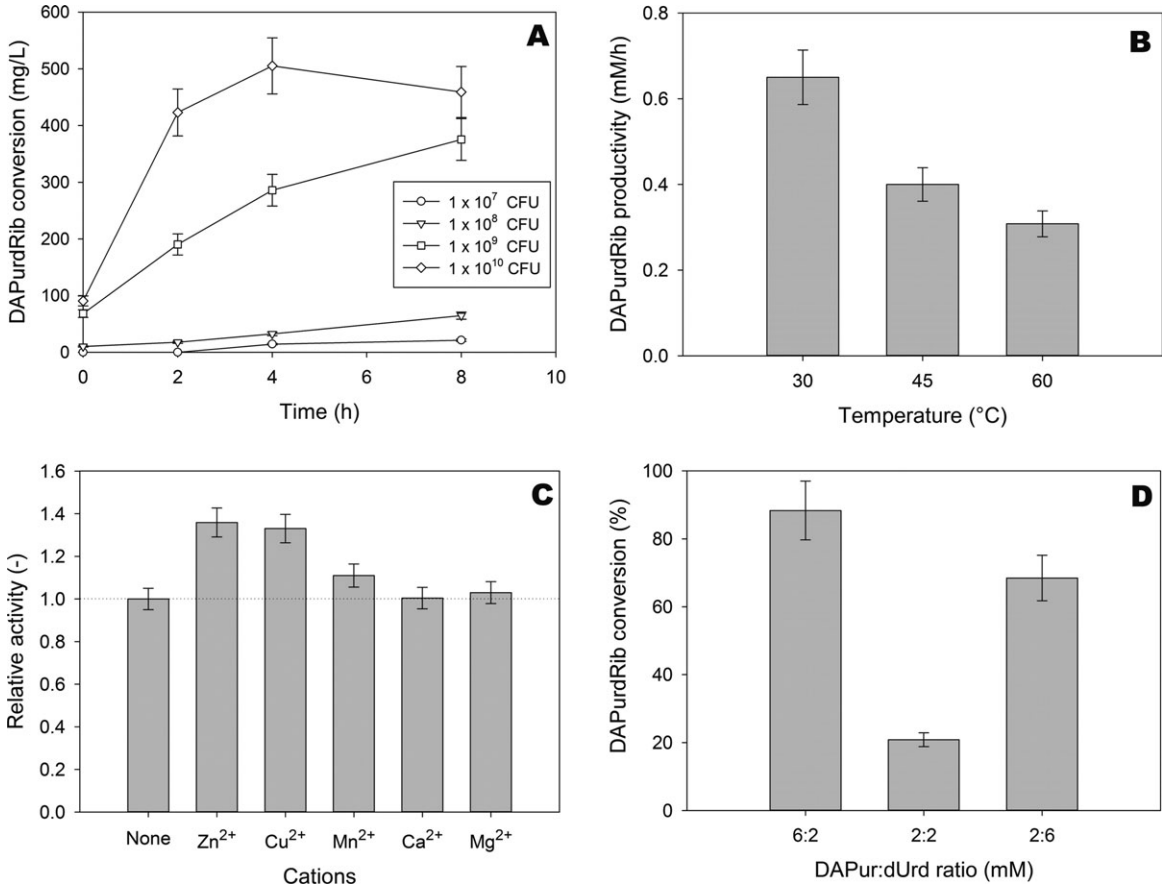


Figure 2. Biotransformation of 2,6-diaminopurine-2′-deoxyribonucleoside.

A: Amount of microorganisms, B: Temperature, C: Cation addition (10 mM), D: Substrate ratio.

Table 1. DAPurdRib Biotransformation and DAPur Deaminase Activity of *G. stearothermophilus* CECT 43 at Different Temperatures

Temperature (°C)	Reaction Time (h)	DAPurdRib Conversion (%)*	DAPur Deamination (%)**
30	1	54	< 5
	2	90	< 5
45	1	30	16
	2	58	33
60	1	25	23
	2	49	37

$$* \text{Conversion } (\%) = \frac{\text{mmol product}}{\text{mmol limiting reagent}} \times 100.$$

$$** \text{Deamination } (\%) = \frac{\text{mmol product}}{\text{mmol initial DAPur}} \times 100.$$

Table 2. DAPurdRib and DAPurRib Biotransformations With Free and Immobilized *G. stearothermophilus* CECT 43

