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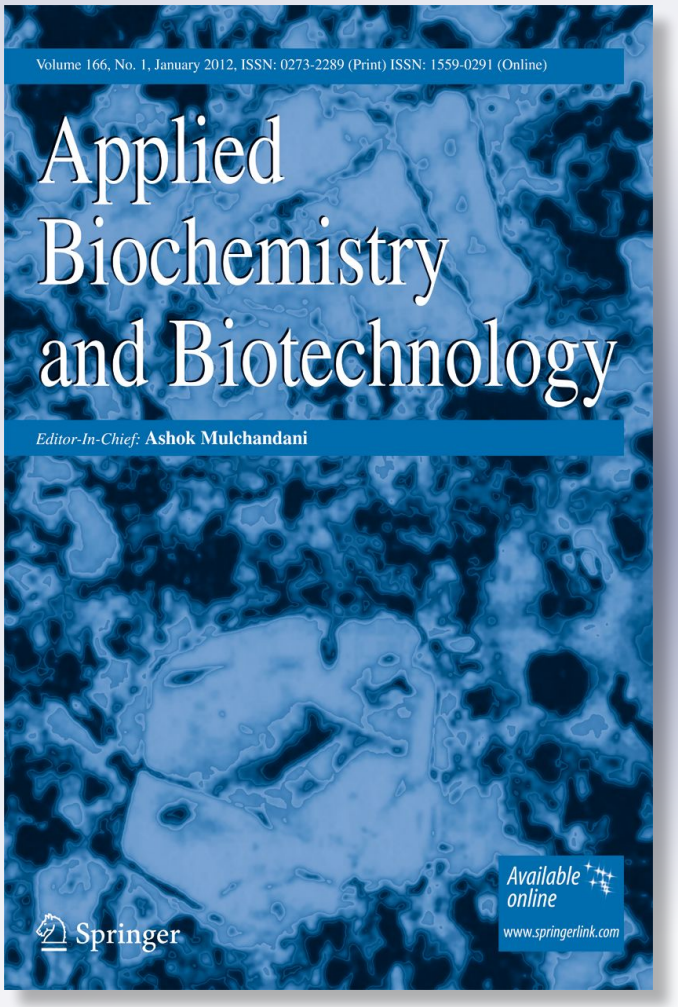

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Selection of a New Whole Cell Biocatalyst for the Synthesis of 2-Deoxyribose 5-Phosphate

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Abstract 2-Deoxyribose 5-phosphate (DR5P) is a key intermediate in the biocatalyzed preparation of deoxyribonucleosides. Therefore, DR5P production by means of simpler, cleaner, and economic pathways becomes highly interesting. One strategy involves the use of bacterial whole cells containing DR5P aldolase as biocatalyst for the aldol addition between acetaldehyde and D-glyceraldehyde 3-phosphate or glycolytic intermediates that in situ generate the acceptor substrate. In this work, diverse microorganisms capable of synthesizing DR5P were selected by screening several bacteria genera. In particular, *Erwinia carotovora* ATCC 33260 was identified as a new biocatalyst that afforded 14.1-mM DR5P starting from a cheap raw material like glucose.

Keywords 2-Deoxyribose 5-phosphate · Aldolases · 2-Deoxyribose 5-phosphate aldolase · Nucleosides · *Erwinia carotovora*

Introduction

Nucleosides analogs are widely used as antiviral and anticancer drugs, since they can act as inhibitors of viral or cellular DNA replication [1–5]. In particular, thymidine is a useful precursor in the chemical synthesis of various antiviral drugs including stavudine and zidovudine, active ingredients in formulations for the treatment of AIDS [6]. 2′-Deoxyribonucleosides (dNs) are also employed for the preparation of the corresponding triphosphates used as PCR reagents.

