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Synthetic Activity of Recombinant Whole Cell Biocatalysts Containing 2-Deoxy-D-ribose-5-phosphate Aldolase from *Pectobacterium atrosepticum*

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In nature 2-deoxy-D-ribose-5-phosphate aldolase (DERA) catalyses the reversible formation of 2-deoxyribose 5-phosphate from D-glyceraldehyde 3-phosphate and acetaldehyde. In addition, this enzyme can use acetaldehyde as the sole substrate, resulting in a tandem aldol reaction, yielding 2,4,6-trideoxy-D-erythro-hexapyranose, which spontaneously cyclizes. This reaction is very useful for the synthesis of the side chain of statin-type drugs used to decrease cholesterol levels in blood. One of the main challenges in the use of DERA in industrial processes, where high substrate loads are needed to achieve the desired productivity, is its inactivation by high acetaldehyde concentration. In this work, the utility of different variants of *Pectobacterium atrosepticum* DERA (*Pa*DERA) as whole cell biocatalysts to synthesize 2-deoxyribose 5-phosphate and 2,4,6-trideoxy-D-erythro-hexapyranose was analysed. Under optimized conditions, *E. coli* BL21 (*Pa*DERA C-His AA C49M) whole cells yields 99% of both products. Furthermore, this enzyme is able to tolerate 500 mM acetaldehyde in a whole-cell experiment which makes it suitable for industrial applications.

Introduction

Aldolases are enzymes that belong to the group of lyases and catalyse the aldol addition between two carbonylic compounds, through a reversible and stereoselective reaction producing chiral β-hydroxy carbonyl compounds (aldols)[1] such as carbohydrates, amino acids, and their analogues.[2] Whereas aldolases can typically use a wide range of aldehydes as acceptors, donor compounds are often structurally invariable.[3] Hence, aldolases can be classified according to their donor specificity.[4] The acetaldehyde-dependent aldolases class contains only one member: 2-deoxy-D-ribose-5-phosphate aldolase (DERA; E.C.4.1.2.4), which is one of the few known aldolases that catalyses the aldol addition between two aldehydes. DERA naturally catalyses the reversible formation of 2-deoxyribose 5-phosphate (DR5P) from D-glyceraldehyde 3-phosphate (G3P), as acceptor, and acetaldehyde as donor[5–7] (Scheme 1a). DR5P has been used as a key intermediate in the biocatalytic preparation of deoxyribonucleosides and their analogues.[8,9] Therefore, DR5P production by simple, clean, and inexpensive routes has become highly interesting.

Mechanistically, DERA is a Class I aldolase, depending on a lysine-mediated enamine formation. It is the only aldolase that accepts three aldehydes in a tandem stereoselective aldol

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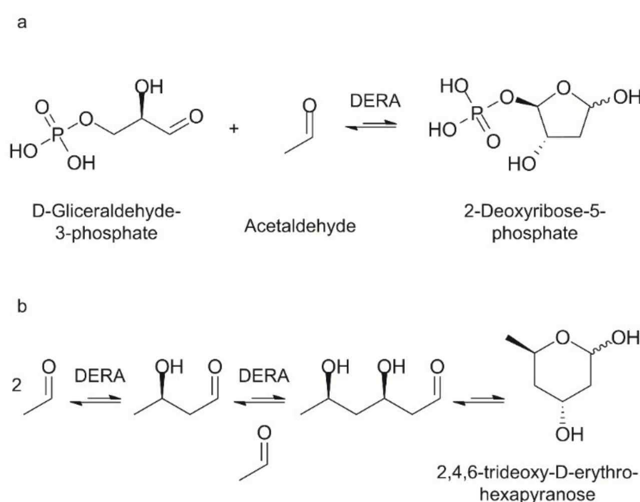

Scheme 1. a) Natural reaction catalysed by DERA. b) Synthetic DERA catalysed tandem reaction.

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addition; the first aldol product can be recognized as a new acceptor substrate for the second reaction (Scheme 1b).