	Matrix	Conversion (%)*	Time (h)	Productivity (mM/h)**
DAPurdRib biotransformation				
Free cells	–	90	2	0.90
Immobilized	Agarose 3 %	97	8	0.48
	Polyacrylamide 20 %	79	24	0.10
DAPurRib biotransformation				
Free cells	–	83	2	0.82
Immobilized	Agarose 3 %	93	8	0.47
	Polyacrylamide 20 %	52	24	0.05

$$* \text{Conversion } (\%) = \frac{\text{mmol product}}{\text{mmol limiting reagent}} \times 100.$$

$$** \text{Productivity } (\text{mM/h}) = \frac{[\text{mM}] \text{ product}}{\text{hour}}.$$

was used, no microorganism release was observed and 518 mg/L of DAPurdRib was produced at 8 h. Additionally, when 20% (w/v) of polyacrylamide was used no bacterial release was detected, but DAPurdRib conversion decreased significantly (164 mg/L) probably due to the toxic effect of acrylamide on microorganisms[25] or to high reticulation degree, which hindered substrate diffusion.[26] Manolov *et al.* also reported *G. stearothermophilus* immobilization using different polyacrylamide concentrations (5–35%) and similarly to our findings, they observed cell release when the polyacrylamide percentages were lower than 20% (w/v).[27]

G. stearothermophilus CECT 43 immobilized in agarose 3% (w/v) was selected for successive tests due to high productivity (Table 2). It is noteworthy that this immobilized biocatalyst was also satisfactorily evaluated for DAPurRib biotransformation, obtaining 521 mg/L at 8 h.

Operational Stability of Immobilized Biocatalyst. G. stearothermophilus CECT 43 immobilized in agarose was tested for its reusability in DAPurdRib biotransformation at 2 h and its relative activity (ra) with respect to the first reuse was determined (Figure 3). This biocatalyst retained full activity for more than 100 reuses, obtaining 23.4 g of

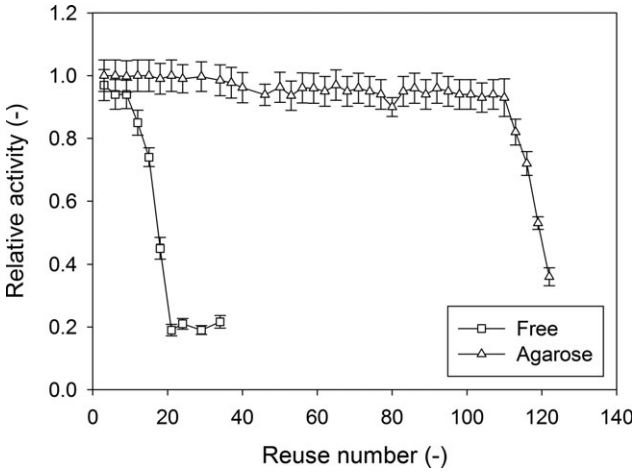


Figure 3. Operational stability of *G. stearothermophilus* CECT 43.

Table 3. Green Chemistry Parameters of 2,6-Diaminopurine Nucleosides Biotransformation by Immobilized *G. stearothermophilus* CECT 43

Product	E-Factor*	C-Efficiency**	A-Economy***
DAPurRib	0.48	62	75
DAPurdRib	0.47	64	70

$$*E-\text{Factor}=\frac{\text{total waste mass}}{\text{product mass}}.$$

$$**C-\text{Efficiency}=\frac{(\text{carbon atoms in product})\times 100}{\text{carbon atoms in reactants}}.$$

$$***A-\text{Economy}=\frac{(\text{product MW})\times 100}{\sum(\text{reactants MW})}.$$

DAPurdRib in 240 h of bioprocess and maintaining activity for more than 6 months at 4°C (storage stability). However, free cells of *G. stearothermophilus* CECT 43 significantly lost their activity in less than 20 reuses and their storage stability at 4°C was only 10 days.

Green Chemical Parameters. Environment-Factor (E-Factor) is a measurement of the environmental impact generated by industries. E-Factor values are around 25-100 for pharmacological compounds.[28] A low E-Factor value shows mass utilization efficiency and a significant decrease of waste production (Table 3). In the present work, E-Factor values for DAPurdRib and DAPurRib biotransformations were lower than 0.5.

Carbon efficiency (C-Efficiency) and atom economy (A-Economy) were designed as parameters to evaluate the efficiency of synthetic reactions. For every biotransformation, C-Efficiency values were greater than 60% and A-Economy values were 70 and 75% for DAPurdRib and DAPurRib, respectively.

Conclusions

G. stearothermophilus CECT 43 was selected to obtain DAPurdRib and DAPurRib by biotransformations. Reaction parameters such as amount of microorganisms, pH, buffer concentration, stirring speed, temperature, cation addition, and substrate ratio were determined to optimize yields and reduce reaction times. In this way, we have been able to improve productivity process, a very important parameter for the subsequent scale-up. Additionally, *G. stearothermophilus* CECT 43 was successfully stabilized in agarose by entrapment techniques. This immobilized biocatalyst showed high operational and storage stabilities and retained full activity at low temperatures reducing the energy cost.

These results indicate that immobilized *G. stearothermophilus* CECT 43 could be used to produce DAPur nucleosides derivatives with high conversion values at short reaction times employing an environmentally friendly methodology.

Acknowledgments

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