Since the chemical synthesis of dNs is complex due to the need of protection and deprotection steps and the absence of the anchimeric effect [7, 8], biotransformations offer an efficient and environmentally clean alternative. We have prepared purine dNs by

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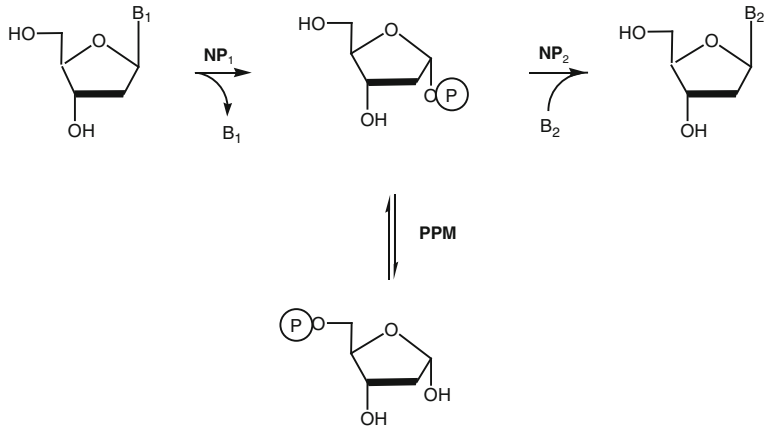
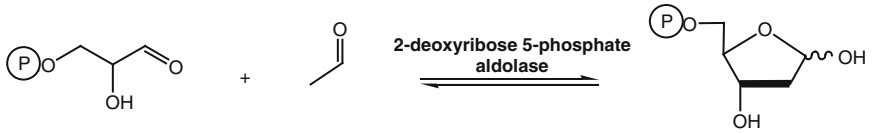
biocatalyzed transglycosylation using nucleoside phosphorylases (NPs) or whole cells that contain these enzymes in a one-pot cascade reaction [9–11]. This methodology requires pyrimidine dNs as starting materials and proceeds through the formation of the corresponding α-furanose 1-phosphate (Scheme 1). A limitation of this approach is the need of the corresponding pyrimidine analogs. A way to overcome this problem is to employ the furanose 1-phosphate intermediates as starting materials, but unfortunately their availability is limited due to the difficulty of synthesizing them and its intrinsic instability [12]. Alternatively, these compounds can be enzymatically obtained from furanose 5-phosphates employing phosphopentomutase (PPM; E.C. 5.4.2.7), an enzyme of the pentose pathway. Therefore, to prepare dNs, a strategy that involves the use of 2-deoxyribose 5-phosphate (DR5P) and the combined action of PPM and one NP was performed [13, 14]. In this process, DR5P synthesis is a key step.

Aldolases are enzymes that catalyze the aldol condensation between two carbonylic compounds by a stereoselective and reversible reaction and constitute an attractive tool in the synthesis of chiral bioactive compounds, such as carbohydrates, amino acids, and their analogs [15]. Whereas aldolases can typically use a broad range of aldehydes as acceptors, the donor compound is often structurally invariable. Hence, aldolases can be classified according to their donor specificity [16]. The class of acetaldehyde-dependent aldolases contains only one member: 2-deoxy-D-ribose-5-phosphate aldolase (DERA; E.C. 4.1.2.4). It is the only known aldolase that catalyzes the aldol reaction between two aldehydes, D-glyceraldehyde 3-phosphate (G3P) and acetaldehyde, affording DR5P [17] (Scheme 2). It has been demonstrated that this enzyme can use some alternative donors, producing 2-substituted deoxyriboses 5-phosphate. In particular, Wong et al. [18] reported the application of a recombinant DERA in the synthesis of a variety of sugar analogs, such as thiosugars and glycolipid precursors.

Howells and Lindstrom [19] used cellular extracts from *Bacillus cereus* as DERA source for DR5P production starting from fructose 1,6-diphosphate (FDP) to study acetaldehyde metabolism. Ogawa et al. [20] reported DR5P synthesis from dihydroxyacetone phosphate (DHAP) and acetaldehyde by using *Klebsiella pneumoniae* whole cells with triose phosphate isomerase and DERA activities. Horinouchi et al. [21] carried out DR5P production from glucose, acetaldehyde, and ATP. They cloned and expressed DERA's gen



Scheme 1 Deoxynucleosides synthesis by biocatalyzed transglycosylation. *NP* nucleoside phosphorylase, *PPM* phosphopentomutase, *B* purine or pyrimidine base or analogs



glyceraldehyde 3-phosphate acetaldehyde 2-deoxyribose 5-phosphate

Scheme 2 Synthesis of 2-deoxyribose 5-phosphate catalyzed by DERA from its natural substrates, D-glyceraldehyde 3-phosphate and acetaldehyde

from *K. pneumoniae* in a strain of *Escherichia coli* without phosphatase activity and used these cells as biocatalyst. This paper describes the hierarchical screening employed to find new DERA-containing bacterial whole cell biocatalysts to prepare DR5P using cheap reagents and the optimization of the reaction conditions.