The 2,4,6-trideoxy-D-erythro-hexapyranose obtained by this double aldol addition can be used, after oxidation to the corresponding lactone, as a side-chain precursor in the synthesis of statin-type cholesterol-lowering drugs.[10,11] The advantage of using this biocatalytic strategy is that it provides the two key chiral centres for the hypocholesterolemic activity of these drugs, with high stereochemical purity.[12] Greenberg et al. synthesized (3*R*,5*S*) 6-chloro-2,4,6-trideoxy-erythro-hexose, as a potential statin precursor, through an interesting and cheap process using an improved DERA, selected by screening of genomic libraries from environmental DNA.[10] Oslaj et al. reported an asymmetric aldol addition process using recombinant *E. coli* DERA whole-cells.[13] These authors used a chemo-enzymatic process to demonstrate, for the first time, that statins can be directly assembled from the lactonized side-chain.[14,15] In addition, Wong et al. reported the large-scale preparation and purification of DERA from a recombinant *E. coli* DH5α strain and its application to the synthesis of a variety of sugar analogues, such as thiosugars and glycolipid precursors.[16] They also used the sequential aldol addition for the synthesis of 2,4-dideoxy and 2,4,6-trideoxyhexoses from different substrates.[17]

A high acetaldehyde concentration is known to inhibit the activity of DERA.[18] The double addition of acetaldehyde generates, as a side reaction, an α,β unsaturated imine with lysine 167 which forms a Michael adduct with cysteine 47. As a result of the formation of this Schiff base intermediate, DERA is irreversibly inhibited.[19] This inhibition can be mitigated by mutating the cysteine 47 to a non-nucleophilic or bulky amino acid, as demonstrated by Jennewein et al.[20] They reported a mutant *E. coli* DERA (C47M) achieved by site-directed mutagenesis, with high tolerance and catalytic efficiency for acetaldehyde and also chloroacetaldehyde. Furthermore, Jiao et al. identified a new DERA from *Lactobacillus brevis* with high activity, good thermostability and tolerance to high concentrations of aldehyde substrates.[21] They modified the enzyme by changing glutamate 78 to lysine, which also provided a significant improvement in both thermostability and aldehyde tolerance. Following this strategy, different recombinant DERAs have been reported with the aim of increasing their tolerance to high aldehyde concentrations.[22]

DERA has been shown to have a low preference for non-phosphorylated substrates.[23] In order to overcome this, De Santis et al., prepared a mutant of *E. coli* DERA (S238D) with improved activity towards 2-deoxy-D-ribose.[24] In addition, Li et al. developed a *Klebsiella pneumoniae* DERA (*Kp*DERA) with higher activity and substrate tolerance towards D-glyceraldehyde, incorporating the mutations F200I and M185V to enhance hydrophobicity in a region of the catalytic centre.[25]

In previous work, we reported a hierarchical screening for DERA activity that allowed us to select the *Pectobacterium atrosepticum* ATCC 33260 strain for its resistance to high acetaldehyde concentrations.[26] Furthermore, this strain proved to be an efficient whole cell biocatalyst for the preparation of DR5P from G3P and also from glucose, a cheap precursor to G3P via the glycolytic pathway. In this paper, we report the use of different recombinant *E. coli* strains containing *P. atrosepticum* DERA with higher activity and aldehyde tolerance than *P. atrosepticum* wild type, with the aim of improving the whole cell biocatalysed synthesis of DR5P and 2,4,6-trideoxy-D-erythro-hexapyranose.[27]

Results and Discussion

DR5P production

DERA, like all aldolases, has attracted much interest due to its ability to catalyse the efficient C–C bond formation with a stereospecific control that is regulated by the enzyme regardless of substrate structure.[4] In particular, DERA recognizes in addition to G3P other aldehydes as acceptors, making it useful for the *de novo* synthesis of 2-deoxysugars.[25] The use of DERA in this synthetic direction involves increasing aldehyde substrate amounts to shift the equilibrium towards product formation. However, this approach is limited by the enzyme inhibition due to high aldehyde concentrations. Therefore, in the last decades considerable attention has been paid to the search for new aldolases from a large diversity of microorganisms with enhanced acetaldehyde resistance.[28,29,22]

