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Chemical stability and catalytic activity of redox enzymes in NADES †

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Abstract: NADES represent a new generation of biocompatible solvents, formed by eutectic mixtures of two or more hydrogen bond donor and hydrogen bond acceptor compounds of natural origin, with a lower melting point compared to that of pure components. The ease of preparation, sustainability, low cost and low toxicity of NADES have allowed these solvents to be investigated in biocatalysis. In this communication we describe the stability and the enzymatic activity of two oxidoreductases, the HLADH and the *Ts*ER, in buffer solution and in mixtures choline-based NADES/buffer. In particular, we report the enantioselective lactonization of 3-methyl-1,5-pentanediol into 4-methyl-δ-valerolactone and the ketoisophorone bioreduction into levodione.

Keywords: NADES; choline cloride; HLADH; TsER.

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1. Introduction

Natural Deep Eutectic Solvents (NADES) are a group of molecular solvents entirely composed by plant primary metabolites such as sugars, alcohols, amino acids, organic acids and choline derivatives.[1] They are composed by a salt such as choline chloride (C) as hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), which are capable of associating through hydrogen bonds and van der Waals interactions, to form eutectic mixtures, with melting points much lower than that of single components. Due to properties such as non-volatility, non-flammability, low toxicity and biodegradability, NADESs are considered as green solvents and have started to be assessed as tools for biocatalysis, either as solvents or as separative agents, with the aim to eliminate multiple steps involved in complex chemical syntheses.[2]

The use of oxidoreductases in industrial organic synthesis has been gaining momentum in the last decade. In this field, the horse-liver alcohol dehydrogenase (HLADH) is one of the most popular biocatalysts for the stereoselective oxidation of alcohols, due to its commercial availability and stereospecificity. This zinc-dependent enzyme performs its oxidant activity using oxidized nicotinamide cofactors ($NAD(P)^+$) as electron acceptors.[3] The HLADH is the catalyst of choice for the direct oxidative lactonization of diols [4]; in particular, the 3-methyl-1,5-pentanediol is the substrate for the synyhesis of (*S*)-3-methyl-δ-valerolactone, an interesting building block present in some complex natural products.[5] The enoate reductases (ERs) are members of the "*old yellow enzyme*" family, a class of flavin-dependent enzymes that catalyze the reduction of C=C bonds conjugated with electron-withdrawing groups with absolute stereospecificity, at the expense of a nicotinamide cofactor. A platform of homologous enzymes has been developed and several industrially relevant molecules could be obtained in enantiomerically active form.[6] α,β-Enones are usually well accepted substrates for ERs; in particular, the

4-ketoisophorone furnishes, after asymmetric bioreduction, the (*R*)-levodione, an important industrial intermediate for carotenoide synthesis.[7]

In this communication, we report the studies on HLADH and ER from *Thermus scotoductus* (*Ts*ER), evaluating the enzyme activity and enantioselectivity in the lactonization of 3-methyl-1,5-pentanediol and ketoisophorone bioreduction, respectively, in XoCH as NADES. Our aim is to associate the biocatalysis and the use of low toxic solvents (NADES) as two important green approaches for the preparation of chiral building blocks.

2. Methods

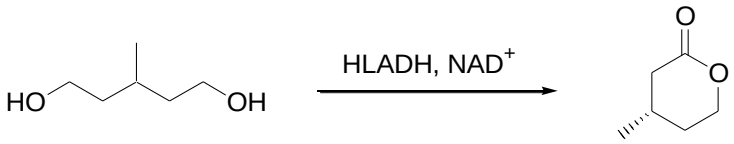
Commercially available reagents were used without further purification unless stated otherwise. All solutions were mixed and thermostated by a Thermomixer comfort (Eppendorf AG, Germany). GC analyses were performed on a Shimadzu GC-2010 plus, columns Chiraldex GTA (30 m x 0.25 mm x 0.12 μm) for HLADH and MEGA-DEX DET beta (25 m x 0.25 mm x 0.25 μm) for *Ts*ER, detector FID.