Materials and Methods

Chemicals and Microorganisms

Reagents and substrates were purchased from Sigma-Aldrich or Fluka. The culture media components were obtained from Anedra, Britania, and Sigma-Aldrich. Solvents for qualitative and quantitative analyses were from Sintorgan and Biopack. Most of the microorganisms were kindly supplied by the *Colección Española de Cultivos Tipo*, Universidad de Valencia (Spain).

Screening

Primary Screening

The microorganisms (109 strains) were cultured in a liquid medium (named MC in this text) with the following composition: KH_2PO_4 0.1% (*w*/*v*), K_2HPO_4 0.1% (*w*/*v*), $MgSO_4{\cdot}7H_2O$ 0.03% (*w*/*v*), yeast extract 0.01% (*w*/*v*), NH_4Cl 0.2% (*w*/*v*), and 2-deoxyribose (DR) 0.3% (*w*/*v*), pH 7 at 28 °C and 200 rpm. Positive cultures were those ones that showed turbidity against a negative control after 48 h of growth.

Secondary Screening

The strains selected in the primary screening were cultured in their optimal liquid media at the temperature (*T*) and time (*t*) detailed in the following, according to the American Type Culture Collection (ATCC) in presence of acetaldehyde 200 mM: *Aeromonas* (*T* 30 °C, *t* 1 day), *Pseudomonas* (*T* 26 °C, *t* 1 day), *Bacillus* (*T* 30 °C, *t* 1 day), *Achromobacter* (*T* 30 °C, *t* 2 days), *Citrobacter* (*T* 37 °C, *t* 1 day), *Enterobacter* (*T* 37 °C, *t* 1 day), *Klebsiella* (*T* 37 °C, *t* 2 days), *Escherichia* (*T* 37 °C, *t* 1 day), *Proteus* (*T* 37 °C, *t* 1 day), *Xanthomonas* (*T* 26 °C, *t* 1 day), *Cellulomonas* (*T* 30 °C, *t* 1 day), *Staphylococcus* (*T* 37 °C, *t* 1 day), *Micrococcus* (*T* 30 °C, *t* 1 day), *Agrobacterium* (*T* 26 °C, *t* 2 days), and *Serratia* (*T* 26 °C, *t* 5 days) were grown in Luria broth medium; *Erwinia* (*T* 30 °C, *t* 1 day) and *Arthrobacter* (*T* 26 °C, *t* 2 days) in Agar II; *Corynebacterium* (*T* 30 °C, *t* 2 days) and *Brevibacterium* (*T* 30 °C, *t* 2 days) in *Corynebacterium* medium; *Lactobacillus* (*T* 37 °C, *t* 1 day) in de Man–Rogosa–Sharpe broth (oxoid CM359); *Streptomyces* (*T* 28 °C, *t* 5 days) in *Streptomyces* medium; and

Nocardia (*T* 30 °C, *t* 1 day) in yeast extract–malt extract (Bennett's agar) medium. Positive cultures were those ones that showed turbidity against a negative control after 48 h of growth.

Biocatalyst Preparation

Microorganisms were cultured in their optimal liquid media as above, until reaching medium exponential phase. Each culture broth (25 mL) was centrifuged at 5,000 rpm for 10 min, and the pellets were resuspended in 250 mL of MC. After 24 h at 28 °C and 200 rpm, these cultures were centrifuged as above, and the pellets were used as biocatalysts.

Biotransformations

DR5P Preparation from Glyceraldehyde 3-Phosphate (MR1)

The reaction mixture comprising 15% (*w*/*v*) biocatalyst, 98.7 mM racemic D,L-glyceraldehyde 3-phosphate, 200 mM acetaldehyde, and 200 mM Tris–HCl buffer pH 9 (final volume 1 mL) was stirred at 200 rpm and 28 °C for 3 h. Samples were centrifuged at 11,000 rpm for 3 min, and the supernatants were analyzed by thin-layer chromatography (TLC).

DR5P Preparation Using Glucose as Starting Material (MR2)

The standard reaction mixture comprising 12.5% (*w*/*v*) biocatalyst, 500 mM glucose, 15 mM $MgSO_4 \cdot 7H_2O$, 15 mM ATP, 1% (*v*/*v*) xylene, 200 mM acetaldehyde, and 100 mM potassium phosphate buffer pH 7.5 (final volume 1 mL) was stirred at 200 rpm and 28 °C for 3 h. Samples were centrifuged at 11,000 rpm for 3 min, and the supernatants were analyzed by TLC.