In a previous work we selected DERA from *P. atrosepticum* (*Pa*DERA) as an efficient wild-type bacterial whole cell biocatalyst for its ability to synthesize DR5P at up to 200 mM of acetaldehyde.[26] In addition, DR5P was further employed in an one-pot multistep enzymatic synthesis to prepare thymidine.[30] In this paper we searched for more efficient DERA variants in order to scale the synthesis of DR5P and to explore the synthesis of deoxynucleoside analogues. The following recombinant *Pa*DERA variants were tested:[27] *Pa*DERA containing *N*-terminal 6×His-tag (*Pa*DERA N-His), *Pa*DERA carrying a C-terminal 6×His-tag (*Pa*DERA C-His), *Pa*DERA containing C-terminal 6×His-tag and 5 amino acid spacer (*Pa*DERA C-His AA) and *Pa*DERA including C-terminal 6×His-tag, the 5 amino acid spacer and the mutation C49M (*Pa*DERA C-His AA C49M). The latest amino acid exchange, equivalent to C47M in *E. coli* DERA (*Ec*DERA), has proved to enhance acetaldehyde resistance by preventing crotonaldehyde, the by-product of the aldol condensation of acetaldehyde, from bridging the catalytically active lysine 169 to the nearby cysteine 49.[18] After purification, the activity of the enzymes was determined by the standard DERA assay based on DR5P hydrolysis. *Pa*DERA N-His showed lower activity and a poorer expression level than *Pa*DERA C-His variants while, among the different *Pa*DERA C-His variants, *Pa*DERA C-His AA C49M displayed the best catalytic efficiency and acetaldehyde resistance.[27] In particular, *Pa*DERA C-His AA C49M exhibited the highest expression level and slightly more than 3 times the activity of *Pa*DERA N-His. However, the storage and operational stability of *Pa*DERA variants, both as purified enzyme and cell free extracts, were low and the obtained results in DR5P synthesis were neither good nor reproducible (data not shown). Therefore, we decided to continue the experiments using whole cells.

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Employing the previously optimized experimental conditions,[25] we carried out the synthesis of DR5P from both G3P and glucose using as biocatalysts the recombinant strains *E. coli* BL21(*Pa*DERA N-His) and *E. coli*BL21(*Pa*DERA C-His AA C49M), and *P. atrosepticum* wild type (*Pa*WT). When G3P was the acceptor, the biotransformations were followed for 4 hours in the presence of 200 mM of the donor substrate, acetaldehyde (Table 1, entries 1, 4 and 7). *Pa*WT afforded 0.13 mol DR5P/mol G3P and *E. coli* BL21(*Pa*DERA N-His) produced 0.43 mol DR5P/mol G3P after 3 h and 4 h respectively, while using *E. coli* BL21(*Pa*DERA C-His AA C49M), 0.99 mol DR5P/mol G3P were obtained after 2 h. The increase in the biocatalysed reaction rate when *E. coli* BL21(*Pa*DERA C-His AA C49M) was used may be due to the higher level of overexpression of the enzyme, 50% more than in *E. coli* BL21(*Pa*DERA N-His). Additionally, a faster reaction probably favours the increase in yield by avoiding the consumption of G3P by metabolic reactions of the whole cells. Notably, the amount of DR5P achieved using *E. coli* BL21(*Pa*DERA C-His AA C49M) as whole cell biocatalyst is the highest yield reported as far as we know.

