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Chiral Polyol Synthesis Catalyzed by a Thermostable Transketolase Immobilized on Layered Double Hydroxides in Ionic liquids

Ghina Ali,[a, b] Thomas Moreau,[a, b] Claude Forano,*[a, b] Christine Mousty,[a, b] Vanessa Prevot,[a, b] Franck Charmantray,[a, b] and Laurence Hecquet*[a, b]

This manuscript is dedicated to Wolf-Dieter "Woody" Fessner on the occasion of his 60th birthday.

In this work we set out to study the activity of a thermostable Transketolase (TK) from *Geobacillus stearothermophilus* (TK_{gst}) in an ionic liquid as cosolvent, which has never been investigated before with this enzyme. 1-Butyl-3-methylimidazolium chloride ([BMIm][Cl]) in the range 30–50% in water maintained the total activity of TK_{gst} and increased the reaction rate in the presence of pentoses as acceptor substrates, particularly D-ribose. To improve the synthetic process, TK_{gst} was immobilized on an inorganic support, layered double hydroxides (LDHs), with excellent immobilization yield and catalytic activity using a simple, eco-compatible, efficient coprecipitation procedure. The biohybrid MgAl@TK_{gst} was tested in 30% [BMIm][Cl] for the synthesis of a rare, very costly commercially available sugar, D-sedoheptulose, which was obtained in one step from D-ribose with an isolated yield of 82%. This biohybrid was reusable over four cycles with no loss of enzymatic activity. The particular activity of free and immobilized TK_{gst} in [BMIm][Cl] holds promise to extend the applications of TK_{gst} in other ionic liquids and unusual media in biocatalysis.

Introduction

Among the challenges in the application of enzymes on a large scale and in industry, particular attention has been paid to the use of ionic liquids (ILs) as an attractive alternative to hazardous volatile organic solvents.[1,2] ILs, based on anion and cation associations, are liquid from room temperature to 150–200 °C and display unique features that make them suitable as solvents or cosolvents for a wide range of applications, such as electrochemistry, catalysis, organic and inorganic synthesis, engineering, and analysis.[3]

In biocatalysis, ILs offer advantages over conventional organic solvents or aqueous media. It is reported that ILs can induce better enzyme activity,[4] high conversion rates[5] and enantioselectivity,[6] changes in substrate specificity, and increased substrate solubility.[7] One of the main interests in ILs is their ability to enhance the solubility of substrates and/or products, although their efficiency is a trade-off between substrate dissolution and the maintenance of enzyme activity. However, many authors have evidenced increased performance if biotransformations proceed in ILs.[8]

ILs have been investigated mainly in enzymatic reactions that involve polar substrates, such as amino acids or carbohydrates in a low-water environment, and hydrolytic enzymes, typically, esterases, lipases, and proteases.[6,9–13] It was shown that the biocatalytic activity was retained even at low water contents. Nonhydrolytic enzymes, particularly oxidoreductases, such as dehydrogenases,[14–17] peroxidases,[18] laccases,[19,20] and oxygenases,[21,22] have also been evaluated in aqueous IL mixtures to test their catalytic activity and stability.

Many factors affect enzyme activity and stability in ILs, especially the intrinsic physical and chemical properties of ILs, such as polarity, hydrophobicity, and viscosity. More particularly, enzyme stability and activity in aqueous IL mixtures are strongly dependent on water activity and kosmotropic versus chaotropic ion properties. Their roles on the protein surface energy or interactions with unfolded proteins affect biocatalytic performance. The immobilization of the enzyme in a protective matrix is an alternative process to stabilize enzyme activity.[23,24]

To overcome enzyme inactivation in IL media and improve activity, various methods, reviewed recently by Zhao,[25] have been studied, such as enzyme immobilization, enzyme modification with polyethylene glycol, water in IL microemulsions, the use of additives, and the design of IL structures. The combination of immobilization and a nonconventional solvent

[a] Dr. G. Ali, Dr. T. Moreau, Prof. C. Forano, Dr. C. Mousty, Dr. V. Prevot, Dr. F. Charmantray, Prof. L. Hecquet
Institut de Chimie de Clermont-Ferrand
Clermont Université, Université Blaise Pascal
BP 10448
63000 Clermont-Ferrand (France)
E-mail: laurence.hecquet@univ-bpclermont.fr
claude.forano@univ-bpclermont.fr

[b] Dr. G. Ali, Dr. T. Moreau, Prof. C. Forano, Dr. C. Mousty, Dr. V. Prevot, Dr. F. Charmantray, Prof. L. Hecquet
CNRS, UMR 6296
ICCF
63177 Aubière (France)

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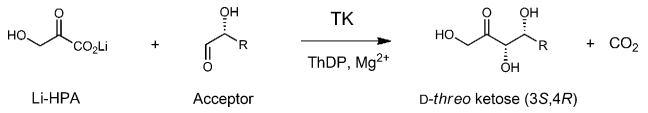
such as an IL offers an innovative strategy to achieve enhanced performance for enzyme-catalyzed reactions.

Our study focuses on transketolase (TK; E.C. 2.2.1.1) an enzyme dependent on thiamine pyrophosphate (ThDP) that catalyzes the synthesis of chiral polyols in one step by the stereospecific formation of a C–C bond. For synthetic purposes, hydroxypyruvate (HPA) is used as a ketol donor substrate, which renders the reaction irreversible through the release of CO_2 (Scheme 1).



Scheme 1. Irreversible reaction catalyzed by TK in the presence of Li-HPA as the donor substrate and an α-hydroxyaldehyde as the acceptor substrate.