The optimized biotransformation involved the addition of 0.4% (*v*/*v*) Tween-20 to MR2 (MR2-T).

DR5P Preparation from Fructose 1,6-Diphosphate (MR3)

The reaction mixture comprising 13% (*w*/*v*) biocatalyst, 50% (*v*/*v*) fructose FDP solution obtained according to Horinouchi et al. [22], 400 mM acetaldehyde, and 1% (*v*/*v*) xylene (final volume 1 mL) was stirred at 200 rpm and 28 °C for 3 h. Samples were centrifuged at 11,000 rpm for 3 min, and the supernatants were analyzed by TLC.

DR5P Preparation from Dihydroxyacetone Phosphate (MR4)

The reaction mixture comprising 15% (*w*/*v*) biocatalyst, 98.7 mM DHAP, and 200 mM acetaldehyde (final volume 0.5 mL) was stirred at 200 rpm and 28 °C for 3 h. Samples were centrifuged at 11,000 rpm for 3 min, and the supernatants were analyzed by TLC.

Synthesis of Thymidine

Once DR5P (14.1 mM) was obtained using MR2-T conditions, the reaction mixture was lyophilized and resuspended in 80 mM Tris–HCl buffer pH 8 (1 mL). This solution (0.25 mL) was added with stirring (160 rpm) to a mixture (1 mL final volume) containing 6 mM thymine, 80 mM Tris–HCl buffer pH 8, 1 mM $MnCl_2$, 200 mM β-mercaptoethanol,

0.15 mL PPM (0.32 mg/mL), and 25 μL thymidine phosphorylase (TP; 1.4 KU/mL), at 37 °C. The reaction was quenched by the addition of 0.1 M HCl. Samples were centrifuged at 10,000 rpm for 3 min, and the supernatants were analyzed by HPLC in order to determine thymidine yield.

Analytical Methods

Qualitative analysis of DR5P was performed by TLC using Silicagel 60 F254 plates (Merck, Rahway, NJ, USA). The mobile phase consisted of *n*-butanol, 2-propanol, and H_2O, 3:12:4 (*v*/*v*/*v*). DR5P was detected (Rf 0.25), using 1% (*v*/*v*) anisaldehyde and 2% (*v*/*v*) H_2SO_4 in acetic acid, as a purple spot. Quantitative analysis of DR5P was performed by the chemical colorimetric procedure described by Burton [23].

HPLC analysis was performed using a C-18 column (150×4 mm) at a flow rate of 0.9 mL min^{-1}. The UV detector was set at 254 nm, and the column was operated at room temperature. The operating condition to analyze ATP permeability was 12-min 100-mM triethylammonium acetate buffer pH 7/acetonitrile (97:3 *v*/*v*) and to quantify thymidine production 15 min water/acetonitrile (90:10 *v*/*v*).

Results and Discussion

DERA is a key enzyme involved in the pentose metabolism and is induced by DR [24, 25]. To select microorganisms containing DERA activity, a hierarchical screening was followed. A primary screening was carried out using MC (see "Materials and Methods") as culture medium, which contained DR as the sole carbon source. DR was metabolized by 41 strains by different species of the genera detailed in "Materials and Methods".

A secondary screening was performed to select those strains that could tolerate the high acetaldehyde concentration required for all assayed biotransformations in order to shift the equilibrium toward the condensation reaction. The strains selected in the primary screening were cultured in their optimum media and temperature, in the presence of 200 mM acetaldehyde. The microorganisms that bore this condition belonged to *Lactobacillus*, *Erwinia*, and *Streptomyces* genera (Table 1).

The selected bacteria were employed as biocatalysts for DR5P synthesis using conditions described as MR1 (see "Materials and Methods"), being racemic G3P and acetaldehyde the starting materials. Among them, *Streptomyces griseus*, *Lactobacillus plantarum*, *Erwinia carotovora* ATCC 33260, and *E. carotovora* ATCC 15713 produced the expected product (Table 1). The other *Streptomyces* strains produced DR instead of DR5P probably due to phosphatase activities.