XoCH preparation: choline chloride (C) was recrystallized from absolute ethanol, filtered and dried under *vacuum*. Xylitol (Xo) was dried under *vacuum* prior use. The components were mixed with the calculated amount of distilled water (H) (1:2:3 ratio) heated at 50 °C and stirred until a clear and viscous liquid was formed (after about 60-90 min). The liquid was filtered under *vacuum*, used without further purification, and stored at 4 °C.

3. Results and Discussion

3.1. HLADH catalyzes the lactonization of 3-methyl-1,5-pentanediol

The stability of HLADH was assessed in XoCH by means of UV/Vis spectroscopy measuring the formation of NADH. XoCH caused a remarkable decrease of enzyme stability when mixed at 25% and 75% in-amino-2-(hydroxymethyl)propane-1,3-diol (TRIS)-HCl. Only in XoCH/TRIS-HCl 75%, the stability of enzyme ameliorated respect to pure buffer. Subsequently, the synthesis of (*S*)-3-methyl-δ-valerolactone from 3-methyl-1,5-pentanediol catalysed by HLADH [4] was performed initially in a watery solution of 2-amino-2-(hydroxymethyl)propane-1,3-diol (TRIS)-HCl, and then introducing the NADES (XoCH/TRIS-HCl mixtures) at various concentrations, until finally reaching the pure NADES (100%). A reaction temperature of 30 °C was chosen in order to optimize the activity of HLADH and to decrease NADES viscosity. The reaction was followed for 24 h by means of GC, highlighting the disappearance of the 3-methyl-1,5-pentanediol, and the formation of the (*S*)-3-methyl-δ-valerolactone (Scheme 1).



Scheme 1. Reaction conditions: [substrate] = [cofactor] = 5 mMn [HLADH] = 0.2 mg/mL, TRIS-HCl or mixtures XoCH/TRIS-HCl, 30 °C, 24 h.

The formation of the lactone in enantiomerically pure form as a function of solvent composition is shown in Figure 1. The curve regarding 100% of XoCH is missing, as no

reaction has occurred.

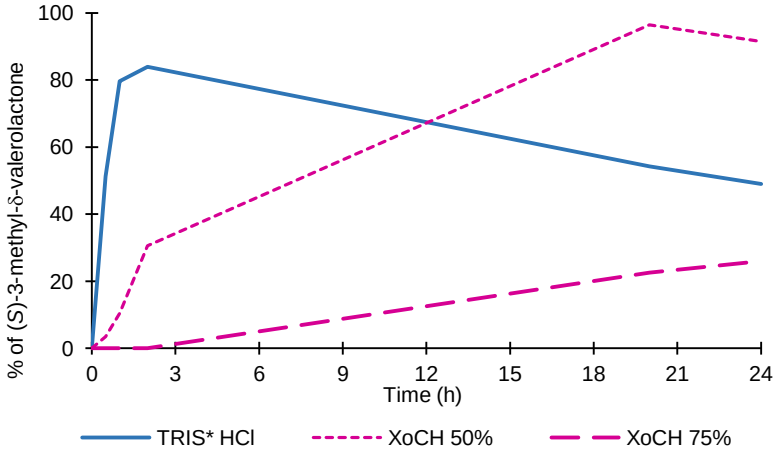
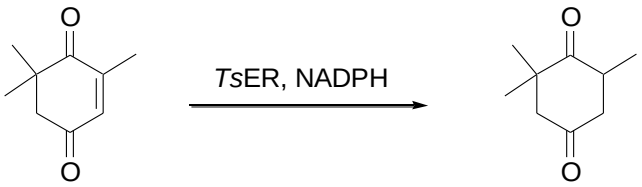


Figure 1. Synthesis of 3-methyl-δ-valerolactone *via* HLADH in TRIS-HCl and XoCH/TRIS-HCl (25%, 50%, and 75% w/w).