Previous studies[26–30] performed with TKs from yeast (TK_{yst}) and *E. coli* (TK_{eco}) showed that TK accepts preferentially, as non-natural acceptor substrates, (2*R*)α-hydroxylated aldehydes with a short carbon chain (up to C_4). The TK products are D-*threo* ketoses (3*S*, 4*R*). The TK reaction has been used for the synthesis of various compounds of biological interest, such as amino alcohols,[31] thiosugars,[32] azasugars,[33] and phosphorylated sugars.[34,35] These properties lend TK a chemical synthetic potential that makes it an attractive biocatalyst for further industrial applications. A thermostable TK from *Geobacillus stearothermophilus* (TK_{gst}) was recently identified and overexpressed, which offers an optimum temperature of 65 °C and a thermostability of five days at 50 °C.[36] To broaden the substrate specificity and modify the stereoselectivity, some interesting TK_{eco}[37–39] and TK_{gst} variants[40] have been obtained. As often reported with other enzymes, thermostability is associated with a high tolerance towards unusual media such as organic solvents and ILs.[41] In parallel, to further optimize the process and to improve storage, thermal and/or pH stability of the enzyme, immobilization on supports is of great interest.[42,43] TK immobilization has been investigated on organic or semiorganic supports, such as activated sepharose,[44] Eupergit-C, Amberlite XAD-7 and Nylon,[45–47] and polyacrylamide gel.[48] We have also reported the immobilization of TK_{yst}[49] and TK_{eco}[50] on an inorganic support, layered double hydroxides (LDHs). LDHs are synthetic materials with positively charged brucite-like layers of mixed metal hydroxides that are described by the abbreviated formula $[M^{2+}M^{3+}A]$, in which M^{2+} and M^{3+} are divalent and trivalent metals, respectively, and A is the interlayer anion that compensates for the positive charge of the metal layers.[51,52] These layered compounds constitute a support that is easy to prepare in a cost-effective way in soft conditions suitable for biomolecule immobilization to maintain the biological activity. The efficient immobilization of amino acids, adenosine 5′-triphosphate (ATP), DNA, and many enzymes on LDH have been reported.[51,53]

The purpose of our study was to improve and extend the applications of TK in biocatalysis to a broader series of substrates and products under nonconventional conditions. To this end, the effects of various ILs on both the stability and activity of the thermostable TK_{gst} were investigated. Notably, the influence of ILs on this family of enzymes has never been described. After an initial screening, a more thorough study was conducted on 1-butyl-3-methylimidazolium chloride ([BMIm][Cl]) aqueous solution to define the optimal proportion of IL in the medium and the substrate specificity of TK_{gst} toward different hydrophilic aldehydes. The immobilization of TK_{gst} on a MgAl-LDH matrix was performed by direct coprecipitation. To further develop an efficient, reusable biocatalyst to produce chiral polyols, TK_{gst} was immobilized on an inorganic LDH. The chemical composition of MgAl-NO_3 was chosen because this matrix had been shown previously to give the best results for the immobilization of TKs from other sources (*S. cerevisiae*, *E. coli*).[49,50] These TKs, like TK_{gst}, are homodimers and have a strong homology of protein sequences and active sites.[36,54,55] MgAl-NO_3, which has a point of zero net charge of approximately 10–11, displays platelets with a net positive charge under experimental conditions (pH 9), whereas TK has an overall negative charge. The activity of the as-prepared biohybrid in IL/water medium was compared with the activity of free TK_{gst}, and the recyclability of the biocatalyst was assessed. Finally, the application of the MgAl-NO_3@TK_{gst} biohybrid for the synthesis of the chiral polyol sugar D-sedoheptulose from D-ribose in aqueous [BMIm][Cl] media was studied. Notably, D-sedoheptulose is rare and, though commercially available, very costly. In plants, the occurrence of free D-sedoheptulose remains a matter of conjecture, in contrast to the well-documented roles of its mono- and bisphosphate esters.[56] In humans, D-sedoheptulose is a key marker of various diseases such as cystinosis, an autosomal recessive lysosomal storage disease,[57] and transaldolase deficiency, a disorder of the carbohydrate metabolism with multisystem involvement discovered recently.[58]

Results and Discussion

TKs in IL/water solution

In a preliminary step, the influence of different ILs based on imidazolium and anions such as Cl^-, $CH_3CO_2^-$, BF_4^-, and ammonium bromide on TK stability was tested (Figure 1 a). Two sources of TK, a new thermostable TK from *G. stearothermophilus*[36] (TK_{gst}) and TK_{eco}, used commonly in biocatalysis, were studied and compared. These TKs, as the other TKs described in the literature, are dimeric enzymes and each individual monomer is not active. The residual activities observed in different aqueous buffered media that contained 0–60 % of ILs are reported in Figure 1 a and b.

Except for [BMIm][Cl], a drastic alteration of TK_{gst} activity was observed systematically. TK_{gst} preserved its total activity at up to 50 % of [BMIm][Cl], whereas for TK_{eco}, a progressive decrease in activity was observed above 20 % of [BMIm][Cl]. Interestingly, even if the incubation time of TK_{gst} in 50 % [BMIm][Cl] was extended to 16 h, 80 % of the enzyme activity was maintained. This resistance of TK_{gst} to high concentrations of [BMIm][Cl] can be correlated with the thermostable properties of TK_{gst}, which make it more robust and stabilize the dimer. A