G3P is a natural metabolite involved in glycolysis; therefore, in order to use cheaper starting materials, it is feasible to start from other glycolytic pathway intermediates [21] (Scheme 3). In this way, the chosen microorganisms were used as biocatalysts for DR5P synthesis using the conditions described in the experimental section as MR2. This reaction mixture comprised ATP and $MgSO_4$ at catalytic amounts to improve G3P synthesis from glucose and xylene to enhance the ATP permeability of the cells. TLC analysis showed that *E. carotovora* ATCC 33260 was the unique strain able to produce only DR5P. *E. carotovora* ATCC 15713 produced DR in addition to DR5P and *Streptomyces griseostramineus* only formed DR (Table 1), again probably due to phosphatase activity. The intermediates generated in glucose metabolism may take part in other metabolic processes which could explain the negative results observed for the rest of the strains.

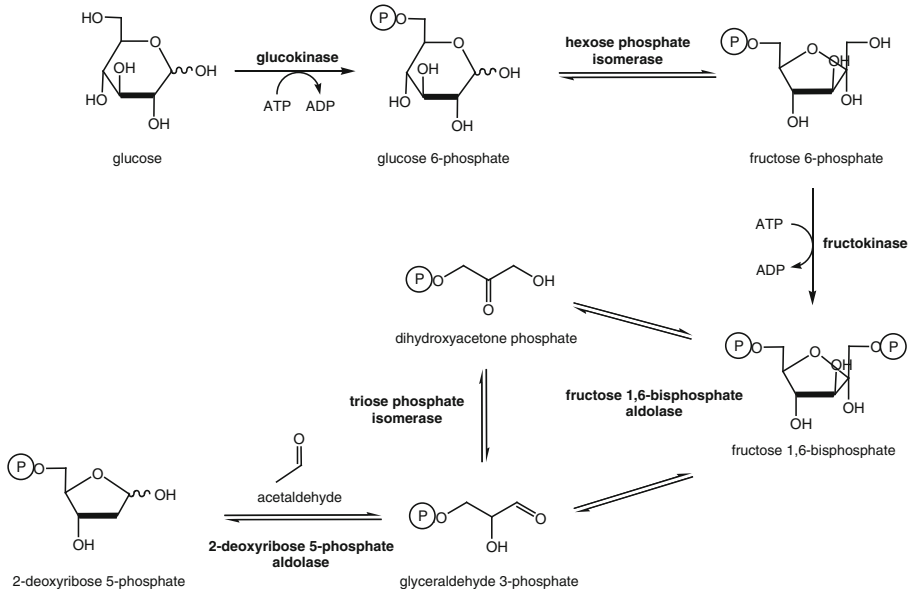
Table 1 Biocatalyzed synthesis of DR5P from different starting materials

	G3P		Glucose		FDP		DHAP	
	DR presence	DR5P (mM)	DR presence	DR5P (mM)	DR presence	DR5P (mM)	DR presence	DR5P (mM)
Streptomyces griseostramineus ATCC 19768	+	–	+	–	–	–	N/D	N/D
Streptomyces griseus ATCC 10137	–	7.5	–	–	–	9.8	N/D	N/D
Streptomyces baldaccii ATCC 27429	+	–	–	–	–	3.5	N/D	N/D
Streptomyces cattleya ATCC 35852	+	–	–	–	–	6.4	N/D	N/D
Streptomyces sp. ATCC 11238	+	–	–	–	–	–	N/D	N/D
Erwinia carotovora ATCC 33260	–	10.1	–	8.6	–	9.7	–	10.5
Erwinia carotovora ATCC 15713	–	9.9	+	17.4[a]	–	4.7	–	7.2
Lactobacillus plantarum ATCC 14917	–	7.8	–	–	–	7.1	N/D	N/D

[a] The amount corresponds to DR+DR5P

N/D not-determined

With the aim of evaluating another glycolytic metabolite, a simple methodology previously reported for FDP production was employed [22]. The reaction mixture containing FDP from baker's yeast was directly used as the starting material for the



Scheme 3 Metabolic pathway for D-glyceraldehyde 3-phosphate generation from glucose

biotransformation with all the microorganisms selected in the secondary screening, employing the conditions described in the experimental section as MR3. Since DHAP, FDP, and G3P take part of a metabolic pool (Scheme 3), G3P consumption catalyzed by DERA produces a displacement of the metabolic flow through a larger decondensation of FDP. Six strains were identified as positives for DR5P synthesis while DR was not observed probably because of the presence of phosphatase inhibitors in yeast mixture.