Since G3P is a natural metabolite that can be obtained in situ within the cells via the glycolytic pathway,[31] glucose was used as an inexpensive starting material. In this experiment, 42.6 mM of DR5P was achieved when *E. coli* BL21(*Pa*DERA N-His) was the biocatalyst and 23.4 mM when *Pa*WT was used. Nevertheless, unexpectedly, the DR5P production by *E. coli* BL21(*Pa*DERA C-His AA C49M) was negligible (Table 1, entries 2, 5 and 8). In addition, in these biotransformations 2,4,6-trideoxy-D-erythro-hexapyranose was obtained but with a yield of less than 10%. The occurrence of this compound is in agreement with the previously mentioned ability of DERA to catalyse a sequential addition of acetaldehyde and the spontaneous formation of a stable cyclic hemiacetal (Scheme 1b).[27,17] The poor yield of DR5P formed in the reaction catalysed by *E. coli* BL21(*Pa*DERA C-His AA C49M) could be a consequence of the lower initial availability of the substrate -G3P- since it must first be formed from glucose. Furthermore, the high expression of an exogenous protein could result in a decrease in the metabolic rate of *E. coli* and therefore, the higher expression level of *E. coli* BL21(*Pa*DERA C-His AA C49M) with respect to the variant N-His could further slow the conversion of glucose to G3P. In a further experiment, prior to the addition of acetaldehyde to the reaction medium, *E. coli* BL21(*Pa*DERA C-His AA C49M) cells were pre-incubated with glucose for 30 min, 1 and 3 h. Only a small increase (less than 10%) was observed in the initial rate (Figure 1). This behaviour could be due to the fact that the G3P formed, when DERA is not present, is redirected to other metabolic pathways and the remaining amount of G3P is not enough to favour aldol addition. Another possibility is that glucose, its metabolic intermediates or any other component of the glucose medium interact with DERA inhibiting its activity.

Table 1. DERA biocatalysed DR5P and 2,4,6-trideoxy-erythro-hexapyranose production.

	Biocatalyst (whole cells)	Acceptor substrate	DR5P[a]	Trideoxy-D-erythro-hexapyranose[b]	Time[c]
1	*Pa*WT	glyceraldehyde 3-phosphate[d]	13.2	n.d.	3
2		glucose[e]	23.4	n.d.	3
3		acetaldehyde[f]	–	n.d.	24
4	*E.coli* BL21 (*Pa*DERA N-His)	glyceraldehyde 3-phosphate[d]	42.9	n.d.	4
5		glucose[e]	42.6	0.1	4
6		acetaldehyde[f]	–	1.6	24
7	*E. coli* BL21 (*Pa*DERA C-His AA C49M)	glyceraldehyde 3-phosphate[d]	98.1	n.d.	2
8		glucose[e]	2.3	5.4	7
9		acetaldehyde[f]	–	33.1	6

[a,b] mM. [c] Hours. Reactions were performed in a final volume of 1 mL using: [d] 98.3 mM of G3P and 200 mM of acetaldehyde. [e] 500 mM of glucose, 15 mM of $MgSO_4 \cdot 7H_2O$, 15 mM of ATP, 1% (v/v) of xylene and 200 mM of acetaldehyde. [f] 100 mM of acetaldehyde. n.d.: no detected.

It is known that modifications at the C-terminal end of DERA produce changes in the activity of the enzyme. Structural studies have shown that the wide substrate tolerance range of DERA is related to the C-terminus which is shorter and more flexible than that of other classes of aldolases.[32] This C-terminal tail generates an equilibrium between an open and a closed state. In the closed state, the C-terminal tyrosine enters to the active site to participate in a catalytic deprotonation step, while the open state allows substrate binding and product release.[33] Furthermore, Schulte et al., used NMR spectroscopy and molecular dynamics simulations to demonstrate the existence of auxiliary phosphate binding residues in the *Ec*DERA C-terminal tail that could facilitate the orientation of phosphorylated substrates in the optimal position for catalysis.[33] This could be the reason why the catalytic efficiency of *Ec*DERA is drastically reduced for non-phosphorylated substrates. However, this is more important in the retro-aldol reaction of cyclic hemiacetals since the presence of the phosphate group would act as an affinity tag that facilitates the binding to the active site and the catalysed opening of the ring, necessary to generate the reactive aldehyde.[34] These mechanistic characteristics, in fact, favour the stability of the cyclic hemiacetal formed with non-phosphorylated aldehydes. In a recent report, Kim et al. designed a DERA for the biosynthesis of (*R*)-1,3-butanediol

