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Natural deep eutectic solvents as thermostabilizer for *Humicola insolens* cutinase

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ABSTRACT

As a new generation of green solvents, deep eutectic solvents (DESs) are considered a promising alternative to current harsh organic solvents and find application in many chemical processing methods such as extraction and synthesis. DESs, normally formed by two or more components via various hydrogen bond interactions, offer high potential as medium for biocatalysis reactions where they can improve efficiency by enhancing substrate solubility and the activity and stability of the enzymes. In the current study, the stabilization of *Humicola insolens* cutinase (HiC) in natural deep eutectic solvents (NADESs) was assessed. The best hydrogen bond donor among sorbitol, xylitol, erythritol, glycerol and ethylene glycol, and the best acceptor among betaine, choline chloride, choline acetate, choline dihydrogen citrate and tetramethylammonium chloride, were selected, evaluating binding energies and molecular orientations through molecular docking simulations, and finally used to prepare NADES aqueous solutions. The effects of component ratio and NADES concentration on HiC thermostability at 90 °C were also investigated. The choline dihydrogen citrate:xylitol, in a 1:1 ratio with a 20 wt% concentration, was selected as the best combination in stabilizing HiC, increasing its half-life three-fold.

Introduction

Cutinases (EC 3.1.1.74) are small extracellular serine hydrolases produced by bacteria and fungi generally catalyzing the hydrolysis of the cutin, an insoluble lipid polyester which acts as structural component of plant cuticles [1]. Compared to other hydrolase family members such as lipases and esterases, cutinases have the smallest molecular weight (20–30 kDa) with a three-dimensional structure characterized by a central parallel β-sheet and surrounding α-helices. Furthermore, although both cutinases and lipases exhibit the Ser-His-Asp catalytic triad in the active site, lipase activity is subjected to interfacial activation due to the presence of a lid which opens at a lipid/water interface. In contrast, cutinases have solvent-exposed catalytic triads and therefore do not undergo this type of conformational change, showing enhanced substrate access and increased activity [2]. Moreover, cutinases present better selectivity than lipases and can act as stereoselective catalysts in esterification and transesterification reactions [3].

Recently, cutinases from several fungal sources have been applied as natural biocatalysts on different synthetic aliphatic polyesters such as polybutylene succinate (PBS), polycaprolactone (PCL), polylactic acid (PLA), poly(butylene succinate-co-adipate) (PBSA) polyethylene terephthalate (PET) [1,4,5], and polyurethanes [6]. Cutinase from *Fusarium sp*. was able to degrade PBS through a surface erosion mechanism [7] whilst *Humicola insolens* cutinase (HiC) was the only enzyme among the several lipases screened to be able to hydrolyze PBS and PBSA [5]. PCL degradation has been reported for several cutinases among others, namely: the above mentioned HiC [5], a cutinase from *Myceliophthora thermophila* (MtCUT) [8], and a bifunctional lipase-cutinase (Lip-Cut)

Abbreviations: Bet, Betaine; ChAc, choline acetate; ChCl, choline chloride; ChDHC, choline dihydrogencitrate; Ery, Erythritol; EtG, Ethylene glycol; Gly, Glycerol; HDA, Hydrogen bond acceptor; HBD, Hydrogen bond donor; NADES, Natural Deep Eutectic Solvents; Sor, Sorbitol; TMACl, tetramethylammonium chloride; Xyl, Xylitol; wt%, weight percentage.

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constructed by end-to-end fusion of the genes from *Thermomyces lanuginosus* lipase and *Thielavia terrestris* cutinase [9].

Cutinases from the thermophilic strains *Thermobifida cellulosilytica* (Thc_Cut1), *Thermobifida fusca*, and *Thermobifida alba* have been reported to hydrolyze the non-biodegradable PET to monomers of terephthalic acid (TPA) and ethylene glycol, allowing reaction conditions close to the glass transition temperature (Tg) of the polymer (75–80 °C) [10].

Interestingly, the Thc_cut1 from *T. cellulosilytica* and HiC cutinases have been shown to catalyze a ring-opening polymerization of lactones as well as efficiently synthesize aliphatic polyesters, opening up the possibility for these enzymes to be employed in the synthesis of polyesters from recycled monomers in novel closed-loop recycling technologies of postconsumer PET waste [11]. Although cutinases can perform degradation of plastics under milder conditions compared to those necessary for the chemical hydrolysis, their activity starts to decrease at temperatures higher than 45 °C due to their low stability [12] generating the need to reengineer the enzyme to improve its properties. To address heat and operational stability of this class of enzymes, several approaches have been pursued using directed or site-specific mutagenesis, metagenomics and immobilization tools [4,13].

