A multienzyme system composed by recombinant dihydroxyacetone kinase from *Citrobacter freundii*, fuculose-1-phosphate aldolase and acetate kinase, allows a practical one-pot C-C bond formation catalysed by dihydroxyacetone phosphate-dependent aldolases from dihydroxyacetone and an aldehyde.

Aldolases have attracted the interest of organic chemists because of their ability to catalyze the formation of C-C bonds with a high degree of stereoselective control. Dihydroxyacetone phosphate (DHAP) dependent aldolases have shown their utility in the synthesis of carbohydrate, carbohydrate-like structures, or non-carbohydrate compounds. A main drawback of these aldolases is the need for DHAP (DHA) as donor substrate, as this compound is expensive and labile at neutral and basic pH's. Several chemical syntheses of DHAP have been described, being the most attractive those starting from the degradation. Here, we describe the over expression in *E. coli* of DHAK from *C. freundii* and its use in a multienzyme system for one-pot C-C bond formation (Scheme 1). Thus, DHA phosphorylation is coupled with the aldolic condensation catalysed by the DHAP-dependent aldolase. The multienzyme system is completed with *in situ* regeneration of ATP catalyzed by acetate kinase (AK).

The system described in this communication has been optimised for fuculose-1-phosphate aldolase (F-1PA) and l-lactaldehyde as acceptor.

The gene encoding DHAK in *C. freundii* was amplified by polymerase chain reaction (PCR), cloned into pRSET-A vector, and expressed at 30 ºC in *E. coli* BL21(DE3) with a 6 X His tag. SDS-PAGE analysis of the expression showed that DHAK represented the 56% of the total soluble protein. The activity in the cell free extract (CFE), measured by enzymatic quantification of the DHAP formed, was 3195 U/L of culture. The recombinant DHAK was purified to 95% in one step by immobilized metal affinity chromatography (IMAC) using Ni²⁺-IDA agarose resins. The main kinetic constants for the recombinant DHAK are summarized in Table 1. The enzyme shows a high catalytic efficiency (*kₐ/Kₐ* = 5.4·10⁴ mM⁻¹·s⁻¹) for DHA, that is 10⁴-fold higher than the catalytic efficiency of the DHAK I from *S. pombe*, DHAK does not show a hyperbolic kinetic pattern with Mg²⁺, but a sigmoidal one. The Hill coefficient, *nₜₐₕ*, is 2.9 indicating a high positive cooperativity. From the Scatchard plot (data not shown), four binding sites for Mg²⁺ per subunit. These results agrees with the crystallographic structure of this enzyme recently published by Siebold et al. 

<table>
<thead>
<tr>
<th>Substrate</th>
<th><em>Kₐ</em> (mM)</th>
<th><em>Vₐₘₐₓ</em> (μmol·min⁻¹·mg of protein⁻¹)</th>
<th><em>kₐ/Kₐ</em> (mM⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHA</td>
<td>0.86·10⁻³</td>
<td>22</td>
<td>5.4·10⁴</td>
</tr>
<tr>
<td>ATP</td>
<td>1.9</td>
<td>35</td>
<td>39.4</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.7</td>
<td>38</td>
<td>47.4</td>
</tr>
</tbody>
</table>

Table 1 Kinetic parameters for recombinant DHAK

F-1PA was obtained from the recombinant *E. coli* strain ATCC # 86984 and purified in a single step using Ni²⁺-IDA agarose resins as previously described. L-lactaldehyde (3a) was synthesized from D-threonine.

ATP *in situ* regeneration was optimised in a DHA phosphorylation reaction (Fig. 1). During the reaction course a drop off in the pH of the mixture leaded to the stop of the reaction. Thus, a continuous adjustment of the pH to 7.5 was necessary to keep working the ATP regeneration system. The concentration needed to feed the system with enough cofactor could be reduced to 2.2 mM (this concentration is close to the *Kₐ* value of DHAK for ATP). In these conditions, DHAP was accumulated at a maximum yield of 82 % after 1 h. Longer reaction times did not increase the yield, but a diminish in the quantity of accumulated DHAP is observed, probably due to its degradation.

Here, we describe the over expression in *E. coli* of DHAK from *C. freundii* and its use in a multienzyme system for one-pot C-C bond formation (Scheme 1). Thus, DHA phosphorylation is coupled with the aldolic condensation catalysed by the DHAP-dependent aldolase. The multienzyme system is completed with *in situ* regeneration of ATP catalyzed by acetate kinase (AK).

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The system described in this communication has been optimised for fuculose-1-phosphate aldolase (F-1PA) and l-lactaldehyde as acceptor.
Afterwards, DHA phosphorylation was coupled with the aldol condensation catalysed by F-1PA. The whole reaction was spectrophotometrically monitored. The amount of DHAP formed can be measured from an aliquot of the reaction by addition of α-glycerophosphate dehydrogenase and after that 4a-g can be measured by the aldolase retro-aldolic activity. The one-pot multienzyme system was firstly assayed in a 3 mL reaction containing DHA (0.1 mmol), L-lactaldehyde (0.1 mmol), acetyl phosphate (0.2 mmol), ATP (6.8 μmol), MgSO$_4$ (25 μmol), ZnCl$_2$ (0.03 μmol), DHAK (3 U), F-1PA (1 U), AK (3 U) and HEPES buffer (50 mM, pH 7.5). The reaction reached the maximum yield (63 %) of fuculose-1-phosphate after 2 h (Fig. 2, white symbols). However, the maximum accumulation of DHAP (48.8 μmol) is reached after 1 h of reaction, and then decreases significantly. These results indicate that the DHAP is produced more rapidly than it is used by the aldolase to form the condensation product, leading to some degree of DHAP degradation. To improve the yield of condensation product the amount of DHAK was reduced to 1.5 U, increasing the ratio F-1PA:DHAK to 1:1.5 (Fig. 2, black symbols). Under these conditions the reaction proceed more slowly, but the amount of accumulated DHAP was maintained between 5-10 μmol along the reaction time. In this way, the yield of fuculose-1-phosphate increased up to 88.8 %. The reaction was scaled-up to 1 mmol of DHA, obtaining a similar yield of condensation product (84 %).

In conclusion, we have optimised a multienzyme system for a facile one-pot C-C bond formation catalysed by DHAP-dependent aldolases from readily available DHA and in which ATP is needed only in catalytic amounts. The recombinant DHAK employed in this work has a catalytic efficiency for DHA very superior to other biocatalysts used for enzymatic phosphorylation of DHA. Also, DHAP is obtained under non-oxidizing conditions that could affect the aldehyde acceptor. The system must be of general application although, for some acceptors the F-1PA:DHAK ratio must be experimentally optimised.

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**Notes and references**

† These authors contributed equally to this work.

12. A complete account of the cloning, over expression and biochemical characterization of the enzyme DHAK will be published elsewhere.
18. TLC (EtOH:NH$_3$ 2.5:3.0) analysis of the reaction showed that after 1 h of reaction the spot corresponding to DHAP ($R_f$=0.31) decreased and a new spot with $R_f$=0.7 appeared.