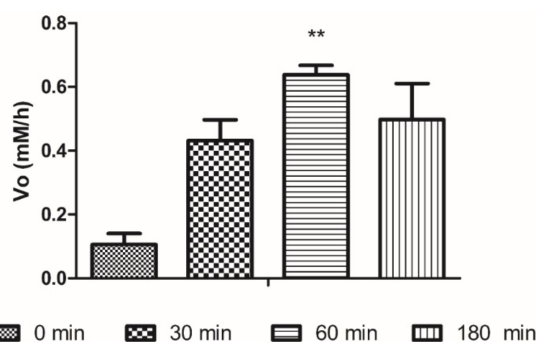

Figure 1. Variation of DERA activity for DR5P synthesis using *E. coli* BL21(*Pa*DERA C-His AA C49M) by the addition of acetaldehyde at different times after incubation with glucose. Results are expressed as mean ± SD one-way ANOVA followed by the Bonferroni's Multiple Comparison Test.

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incorporating modifications near the C-terminus achieving that the acetaldehyde condensation reactions of this mutant enzyme were not affected while its activity for the synthesis of DR5P was greatly reduced.[35]

In order to check the effect of glucose medium on DERA activity in *E. coli* BL21(*Pa*DERA C-His AA C49M) cells, additional experiments were carried out. After incubation with glucose medium for 30 min, a fresh solution of G3P and acetaldehyde was added and the reaction was followed by 7 h. Under these conditions only slightly more than 4 mM of DR5P was obtained, unlike the result obtained in the absence of glucose medium (Table 1, entry 7). In a similar experiment, but incubating the *E. coli* BL21(*Pa*DERA C-His AA C49M) cells with glucose without the rest of the components of the glucose medium, DR5P was not formed, as expected. Finally, the activity of the purified enzyme was tested in the presence of glucose. The retro-aldol reaction of DR5P was also affected since 50% reduction of activity was determined. These results support the hypothesis of the inhibition of DR5P synthesis of this variant by glucose. However, further studies are required to elucidate the structural features responsible for the different activity of *E. coli* BL21(*Pa*DERA N-His) and *E. coli* BL21(*Pa*DERA C-His AA C49M) in presence of glucose.

2,4,6-Trideoxy-D-erythro-hexapyranose production

As mentioned above, DERA has been used for the production of key chiral side chain intermediates of statins due to its ability to form cyclic hemiacetals with two chiral centres starting from acetaldehyde and a wide variety of non-chiral aldehydes.[10]

E. coli BL21(*Pa*DERA N-His) and *E. coli* BL21(*Pa*DERA C-His AA C49M) were tested to carry out the synthesis of 2,4,6-trideoxy-D-erythro-hexapyranose using acetaldehyde as both the donor and acceptor substrate (Table 1, entries 6 and 9). In a previous work, we reported the synthesis of 2,4,6-trideoxy-D-erythro-hexapyranose using 100 mM acetaldehyde as both acceptor and donor substrate and 3 $mg\,mL^{-1}$ *E. coli* BL21(*Pa*DERA C-His AA C49M) whole cells achieving 50% yield in 48 h.[27] In order to improve productivity, the synthesis was optimized by increasing the biocatalyst load to 150 $mg\,mL^{-1}$. Using *E. coli* BL21(*Pa*DERA N-His) as biocatalyst, 4.8% yield of 2,4,6-trideoxy-erythro-hexapyranose was obtained in 24 h while when *E. coli* BL21(*Pa*DERA C-His AA C49M) was used, the 2,4,6-trideoxy-D-erythro-hexapyranose formation proceeded much faster (6 h) and in 99% yield. Taking into account these results, *E. coli* BL21(*Pa*DERA C-His AA C49M) was selected to continue with the studies of production of 2,4,6-trideoxy-D-erythro-hexapyranose.