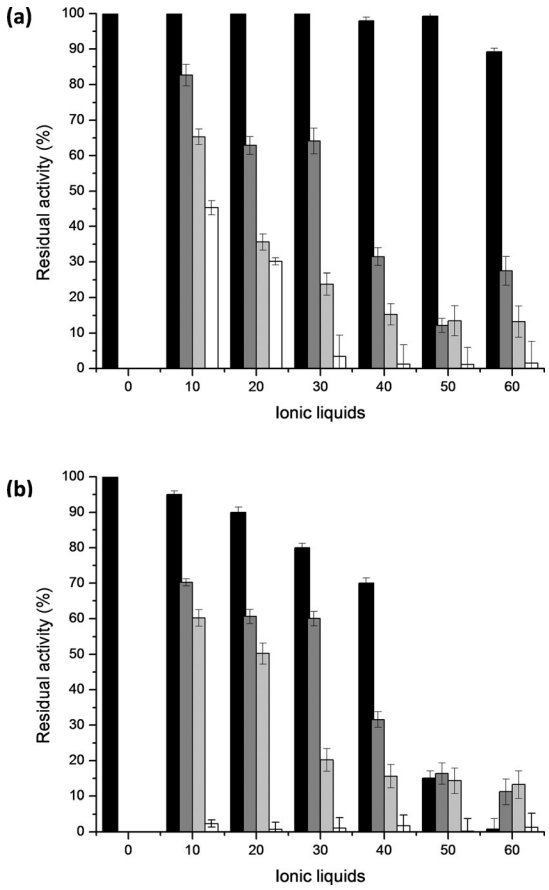


Figure 1. Activities of a) TK_{gst} and b) TK_{eco} after incubation (5 min) of enzymes in ILs (0–60%). [BMIm][Cl] (black), [BMIm][CH_3COO] (dark gray), [BMIm][BF_4] (light gray), and [$CH_3(CH_2)_9N(CH_3)_3$][Br] (white). Residual activities of TKs were determined with L-erythrulose and D-ribose-5-phosphate as substrates to yield D-sedoheptulose-7-phosphate and glycolaldehyde. The glycolaldehyde released was measured spectrophotometrically at 340 nm by NADH consumption by alcohol dehydrogenase.

high ion concentration can modify the water structure and hence influence the protein hydration environment. In particular, hydrophilic and water-miscible ILs might remove internally bound water from enzymes and lead to a loss of enzyme activity. Thermostable enzymes are generally characterized by greater rigidity and a more compact structure, which could induce a stronger resistance to water abstraction by ILs.[41–43]

We went on to study the effect of [BMIm][Cl] as a water-miscible IL mainly in the presence of TK_{gst}. We underline that [BMIm][Cl] is tolerated by many enzymes and is able to dissolve numerous carbohydrates.

Substrate specificity of TK_{gst} for different aldehydes

For this study, we used lithium β-hydroxypyruvate (Li-HPA) as a donor substrate and a broad range of α-hydroxylated acceptor substrates, with a variation of the length of the carbon chain at both 30 and 50% IL as the enzymatic activity is fully maintained at these IL proportions (Table 1, Scheme 1). The TK_{gst}-catalyzed conversion of Li-HPA in the presence of each aldehyde was measured over time using lactate dehydrogenase and NADH by spectrophotometry at 340 nm. We checked that

Table 1. Hydroxylated acceptor substrates used for the study of TK_{gst} substrate specificity.

CHO–R with R=	Name
$-CH_2OH$	glycolaldehyde
$-CHOH(R)-CH_2OH$	D-glyceraldehyde
$-CHOH(R)-CHOH(R)-CH_2OH$	D-erythrose
$-CHOH(R)-CHOH(R)-CHOH(R)-CH_2OH$	D-ribose
$-CHOH(R)-CHOH(S)-CHOH(R)-CH_2OH$	D-xylose
$-CHOH(S)-CHOH(R)-CHOH(R)-CH_2OH$	D-arabinose
$-CHOH(R)-CHOH(S)-CHOH(R)-CHOH(R)-CH_2OH$	D-glucose

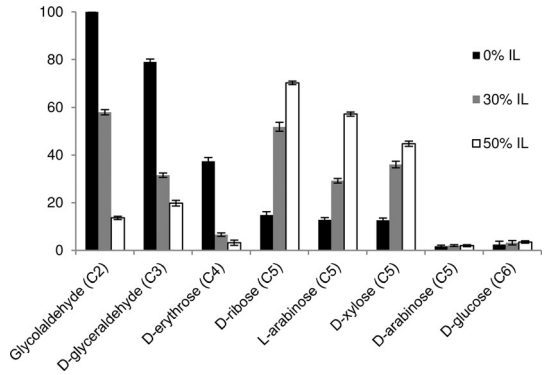


Figure 2. Consumption of Li-HPA [%] as a function of substrate range with TK_{gst} at 0% (dark gray), 30% (light gray), and 50% (white) of [BMIm][Cl] with TK_{gst} (0.25 U mL^{-1}) after 3 h at 25 °C and pH 7.5. The acceptor substrate was C5 pentose with (2*R*) configuration (L-arabinose, D-xylose and D-ribose) and (2*S*) configuration (D-arabinose). Li-HPA conversion was measured using lactate dehydrogenase and NADH by spectrophotometry at 340 nm.

the stability of Li-HPA without TK_{gst} was not modified by the presence of [BMIm][Cl].