A full conversion of substrate was obtained in 100% TRIS-HCl after 2 h. Subsequently, a slight decrease in the concentration of the final product was observed. With 50% XoCH/buffer, the formation of the product was more gradual, reaching a maximum concentration value after 20 h. As the percentage of NADES increases (75%), the concentration of final product is greatly reduced. The evaluation of the enantioselectivity also showed that the reaction was stereospecific; in particular, going from 0 to 50% of NADES there was a substantial increase of stereoselectivity (99%), which was maintained until finally reaching 75% of NADES.

3.2. TsER catalyzes the reduction of ketoisophorone

Similarly to what was conducted for the previous enzyme, the stability of *Ts*ER was assessed in XoCH at concentrations to be used accordingly. The results were satisfactory and no substantial changes of enzyme were detected. The bioreduction of ketoisophorone catalyzed by *Ts*ER give the (*R*)-levodione as the prevailing product when the reaction was conducted in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer with $CaCl_2$ at pH 7.0 [8], and in MOPS with increasing concentrations of XoCH, until finally reaching the pure XoCH at 40 °C. The reaction was followed for 24 h by means of GC, highlighting the disappearance of the ketoisophorone and the formation of the levodione as the sum of both enantiomers (Scheme 2).



Scheme 2. Reaction conditions: [substrate] = 10 mM, [NADH] = 15 mM, [*Ts*ER] = 1 mg/mL, MOPS, mixtures XoCH/MOPS or pure XoCH, 40 °C, 24 h.

The formation of the levodione as a function of solvent composition is reported in

Figure 2.

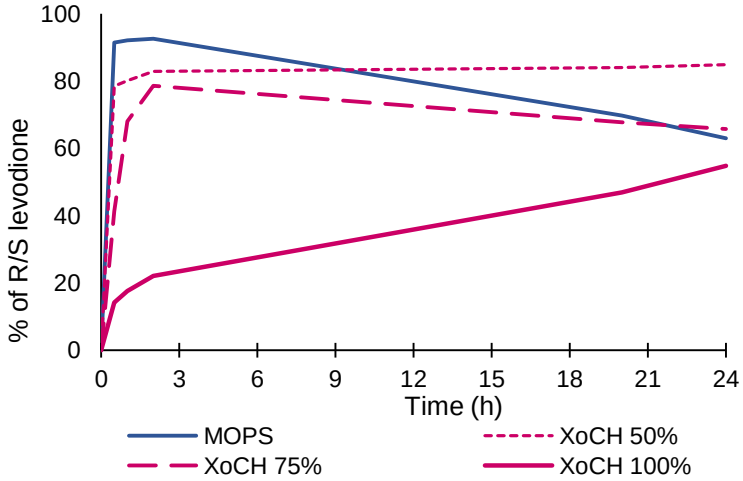


Figure 2. Synthesis of levodione *via Ts*ER in MOPS, XoCH in MOPS (50%, 75% w/w) and pure XoCH.

The full conversion of ketoisophorone into *R*/*S*-levodione was obtained with pure MOPS. With 50% and 75% of XoCH/buffer, the formation of the product was quite complete in 2 h, with a decrease in the concentration of final product in the case of 75% of NADES after 20 h. With pure XoCH, the formation of levodione decreased, although a progressive increase in concentration was observed over the 24 h of reaction. The evaluation of the enantioselectivity of the reaction also showed that the *R*/*S* ratio of levodione slightly increased with NADES concentration, reaching high values above 75% of XoCH.

4. Conclusions

In summary, we conducted two enantioselective reactions: the lactonization of 3-methyl-1,5-pentanediol and the reduction of ketoisophorone catalyzed by the redox enzymes HLADH and *Ts*ER, respectively, with a XoCH as solvent at various concentrations (mixed with buffer). In both cases, we have noticed that NADES favors the activity of the two enzymes and stereoselectivity, but only at defined concentrations. Overall, these results suggest that NADES possess good biocompatibility with studied enzymes and could contribute to create environmentally friendly processes.

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