To confirm the aldehyde tolerance of the enzyme in a whole cell-experiment, the synthesis was performed testing acetaldehyde concentration from 100 to 500 mM. The biotransformation was followed for 48 h and the time courses of reactions are shown in Figure 2. *E. coli* BL21(*Pa*DERA C-His AA C49M) whole cells showed excellent activity in the presence of up to 500 mM of acetaldehyde, achieving same quantitative yields albeit slower than using 100 mM. In addition, this

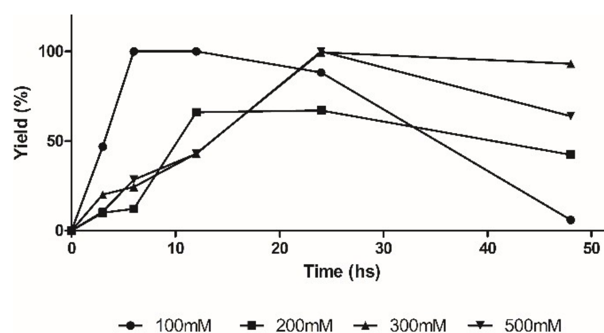

Figure 2. Time course of 2,4,6-trideoxy-D-erythro-hexapyranose formation using different initial acetaldehyde concentrations (100 mM–500 mM).

biocatalyst was more tolerant to acetaldehyde than the free enzyme (500 vs 300 mM).[27] This result is in line with that reported by Li et al.,[25] who also observed improved tolerance to acetaldehyde when using *E. coli* BL21(*Kp*DERA12) whole cells instead of free enzyme. In view of an industrial process, these results entail an advantage in cost and quantity of product since 22 g of 2,4,6-trideoxy-D-erythro-hexapyranose/L of reaction were obtained, the highest concentration of this product reported to date employing whole cells.

The inhibitory effect of glucose was also analysed and confirmed in this biotransformation. After 30 min of *E. coli* BL21(*Pa*DERA C-His AA C49M) whole cells incubation with glucose, 100 mM of acetaldehyde was added. A strong decrease in the 2,4,6-trideoxy-D-erythro-hexapyranose production was observed, only negligible amounts were obtained.

Finally, in a preliminary experiment, different non-phosphorylated substrates such as propionaldehyde and phenylacetaldehyde were tested as acceptor substrates of *E. coli* BL21(*Pa*DERA C-His AA C49M) whole cells, affording the corresponding 6-substituted 2,4,6-trideoxy-D-erythro-hexapyranoses (Figure S5). An exhaustive analysis of the substrate specificity of this biocatalyst is in progress.

Conclusion

The use of a whole cell biocatalyst is an economical approach, especially when enzymes with limited stability such as DERA are involved. Two recombinant variants of *Pectobacterium atrosepticum* ATCC 33260 DERA (*Pa*DERA) were prepared by cloning and expression in *E. coli* BL21. *Pa*DERA N-His contains a 6×His N-terminal tag while *Pa*DERA C-His AA C49M includes a 6×His C-terminal tag, a 5 amino acid spacer and the C49M mutation. *E. coli* BL21(*Pa*DERA N-His) was used as a whole cell biocatalyst to prepare DR5P from G3P and glucose achieving acceptable yields. On the other hand, *E. coli* BL21(*Pa*DERA C-His AA C49M) provided an excellent yield of DR5P but only from G3P. Furthermore, this latter biocatalyst was the best for preparing 2,4,6-trideoxy-D-erythro-hexapyranose by a tandem aldol addition of acetaldehyde. 99% Yields were obtained even at 500 mM acetaldehyde concentration, which makes this biocata-

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lyst promising to be used in the large scale synthesis of statin precursors, drugs involved in the reduction of blood cholesterol levels.

Experimental Section

Chemicals and microorganisms: Reagents and substrates were purchased from Sigma-Aldrich. The culture media components were obtained from Anedra, Britania and Sigma-Aldrich. Solvents for qualitative and quantitative analyses were from Sintorgan and Biopack. *Pectobaterium atrosepticum (*ATCC 33260) was kindly supplied by the Colección Española de Cultivos Tipo, Universidad de Valencia (Spain). *Escherichia coli* DH5α (Invitrogen) and *E. coli* BL21 (DE3) (ATCC 47092, acid phosphatase deficient) strains were used in cloning and expression experiments.