In the presence of TK_{gst} in pure aqueous solution, as reported in the literature,[26–30] the hydroxylated aldehydes with a C_2–C_3 chain such as glycolaldehyde and D-glyceraldehyde exhibited the best Li-HPA consumption (Figure 2). The TK_{gst} substrate specificity profile was modified at 30 and 50% [BMIm][Cl]. A drastic decrease in Li-HPA conversion in the presence of hydroxylated aldehydes with a short carbon chain such as glycolaldehyde or D-glyceraldehyde was observed, whereas there was no particular effect of the IL on hydroxylated aldehydes composed of four and six carbon atoms (D-erythrose and D-glucose). Strikingly, for D-ribose and other pentoses with a (2*R*) configuration, a two- to threefold increase in Li-HPA conversion was obtained with 30 and 50% of [BMIm][Cl] compared with the results obtained without IL (Figure 2). For D-arabinose with a (2*S*) configuration, which is not accepted by TK_{gst} under usual conditions, we observed a slight increase in activity with 50% [BMIm][Cl]. These results highlight that the TK_{gst} substrate profile obtained in [BMIm][Cl] aqueous solution is completely different from that obtained in water. This modification of TK_{gst} substrate scope by [BMIm][Cl] might be related to the physical and chemical parameters of the complex IL/water medium.[59] The best enhancement of Li-HPA conversion with pentoses could be explained by the physical and chemical properties of IL, which favors suitable arrangements of IL and H_2O that might be able to modify the chemical and con-

formational properties of pentoses and/or enzyme and favorably influence the TK_{gst} reaction rate. Other reports have described the effects of ILs in aqueous solution on the solvation behavior of monosaccharides favored by the hydrogen-bonding ability of IL anions and on enzymatic or chemical reactions that involve monosaccharides.[59–61]

The effect of [BMIm][Cl] (30%) on TK_{gst} affinity toward D-ribose ($K_{M(app)}$) and TK velocity ($v_{max(app)}$) was studied and compared with values obtained without the IL. The values given by the Lineweaver–Burk model are given in Table 2. Substrate affinities for TK_{gst} were close to those obtained without IL, which shows a slight improvement in D-ribose affinity at 30% [BMIm][Cl]. The values of $v_{max(app)}$ of TK_{gst} for the Li-HPA substrate increased slightly at 30% [BMIm][Cl] and more significantly with D-ribose to twice the $v_{max(app)}$ without [BMIm][Cl]. These results suggest a beneficial effect of [BMIm][Cl] on D-ribose kinetic constants, particularly on $v_{max(app)}$, that could be correlated with the results reported above on the TK_{gst} acceptor substrate specificity, which shows a twofold increase in Li-HPA conversion toward (2*R*)-pentoses with 30% IL.

Table 2. Kinetic parameters obtained with free TK_{gst} for Li-HPA and D-ribose.

[BMIm][Cl]	Donor: Li-HPA		Acceptor: D-ribose	
	$K_{M(app)}$[a] [mM]	$v_{max(app)}$[a] [U mg^{-1}]	$K_{M(app)}$[a] [mM]	$v_{max(app)}$[a] [U mg^{-1}]
0%	3.8 ± 0.4	100 ± 9	9.5 ± 0.8	3.1 ± 0.3
30%	3.7 ± 0.6	140 ± 11	7.2 ± 1	6 ± 0.7

[a] Li-HPA and D-ribose with 0 and 30% [BMIm][Cl] in the medium. Initial rates were measured by varying Li-HPA and D-ribose, respectively. Li-HPA was measured from aliquots using LDH enzyme and NADH by spectrophotometry at 340 nm. One unit [U] of TK releases 1 μmol of D-sedoheptulose per minute at pH 7.5 at 25 °C. Hanes–Woolf plots were used to determine v_{max} and K_M values. For K_M, the cosubstrate was applied in excess.

Immobilization of TK_{gst} on LDH

The immobilization of TK_{gst} was performed as described previously.[49,50] Typically, MgAl@TK_{gst} biohybrid was prepared directly in the presence of TK_{gst} by the coprecipitation of metal salt at a constant pH of 9 at a TK_{gst}/LDH ratio of 0.5. This approach is a tunable, cheap, eco-friendly process for enzyme immobilization.[51,53]

The XRD patterns of both MgAl-TK_{gst} biohybrid and MgAl-NO_3 reference material are presented in Figure 3. They show the typical patterns of layered structures with a hexagonal lattice and a rhombohedral symmetry ($R\bar{3}m$). The diffractograms contain the 00*l* reflection series ($2\theta < 30°$), the reticular distances (d_{00l}) of which depend on both the size of the intercalated anion and the hydration rate, and the characteristic *hk*0 and *hkl* diffraction lines ($2\theta > 30°$) related to the cation ordering inside the metal hydroxide layers. The interlamellar distance ($1/3\,d_{003}$) of MgAl-NO_3 is consistent with the presence of intercalated NO_3^-.[52] The XRD pattern of MgAl@TK_{gst} biohybrid shows the same characteristic diffraction peaks as the reference material, which indicates that the LDH lamellar structure is also well formed in the presence of TK_{gst}. As TK_{gst} immobilization does not affect the LDH structure and the *d* spacing remains the same as the reference material MgAl-NO_3, we can conclude that there is no intercalation of TK_{gst} between the LDH layers. In parallel, the FTIR spectra of the materials (see Supporting Information) led to similar conclusions with regard to the formation of the LDH materials. The spectra display vibration bands typical of the LDH structure: ν_{OH} vibration bands at $\tilde{\nu} = 3000–3700$ cm^{-1} attributed to OH groups of hydroxylated layers and water molecules, vibration bands in the range $\tilde{\nu} = 400–800$ cm^{-1} assigned to the lattice vibrations ν_{MO} and δ_{OMO}, and a peak at $\tilde{\nu} = 1300–1400$ cm^{-1} attributed to the NO_3^- group. The MgAl@TK_{gst} biohybrid displays additional bands observed in the range of $\tilde{\nu} = 2700–2800$ and 1200–1800 cm^{-1} characteristic of the CH and amide elongation bands of the TK_{gst}, respectively. The presence of TK_{gst} in the LDH structure is confirmed.