Deep eutectic solvents (DESs) are a prominent class of liquid solvents obtained combining two or more components, which results in a significantly lower melting temperature compared to either one of its individual components due to strong interactions [14]. DESs are classified into five categories (Types I to V), where mixtures composed of a hydrogen bond acceptor (HBA), such as an ammonium salt, and hydrogen bond donor (HBD), such as polyols and sugars (Type III) are the most studied [15]. When DESs are prepared solely using naturally occurring compounds (such as sugars and amino acids), these are termed NADESs [16,17]. Besides their low flammability, low volatility, high thermal stability, facile preparation, reduced cost, and biodegradability, the main interest in NADESs originates from the wide scope of potential applications as cosolvent or reaction medium in a broad range of enzymatic reactions, resulting in higher product conversion and enhanced enantioselectivity [14,18]. Furthermore, incubation of enzymes in NADESs can be suggested as an alternative *post-synthesis* approach to avoid their thermal inactivation at high temperatures [19,20], which is typically observed in organic solvents [21].

One of the most useful property of NADES solvents is their tunability, meaning that numerous combinations of HBA and HBD in different ratios can be designed, giving the opportunity for rational solvent design to meet specific aims [22,23]. In this regard, the ratios between HBA and HBD components has been found to affect both the activity and stability of different class of enzymes [24,25]. Although there are several reports on the stabilization of lipases when incubated in NADESs [22,26,27], there is a lack of data regarding the study of cutinase behaviour in these solvents. One report [2] described a 200% activation of AmCut cutinase from *Amycolatopsis mediterannei* in the presence of 10% v/v cholinium DES based on glycerol as HBD. Moreover, AmCut maintained 100% activity after incubation at 50 °C for 2 h when incubated in 50% DES (choline chloride: glycerol; 1:1 molar ratio).

Based on our previous results on laccase stabilization using NADESs aqueous solutions [19], a computer aided approach was applied to select NADES components for the thermal stabilization of HiC cutinase. The latter was chosen as one of the most studied members of the cutinase family, finding promising applicability in the hydrolysis of a variety of polyesters such as PBS, PBSA and PCL [5,10], insoluble triacylglycerols and low-molecular weight soluble esters [28]. The best hydrogen bond donors and acceptors were selected from different candidates, evaluating binding energies and molecular orientations through molecular docking simulations and then used to prepare NADESs aqueous