Culture conditions: *Pectobaterium atrosepticum* wild type was cultured in YDC liquid medium ($CaCO_3$ 2% (w/v), glucose 2% (w/v) and yeast extract 1% (w/v), pH 7.2) at 28 °C and 200 rpm until reaching mid-exponential phase. The culture broth (25 mL) was centrifuged at 5000 rpm for 10 min, and the pellet was resuspended in 250 mL of induction medium (MI: 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 for 24 h. The bacterial cells were harvested by centrifugation (5000 rpm, 10 min, 4 °C), washed with 0.1 M potassium phosphate buffer pH 7, recentrifuged and used as biocatalyst.

Plasmids encoding *P. atrosepticum* DERA (*Pa*DERA containing N-terminal 6×His-tag (*Pa*DERA N-His), *Pa*DERA containing C-terminal 6×His-tag (*Pa*DERA C-His), *Pa*DERA containing C-terminal 6×His-tag and a 5 amino acid spacer (*Pa*DERA C-His AA) and *Pa*DERA including C-terminal 6×His-tag, the 5 amino acid spacer and the mutation C49M (*Pa*DERA C-His AA C49M)[27] were transformed into chemically competent *E. coli* BL21(DE3) for expression. Each recombinant *E. coli* strain was grown in LB medium supplemented with kanamycin 30 $\mu g\,mL^{-1}$, at 37 °C and 200 rpm, overnight. The pre-cultures (25 mL) were then used to inoculate a fresh LB medium containing kanamycin 30 $\mu g\,mL^{-1}$ (25O mL) and cultured at 37 °C, 200 rpm until OD_{600} reached 0.6–0.8; at which point the protein expression was induced with isopropyl-β-thiogalactoside (IPTG, final concentration 0.1 mM). The cultures continued growing at 28 °C and 180 rpm overnight. Subsequently, the cells were harvested by centrifugation (5000 rpm, 10 min, 4 °C), washed with 0.1 M KPi buffer pH 7, recentrifuged and used as biocatalyst (2.5×10^9 cells mg^{-1} wet weight).

Protein purification: Cells were resuspended in 0.1 M KPi buffer pH 7 to achieve a final concentration of 30% w/v and further disrupted by ultrasonication using the Vibra-cell disruptor, VCX130 (Sonics, USA), at 50% amplitude for 5 cycles at 4 °C. The cell free extract (CFE) was obtained as supernatant after centrifugation at 4 °C and 9000 rpm for 15 min.

For all the purifications, 20 μl of Ni Sepharose TM 6 Fast Flow (obtained from GE Life Science) and 200 μl of CFE were used. The elution was carried out with a gradient of imidazole (5 mM–500 mM) in 0.1 M KPi buffer pH 7. Relevant fractions of the eluted proteins were combined and desalted using a PD MidiTrap G-10 column and 2 mL of 0.1 M KPi buffer pH 7.

The protein purity was analysed by SDS-PAGE using 15% acrylamide resolving gels running at 130 V for 30 min. The gels were stained with Coomassie Brilliant Blue R250 and destained. Molecular weights were estimated by comparison with the migration rates of ARCOIRIS Pre-stained Protein Marker (PB-L, Argentina) (Figure S1). The protein concentration was determined by the Bradford assay.[36] *Pa*DERA N-His: 0.66 $mg\,ml^{-1}$ CFE and *Pa*DERA C-His AA C49M: 0.98 $mg\,ml^{-1}$ CFE.

DERA activity assay: The hydrolysis activity of *Pa*DERA N-His and *Pa*DERA C-His AA C49M was determined measuring the oxidation of NADH as described previously.[37] The assay mixture contained 100 mM KPi buffer pH 7, 0.2 mM NADH, 0.4 mM DR5P, 4 μL of α-glycerophosphate dehydrogenase/triosephosphate isomerase mixture (α-GDH-TPI) and 5 μL of freshly purified DERA, in a final volume of 0.5 mL. The reaction was initiated by the addition of DERA and the subsequent decrease of NADH concentration was monitored at 340 nm for 1 min at 28 °C with continuous shaking. 1 U was defined as the amount of enzyme required for cleavage of 1 μmol of DR5P per minute. *Pa*DERA N-His: 9.2 $U\,mg^{-1}$ and *Pa*DERA C-His AA C49M: 28.9 $U\,mg^{-1}$.