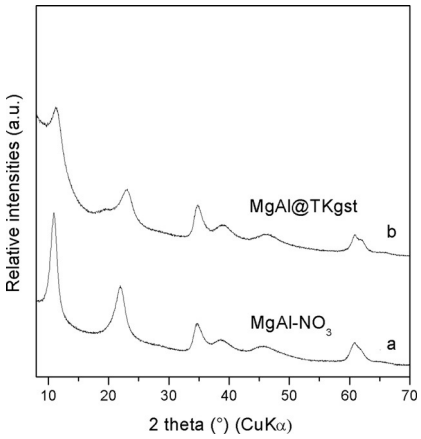


Figure 3. XRD patterns of a) MgAl-NO_3 and b) biohybrid MgAl@TK_{gst}.

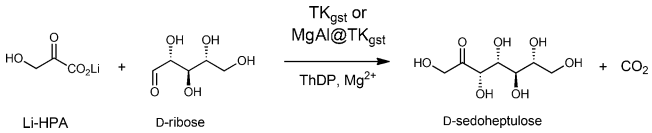
Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was performed on all samples to determine the Mg/Al chemical composition. An experimental Mg/Al ratio of 2.5 and 2.3 was obtained for MgAl-NO_3 and MgAl@TK_{gst} biohybrids, respectively. The analysis of supernatants evidenced that the slight difference between experimental and theoretical values can be assigned to the incomplete precipitation of Mg because of the lower pH value used compared to the optimal one.[52]

The amount of TK_{gst} immobilized in the LDH was quantified using thermogravimetric analysis. As the thermal decomposition of the biohybrid LDH proceeds through successive events, the exothermic combustion of the immobilized enzyme is evidenced clearly on the thermogram (see Supporting Information). Indeed, the MgAl@TK_{gst} biohybrid decomposes in three successive thermal steps: (1) the dehydration of bound water molecules from 100 °C, (2) the decomposition of the hydroxylated layers and their breakdown from 200 °C, and (3) the combustion of organic molecules. If we determined the total mass loss of the three samples TK_{gst}, MgAl-NO_3, and MgAl-TK_{gst}, we can access the experimental coprecipitation yield (LDH/TK_{gst}) of 1.75, very similar value to the theoretical value of 2.

For a protein-to-LDH weight ratio of 0.5,[50] the immobilization yield of TK_{gst} was 98–99%, and the specific activities of free and immobilized TK_{gst} on the LDH matrix ($MgAl@TK_{gst}$) were compared. The specific activity of free TK_{gst} was 1 U mg^{-1}, compared with 0.96 U mg^{-1} for the immobilized TK_{gst} (activities measured with L-erythrulose and D-ribose-5-phosphate as substrates, see Experimental Section). We conclude that TK_{gst} kept 96% of its activity once immobilized on LDH using the direct coprecipitation process. The supported enzyme kept its total activity for two months. The high retention activity of TK_{gst} on the MgAl LDH matrix shows the strong affinity of LDH toward TK and the efficiency of the immobilization support. The influence of immobilization on the activity and stability of multimeric enzymes has been reviewed by Lafuente et al.[42b,c] The total activity of TK_{gst} was recovered after immobilization, and the enzyme was not dissociated as the individual monomers are not active. The enzyme was not released in spite of the immobilization treatments (stirring, pH variation, presence of salts) as no protein was detectable in solution after centrifugation.

Synthesis of D-sedoheptulose catalyzed by $MgAl@TK_{gst}$

To examine the potential efficiency of the biohybrid $MgAl@TK_{gst}$ in H_2O/[BMIm][Cl], the conversion of Li-HPA over time in the presence of D-ribose as the acceptor substrate was followed (Scheme 2), and the results were compared with those obtained with free TK_{gst} (Figure 4).



Scheme 2. Irreversible reaction catalyzed by free TK_{gst} or $MgAl@TK_{gst}$ in the presence of hydroxypyruvate (HPA) as the donor substrate and D-ribose as the acceptor substrate.

The profiles of the Li-HPA conversion with $MgAl@TK_{gst}$ biohybrid and free TK_{gst} were the same, and showed an increase in Li-HPA conversion with the proportion of [BMIm][Cl] in the reaction medium. In both cases, at 50% of IL, the Li-HPA conversion increased by almost a factor of two compared with the reaction performed without [BMIm][Cl]. It appears that TK_{gst} immobilization does not modify the enzyme behavior toward the IL.

To gain greater insight into the enzyme activity and the possibility to reuse the biohybrid, the $MgAl@TK_{gst}$ compound was centrifuged, washed, and reused at the end of each cycle. The Li-HPA conversion for three different percentages of [BMIm][Cl] in the medium (0, 30, and 50%) in the presence of TK_{gst} immobilized on LDH is shown in Figure 5. Interestingly, the Li-HPA conversion rate was almost unmodified (70±5)% over four cycles whatever the percentage of [BMIm][Cl] in the medium. No contamination by proteins from the reaction mixture after each cycle was observed, which shows that TK was reusable without release. In addition, the activity of immobilized TK was not modified overtime. This suggests that TK was not dissociated. Notably, as the activity of TK_{gst} increases with the concentration of [BMIm][Cl] and, consequently, with Cl^-, to a certain extent (up to 50% w/w in water), there is no anion exchange of Cl^-, which could affect the stability of $MgAl@TK_{gst}$. Clearly, this evidences the maintenance of the enzyme activity and the utility of the combination of enzyme immobilization and the presence of IL in the medium.