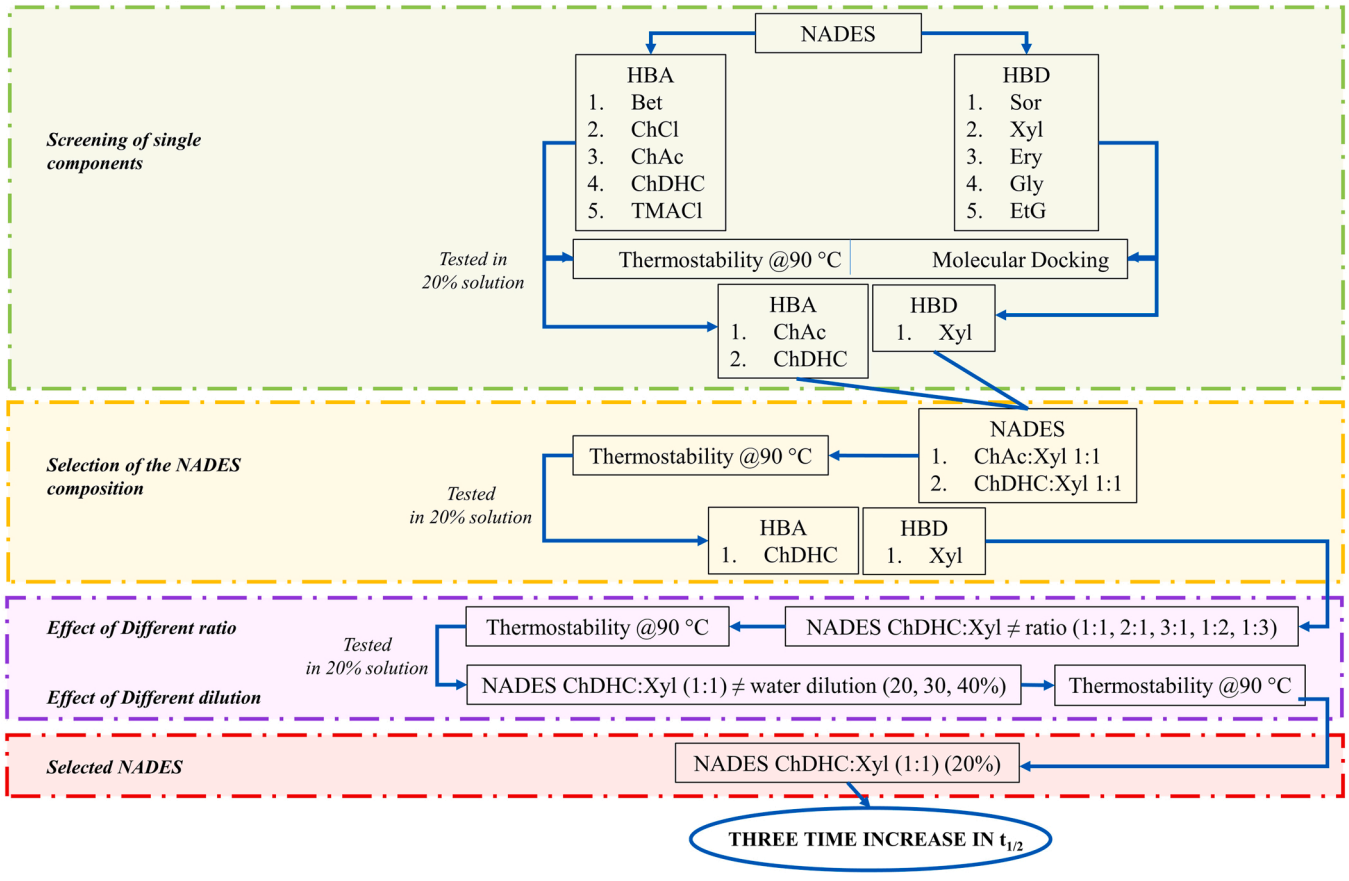


Fig. 1. Flowchart showing the rationale of the study and the main output.

solutions. The best ratio between selected molecules and the NADES percentage in water were also determined to improve HiC thermostability at 90 °C. The rationale of the study, as well as the main outputs, are showed in the flowchart in Fig. 1.

Materials and methods

Materials

Sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), betaine, choline chloride, choline acetate, choline dihydrogen citrate, tetramethyl ammonium chloride, ethylene glycol, glycerol, erythritol, xylitol, sorbitol, p-nitrophenyl butyrate (p-NPB, ≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cutinase from *Humicola insolens* (product Novozym©51032) was provided by ChiralVision as a solution (activity 28,000 U/mL). All reagents were used as received.

Preparation of NADES

For each hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) (Table 1), a 20 wt% solution in 0.1 M sodium phosphate buffer (pH 7.8) was prepared. HBA and HBD were chosen based on literature on the molecules used most frequently in NADES preparation. Betaine was considered as a zwitterion.

To prepare NADES, the HBA and HBD mixtures were heated for 10 min at 90–100 °C in an oil bath and shaken; this was repeated until a homogeneous clear liquid formed. In particular, the following NADES were prepared (Table 1): choline acetate/xylitol (ChAc:Xyl 1:1 molar ratio) and choline dihydrogen citrate/xylitol (ChDHC:Xyl 1:1). Once selected the ChDHC:Xyl from the first screening, different ratios of ChDHC:Xyl (1:2, 1:3, 2:1, 3:1 molar ratio) were prepared and tested. Then, solutions of aqueous ChDHC:Xyl (1:1) in 0.1 M sodium phosphate buffer (pH 7.8) were prepared in various concentrations (20, 30 and 40 wt%).

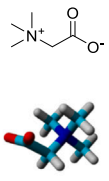
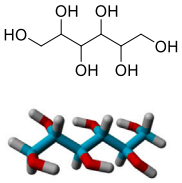
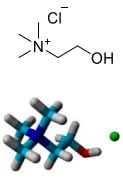
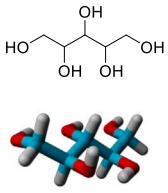
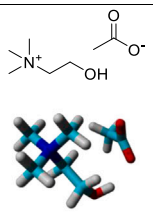
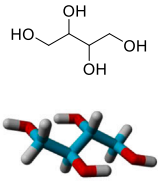
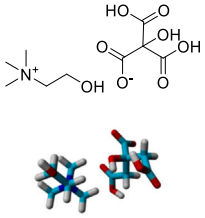
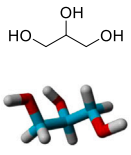
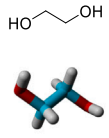
Cutinase stability in aqueous NADES solutions

A diluted enzyme solution was prepared in 0.1 M sodium phosphate buffer pH 7.8 (1: 100 cutinase stock solution). A small volume (50 µL) of the same diluted enzyme solution was added to phosphate buffer aqueous solutions with or without HBDs, HBAs and NADESs (5 mL). The initial enzymatic activity of all cutinase solutions was measured via the continuous spectrophotometric method using 4-hydroxy phenyl butyrate (p-NPB) as substrate. The solutions were incubated in a water bath previously thermostated at 90 °C. After 30 min, the solutions were cooled immediately on ice for few seconds and the residual enzymatic activity was measured and compared to the initial activity. All tests were run in duplicate and Tukey's test was used to compare and find means that were significantly different from each other.