Biotransformations

Whole-cell biotransformation of G3P to DR5P: The reaction mixture containing whole-cell biocatalyst 15% (w/v), G3P 98.7 mM and acetaldehyde 200 mM in 100 mM KPi buffer pH 7 (final volume 1 mL), was stirred at 200 rpm and 28 °C for 3 h. Samples were collected every 30 min, centrifuged at 11,000 rpm for 3 min, and the supernatants were qualitatively analysed by TLC against commercial standards (Figure S2) and quantified through a colorimetric assay, as described below.

Whole-cell biotransformation of glucose to DR5P: The reaction was performed at 28 °C and 200 rpm for 7 h using biocatalyst 15% (w/v), glucose medium (glucose 500 mM, $MgSO_4 \cdot 7H_2O$ 15 mM, ATP 15 mM, xylene 1% (v/v)) and acetaldehyde 200 mM in 100 mM KPi buffer pH 7, final volume 1 mL. Samples were withdrawn at regular time intervals and analysed as previously mentioned.

Whole-cell biotransformation of acetaldehyde to 2,4,6-trideoxy-D-erythro-hexapyranose: The reactions, performed in 1 mL of 100 mM KPi buffer pH 7 containing 100–500 mM acetaldehyde and biocatalyst 15% (w/v), were shaken at 28 °C and 200 rpm. Samples were withdrawn at regular time intervals, centrifuged at 11,000 rpm for 3 min and the supernatants were quenched with 3 volumes of acetonitrile. The precipitates were removed through ultrafiltration using a 0.22 μm filter and 250 mg NaCl was added to the filtrates. After 5 min at 6–8 °C, the upper organic phases were collected, dried over $MgSO_4$, qualitatively analysed by TLC and quantified by GC (Figure S4) as described below. After vacuum concentration, the extracts were purified by silica gel column chromatography using acetonitrile/ethyl ether 1:1 (v/v) to give the desired product in 1:1 alpha:beta anomeric ratio as a yellow oil. ^1H and ^{13}C-NMR spectra were in agreement with our previously reported data (Figure S3).[27]

Analytical methods: ^1H and ^{13}C NMR experiments were recorded on a Bruker Avance II 500 spectrometer (Madison, WI, USA) at 500 MHz and 125 MHz respectively, using $CDCl_3$ as solvent. TLC analysis was performed using silica gel 60 F254 aluminium plates from Merck (Darmstadt, Germany). GC analysis was performed in a Thermo Scientific Trace 1300 using a Thermo Scientific TR-5 (30 m× 0.25 mm×0.25 μm) column.

Qualitative analysis of DR5P was performed by TLC using n-butanol:2-propanol:H_2O, 3:12:4 (v/v/v) as the mobile phase. DR5P (Rf: 0.2) was detected 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 in a BioTek Cytation 5 instrument by the chemical colorimetric procedure described by Burton using 100 μl of freshly prepared reagent [20 mL solution A (1.5 g diphenylamine, 98.5 mL glacial acetic acid, 1.5 mL H_2SO_4) + 100 μL solution B (16 $mg\,mL^{-1}$ acetaldehyde aq.)] and 50 μL of the test sample.[38]

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Qualitative analysis of 2,4,6-trideoxy-D-erythro-hexapyranose was performed by TLC using acetonitrile : ethylether 1:1 (v/v) as a solvent and the spots were visualized by heating after spraying with 5% v/v H_2SO_4 in ethanol (Rf: 0.5). Quantitative analysis was performed by GC using He as gas carrier (1 mL min^{-1}) and injector temperature 200 °C. The oven temperature was set at 50 °C (5 min), increased to 250 °C (10 °C min^{-1}) and maintained at the final temperature for 5 min (Rt: 14.5 min, Figure S4).

All measurements were performed in duplicate. Data fitting was performed using Prism (GraphPad).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: aldolases · aldol addition · 2-deoxy-D-ribose-5-phosphate aldolase · 2-deoxyribose 5-phosphate · 2,4,6-trideoxy-D-erythro-hexapyranose

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