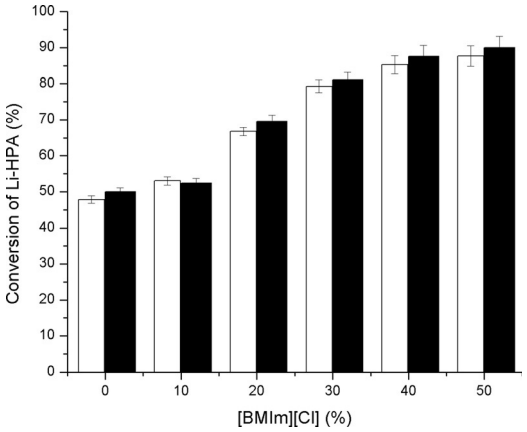


Figure 4. Comparison of Li-HPA conversion catalyzed by free TK_{gst} (white) and biohybrid $MgAl@TK_{gst}$ (black) at 0–50% of [BMIm][Cl] (TK_{gst} 2.10^{-3} U mL^{-1}, 25 °C, 24 h, pH 7.5). Li-HPA (25 mM) conversion in the presence of D-ribose (25 mM) was measured from aliquots using lactate dehydrogenase and NADH by spectrophotometry at 340 nm.

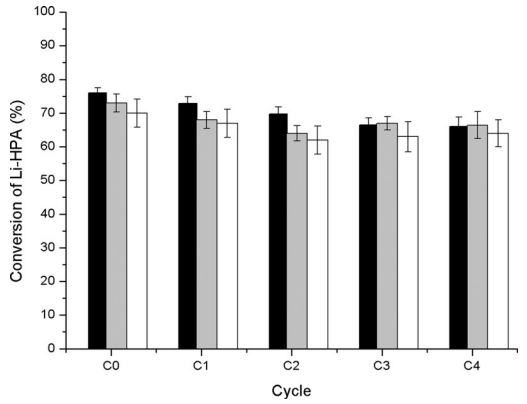


Figure 5. Reuse of biohybrid $MgAl@TK_{gst}$ in 0–50% of [BMIm][Cl]: 0% (black), 30% (gray), and 50% (white). For each cycle (C0–4), $MgAl@TK_{gst}$ was centrifuged and washed with glycylglycine buffer at 25 °C. Li-HPA (25 mM) conversion in the presence of D-ribose (25 mM) was measured from aliquots using lactate dehydrogenase and NADH by spectrophotometry at 340 nm.

The reaction catalyzed by $MgAl@TK_{gst}$ was performed with Li-HPA as the donor and D-ribose as the acceptor in 30% [BMIm][Cl] on a preparative scale to obtain the corresponding high-value-added product, D-sedoheptulose. Li-HPA was added progressively at a low constant rate to the reaction medium to limit its instability or decomposition, which permitted its total and sole conversion by the biocatalyst $MgAl@TK_{gst}$. To avoid the increase in pH during synthesis caused by bicarbonate ion formation from Li-HPA decarboxylation and to maintain the pH at 7.5, an acid solution (0.1 M HCl) was added slowly over the course of the reaction by using a pHstat. At the end of the reaction, the biohybrid was removed easily by centrifugation.

The chloride anion of [BMIm][Cl] was then exchanged with BF_4^- by adding $NaBF_4$ salt to give a biphasic medium. Once purified from the aqueous phase, the structure of the product was confirmed by NMR spectroscopy, which evidenced that D-sedoheptulose was formed and that [BMIm][Cl] was removed efficiently. Finally, D-sedoheptulose was produced with an isolated yield of 82%, which is similar to that obtained with free TK_{gst}, and an optical rotation of $[\alpha]_D^{20} = +8.6$ comparable to published values. The presence of 30% [BMIm][Cl] in the reaction medium increased the conversion rate without altering the efficiency of the biohybrid or the TK_{gst} stereospecificity.

Conclusions

This work offers a new approach for the synthesis of chiral polyols using a reaction process catalyzed by Transketolase (TK) under unusual conditions. TK from *Geobacillus stearothermophilus* (TK_{gst}) kept its activity for 16 h in H_2O/1-butyl-3-methylimidazolium chloride ([BMIm][Cl]). Proportions of 30 and 50% [BMIm][Cl] modified the TK_{gst} substrate profile and enhanced TK_{gst} activity towards pentoses, particularly D-ribose. To improve the TK_{gst}-catalyzed reaction process, TK_{gst} was immobilized successfully on an inorganic, eco-compatible layered double hydroxide support, which offers a high immobilization yield and activity. Finally, we used the biohybrid MgAl-NO_3@TK_{gst} for the one-step synthesis of the very costly commercially available sugar D-sedoheptulose from the cheap acceptor substrate D-ribose. The removal of [BMIm][Cl] and the recovery of the pure product were performed successfully, and a yield of 85% was achieved. In addition, MgAl@TK_{gst} could be reused easily with no significant loss of the lithium β-hydroxypyruvate conversion rate over four cycles. Finally, this work shows the potential of free and immobilized TK_{gst} in [BMIm][Cl]/water to improve the reaction process. The influence of [BMIm][Cl] in water on TK_{gst} activity and substrate specificity involves numerous physicochemical parameters, and a better understanding will require further investigations. These advances should offer new perspectives for increasing wild-type or variant TK_{gst} activity towards poorly water-soluble substrates by extending the study to hydrophobic ionic liquids.

Experimental Section

Materials

Chemicals of a reagent grade were purchased from Sigma Aldrich. Bradford reagent was obtained from Bio-Rad. The enzymes alcohol dehydrogenase (ADH), L-lactate dehydrogenase, and [BMIm][Cl] were purchased from Sigma Aldrich. $Mg(NO_3)_2{\cdot}6H_2O$ and $Al(NO_3)_3{\cdot}9H_2O$ were obtained from Acros Organics. All solutions were prepared in deionized distilled water.