Finally, DHAP was employed as starting material for G3P in situ generation using the conditions describes as MR4. The microorganisms employed as biocatalysts were only those ones that produced DR5P in all of the previous biotransformations: *E. carotovora* ATCC 33260 and *E. carotovora* ATCC 15713. As expected, all of them formed DR5P since the DHAP isomerization shifts to G3P formation (Scheme 3).

Summarizing the results shown in Table 1, *E. carotovora* strains were the most versatile biocatalysts based on the fact that they generated DR5P from all the starting materials tested, affording product concentrations higher than 5 mM. *L. plantarum* and *S. griseus* produced DR5P only from G3P and FDP, while *Streptomyces baldaccii* and *Streptomyces cattleya* were able to afford DR5P only from FDP and gave DR instead of DR5P using MR1. Employing *S. griseostramineus* or *Streptomyces* sp. as biocatalysts only DR was obtained.

Considering the cost of starting materials and the ease and simplicity of the experimental conditions, glucose (MR2) was the G3P precursor chosen for the biotransformation, and *E. carotovora* ATCC 33260 was finally selected as a potentially useful biocatalyst to further explore its properties. *E. carotovora* ATCC 15713 was ruled out because of the lack of a proper DR5P quantification method.

In order to optimize biotransformation conditions, the influence of induction and reaction times was evaluated using the selected biocatalyst. *E. carotovora* ATCC 33260 was cultured in its optimum medium and subcultured in MC. First, the incidence of the induction time over DERA production was assessed at 0, 12, 24, 48, and 72 h. Next, in order to identify the best reaction time, each fraction was used as biocatalyst using the conditions described as MR2, and samples were taken at 0.5, 1, 2, 3, and 4 h. TLC analysis demonstrated that 24-h induced cells afforded the best yield of DR5P after 3 h of reaction.

The use of non-ionic detergents has been previously reported to improve cell permeation [20]. Therefore, Tween-20, Triton X-100, and Nonidet P-40 were tested to increase ATP entrance into *E. carotovora* ATCC 33260 whole cells using MR2 conditions. The results showed that Tween-20 and Nonidet P-40 were able to enhance ATP permeability affording higher yields (14.1 and 10.6 mM, respectively) (Table 2). Furthermore, HPLC analysis demonstrated the decrease of ATP concentration and the presence of larger quantities of adenosine diphosphate, monophosphate, adenosine and adenine compared to the biotransformation without surfactants, due to ATP cell metabolism that involves kinases and nucleoside phosphorylases. In the experiments performed with the addition of Triton X-100, a turbidity of the reaction media was systematically observed. This fact together with the lower yields achieved suggests that cell disruption took place producing the consequent partial loss of activity.

Table 2 Effect of surfactants on DR5P production with *E. carotovora* ATCC 33260 as biocatalyst. Surfactant 0.4% (*v*/*v*) was added to MR2 condition

Surfactant	DR5P (mM)
None	8.6
Tween-20	14.1
Triton X-100	6.5
Nonidet P-40	10.6

Finally, nucleoside synthesis using the coupled action of DERA, an over-expressed PPM [14], and a commercially available thymidine phosphorylase was carried out. Employing thymine and DR5P obtained with the selected biocatalyst and MR2-T medium, 3.5 mM thymidine (100% yield based on DR5P) was achieved.

As far as we know, only one previous report used MR2 conditions employing as whole cell biocatalyst the microorganism *K. pneumoniae* [21]. They constructed a *K. pneumoniae* DERA-expressing alkaline phosphatase-defective *E. coli* 10B5 cell and obtained 6.3 mM of DR5P from MR2 and 12.3 mM from MR3. Using DR5P prepared from MR3 and adenine, deoxyinosine (9.9 mM, 80.2% yield based on DR5P) was synthesized after treatment with PPM and purine nucleoside phosphorylase [26]. In our case, using a non-pathogenic wild-type microorganism *E. carotovora* ATCC 33260, under MR2-T condition, 14.1-mM DR5P was obtained and thymidine was achieved in 100% yield based on DR5P after the action of PPM and TP in presence of thymine.

Conclusions

In this paper, a hierarchical screening allowed the identification of strains belonging to *Lactobacillus*, *Erwinia*, and *Streptomyces* genera with DERA activity, and *E. carotovora* ATCC 33260 was identified as a potentially useful biocatalyst. Different substrates for in situ G3P generation were assessed to carry out the synthesis of DR5P, and the condition MR2-T that uses glucose as starting material resulted as the most appropriate in terms of cost and handling.

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