Analytical methods

Powder XRD patterns of solids were recorded by using a Philips X-Pert Pro diffractometer equipped with a graphite monochromator using CuK_α radiation with $\lambda = 0.15415$ nm. The patterns were recorded over $2\theta = 2.0$–70.0° in steps of 0.033° with a counting time per step of 219 s. Attenuated total reflectance (ATR) FTIR spectra were obtained by using a Nicolet 5700 spectrometer from Thermo Electron Corporation using KBr pellets. Thermogravimetric analyses (TGA) were measured by using Setaram Instrumentation. UV/Vis absorbance was measured by using a microplate reader Safire II TECAN spectrophotometer. NMR spectra were recorded in D_2O by using a Bruker Advance 400 spectrometer. The optical rotatory power of D-sedoheptulose was determined by using a polarimeter P-2000 (Jasco PTC-262).

Production and purification of TK_{gst}

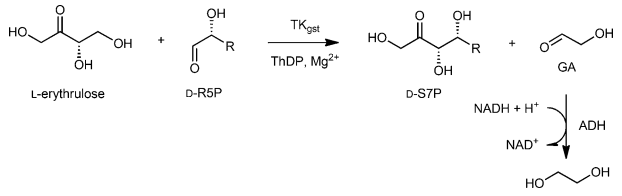
The expression of TK_{gst} was performed in *E. Coli* BL21 (DE3) Gold strain, which was first transformed by electroporation with pGEN717.[36] The preculture (100 mL) was grown at 37 °C in lysogeny broth (LB) medium overnight with Kanamycin (30 μg mL^{-1}). The preculture was inoculated with a culture medium (1 L) that contained 30 μg mL^{-1} of Kanamycin. If the absorbance reached 0.8, isopropyl β-D-thiogalactoside (1 mM) was added. Cells were grown overnight at 30 °C and harvested. TK_{gst} was extracted from the cell pellets after centrifugation. After the suspension of the cell paste in lysis phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8), the cell lysis was conducted by ultrasonication for 30 min on ice, and the insoluble pellet was discarded after centrifugation (13000 × *g*, 20 min, at 4 °C). TK_{gst}, which has a His_6 tag on the N-terminal, was purified from the cell-free extract with Ni-NTA affinity column resin equilibrated with phosphate buffer. The fractions that contained TK_{gst} (control by Bradford and activity enzyme assay) were collected after elution by imidazole (250 mM), dialyzed against water, lyophilized, and stored at 4 °C. Purified TK_{gst} has a specific activity of 1.1 U mg^{-1} determined by the Bradford test, and the activity assay described in the next section.

Protein assay

The Bradford assay was used to determine the protein concentration. Aliquots (25 μL) of the TK_{gst} extracts were added to the Bradford reagent (225 μL) and incubated in the dark 30 min at 25 °C. A standard curve was obtained using bovine serum albumin (BSA) as the standard at concentrations of 10–100 μg mL^{-1}. The absorbance was measured at two wavelengths (450 and 590 nm), and the ratio of both values varied linearly with protein concentrations, which are expressed in mg mL^{-1}. The immobilization yield was calculated by subtracting the protein amount present in the supernatant (determined by the Bradford assay) to the total amounts of proteins.

TK enzymatic activity

TK_{gst} activity was determined at 27 °C in glycylglycine buffer (100 mM, pH 7.5) containing 0–60% [BMIm][Cl] with cofactors thiamine pyrophosphate (ThDP; 0.1 mM) and $MgCl_2$ (0.5 mM) using L-erythrulose as the donor substrate (100 mM) and D-ribose-5-P (D-R5P) as the acceptor substrate (10 mM), which led to D-sedoheptulose-7-phosphate (D-S7P) and glycolaldehyde (GA) as products [Eq. (1)]. The GA formed is reduced by ADH (5 U mL^{-1}) to glycol in the presence of NADH (0.75 mM) and was measured spectrophotometrically at 340 nm by NADH consumption by ADH ($\varepsilon_{NADH\,340nm} = 6220\ M^{-1}\,cm^{-1}$). TK_{gst} activity was expressed in U mL^{-1} and the specific activity in U mg^{-1} of protein. In the presence of ILs, the activity of ADH was controlled and a large excess of ADH was used to deal with its loss of activity. The amount of ADH was



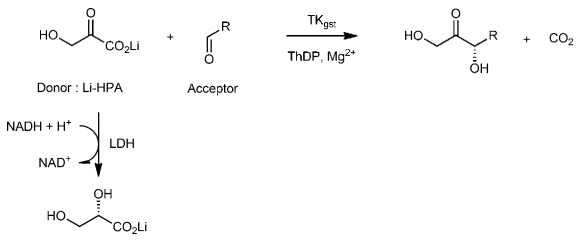
adjusted to obtain a comparable activity than that obtained without ILs in the medium.

Immobilization of TK$_{gst}$ on LDH

TK$_{gst}$ was immobilized on LDH using a coprecipitation method to form biohybrid-labeled MgAl@TK$_{gst}$. The choice of immobilization method and matrix were described earlier for TKs from other sources.[49,50] TK$_{gst}$ per LDH amount (w/w) was fixed at 0.5. A mixed aqueous solution of $Mg(NO_3)_2{\cdot}6H_2O$ and $Al(NO_3)_3{\cdot}9H_2O$ with a Mg^{2+}/Al^{3+} molar ratio of 4 and total molar concentration of cations of 0.1 M was added at a flow constant rate of 0.063 mL min^{-1} to the TK$_{gst}$ solution (2.5 mg mL^{-1}, pH 7.5, 4 °C) in a four-necked glass vessel under vigorous magnetic stirring over 3 h. The pH of the solution was maintained constant at 9 during the immobilization process by the addition of NaOH solution (0.1 M). The experiment was performed under N_2 pressure to prevent any competitive carbonatation. The suspension obtained was centrifuged at 12000×*g* for 20 min and washed three times with decarbonated water at pH 7.5. The recovered precipitate was either stored at 4 °C in glycylglycine buffer for biocatalysis or dried for physical characterization. The supernatant was analyzed to determine the immobilization yield by quantification of residual protein concentration by a Bradford assay.

TK$_{gst}$ substrate specificity in [BMIm][Cl]

The reaction was performed at 27 °C in glycylglycine buffer (0.1 mM, pH 7.5) that contained 0, 30, or 50% of [BMIm][Cl], ThDP (0.1 mM) and $MgCl_2$ (0.5 mM), Li-HPA (25 mM), and aldehydes (25 mM) [Eq. (2)]. After the addition of TK$_{gst}$, the residual Li-HPA concentration was determined from different aliquots of reaction mixture taken at definite times and added to a reaction mixture that contained NADH (10 mg mL^{-1}), LDH (100 U mL^{-1}), and glycylglycine buffer (0.1 mM, pH 7.5). The disappearance of NADH was followed by spectrophotometry at 340 nm, and the difference between initial absorbance and final absorbance gave the Li-HPA concentration using the Beer–Lambert law ($\varepsilon_{NADH\,340nm}$ = 6220 M^{-1} cm^{-1}). Control reactions with Li-HPA and each acceptor substrate without TK$_{gst}$ and with 30 and 50% of [BMIm][Cl] were performed.



Determination of TK$_{gst}$ kinetic constants

$K_{M(app)}$ and $v_{max(app)}$ of TK$_{gst}$ towards its donor substrate, Li-HPA, and acceptor substrate, D-ribose, in [BMIm][Cl] at 30% were determined in the presence of NADH and lactate dehydrogenase using the enzyme assay described above. Initial velocities of TK$_{gst}$ were obtained by varying each substrate concentration in a range of 1–50 mM for Li-HPA and 5–70 mM for D-ribose. For each $K_{M(app)}$, the cosubstrate was present in excess. Lineweaver–Burk plots were used to determine the kinetic constants values. Control reactions that contained Li-HPA and D-ribose without TK$_{gst}$ and with 30% of [BMIm][Cl] were also performed.

Synthesis of D-sedoheptulose in [BMIm][Cl]

The synthesis of D-sedoheptulose was performed from Li-HPA and D-ribose. A solution of H_2O (17.5 mL, 70%)/[BMIm][Cl] (7.5 mL, 30%) was prepared and adjusted to pH 7.5 by the addition of 0.1 M NaOH. $MgCl_2$ (0.3 mM) and ThDP (0.08 mM) were dissolved in H_2O/[BMIm][Cl], and MgAl@TK$_{gst}$ biohybrid (2 U mL^{-1}) was added to the mixture and stirred for 20 min after the adjustment of the pH to 7.5. D-Ribose (100 mM) solution was prepared in H_2O/[BMIm][Cl], added to the enzyme/cofactors mixture, and adjusted to pH 7.5. Li-HPA solution (140 mM) incubated in ice was added at a low constant flow to the mixture, which was stirred for 150 min at 25 °C. During the synthesis, the pH was maintained at pH 7.5 by adding 0.1 M HCl by using a pH stat (Radiometer Analytical). The reaction was monitored by TLC to follow the product formation using ethanol/butanol/H_2O 110:10:20. After the total conversion of D-ribose, MgAl@TK$_{gst}$ was eliminated by simple centrifugation (12000×*g*, 20 min), and the supernatant was recovered. [BMIm][Cl] was removed from the supernatant solution by adding $NaBF_4$ to give [BMIm][BF_4], which formed a heterogeneous phase with H_2O according to the method described by Creary and Willis.[62] The aqueous phase that contained D-sedoheptulose was then recovered and washed with CH_2Cl_2, which was then eliminated by using a separatory funnel. The aqueous phase was concentrated in vacuo to give D-sedoheptulose as a light brown syrup (370 mg, 82% yield). $[\alpha]_D^{20} = +8.6$ ($c = 0.1$, H_2O), lit. $[\alpha]_D^{20} = +8$[63] and +8.2.[64] The ^{13}C NMR spectrum was identical to that described previously.[56] ^{13}C NMR (101 MHz, D_2O) α-furanose, β-furanose, and α-pyranose forms, respectively: $\delta = 62.3$, 62.4 and 64.17 (C-1), 97.51, 98.9, and 102.6 (C-2), 68.3, 75.8 and 81.79 (C-3), 70.98, 75.4 and 76.4 (C-4), 63.49, 80.17 and 81.7 (C-5), 68.74, 70.98 and 72.68 (C-6), 61.3, 62.3 and 62.6 ppm (C-7).

Reusability of biohybrid MgAl@TK$_{gst}$ in [BMIm][Cl]

To test the reusability of biohybrid, the reaction mixture that contained H_2O (17.5 mL, 70%)/[BMIm][Cl] (7.5 mL, 30%) adjusted to pH 7.5 by 0.1 M NaOH, $MgCl_2$ (0.3 mM), ThDP (0.08 mM), Li-HPA (25 mM), D-ribose (25 mM), and MgAl@TK$_{gst}$ biohybrid (2 U mL^{-1}) was centrifuged after 3 h, and the precipitate was washed with glycylglycine buffer pH 7.5 and then recovered. The supernatant was analyzed to determine the residual protein concentration by the Bradford assay. The Li-HPA conversion was determined as described before. A new batch of substrates was added, and the experiment was repeated four times.

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