In greater detail, 0.1 mL of each sample was added to 3 mL of sodium potassium phosphate buffer (0.1 M, pH 7.8) containing 1 mM of 4-NPB. In the presence of the enzyme, this substrate is rapidly hydrolysed into butyric acid and 4-nitrophenol, formation of which can be spectrophotometrically measured at 410 nm over time. In particular, the absorbance (Abs410nm) was continuously monitored using a UV-Vis spectrophotometer thermostated at 25 °C, and the absorbance per min was obtained using the maximum linear rate. One unit of enzyme is defined as the amount that hydrolyses 1 µmol of 4-nitrophenyl butyrate to butyric acid and 4-nitrophenol per min under the assay conditions.

For the evaluation of the half-life, after different incubation times (0.5, 1, 3, 5, 9, 15, 22.5 and 30 min) at 90 °C, an aliquot (500 µL) of the solution was taken and cooled immediately on ice for few seconds, and the residual activity [A] was measured by p-NPB assay and compared to the initial activity [A^0]. Half-life of enzyme was calculated according to a second order kinetic ($r^2 = 0.99$) as described in Eq. (1):

Table 1
Chemical structure, composition, nomenclature, and molar ratio of HBAs, HBDs and NADESs applied in this study.

HBA	HBD
 Betaine (Bet)	 Sorbitol (Sor)
 Choline Chloride (ChCl)	 Xylitol (Xyl)
 Choline Acetate (ChAc)	 Erythritol (Ery)
 Choline Dihydrogencitrate salt (ChDHC)	 Glycerol (Gly)
 Tetramethylammonium Chloride (TMACl)	 Ethylene glycol (EtG)

NADES	Molar ratio
ChAc:Xyl	1:1
ChDHC:Xyl	1:1; 1:2; 1:3; 2:1; 3:1

$$t_{1/2} = \frac{1}{(k \times [A^0])} \quad (1)$$

where k is the kinetic constant, i.e., slope obtained by plotting 1/[A] versus time; A^0 is the initial enzyme activity [29].

Computational analyses of cutinase -HBA and -HBD interactions

Small molecule docking (SMD) and protein visualization were performed using the YASARA Structure [30]. The crystal structure of *Humicola insolens* cutinase (PDB: 4OYY) was energy minimized, optimizing the amino acid side chains to obtain a lower free energy of the protein by removing van der Waals clashes and negative contacts. Receptors were then cleaned, and their structures were energy minimized using AMBER14 force field. The ligand 3-dimensional structures of HBD (sorbitol, xylitol, glycerol, ethylene glycol, and erythritol) and HBA (betaine, choline chloride, choline acetate, choline dehydrogenate salt, tetramethylammonium chloride) were built from their SMILES strings and cleaned, and their geometry was optimized by the YASARA Structure. The binding energies were calculated for all the ions constituting the HBAs ($[DHC]^-$, $[Ch]^+$, $[Ac]^-$, $[Cl]^-$, $[TMA]^+$) [24]. SMD was applied on cutinase by Autodock VINA performing 25 docking runs per simulation which are clustered into distinct conformations, differing by at least 5.0 Å heavy atom RMSD after superposing on the receptor. The best binding model was evaluated in terms of binding energy (more positive energies indicate stronger binding, and negative energies mean no binding) and dissociation constant, both expressed as kcal mol^{-1} [31].

Statistical analysis

Pearson correlation coefficients (r) to measure the strength of the linear relationship between residual activity of cutinase incubated in NADESs at 90 °C and binding energy spread of the best cutinase-NADES molecules complex conformation were calculated using the Statistical Package for the Social Sciences (SPSS19, SPSS Inc., USA) software.

Results and discussion

Effect of HBAs and HBDs on cutinase thermostability

The NADES effect on thermostability of several enzymes has been widely studied in the past few years, but it is difficult to find a clear trend between the different NADESs, since this stability is highly influenced by the nature of NADES components [32]. In the present study, it was decided to start the screening from the evaluation of cutinase thermostability in the individual NADES components. To the best of our knowledge, this is the first time that a similar approach has been pursued for the evaluation of cutinase stabilization in NADES.

Different HBAs and HBDs were tested (Table 1), namely betaine, tetramethylammonium chloride, choline chloride, choline acetate and choline dihydrogen citrate as HBAs, and ethylene glycol, glycerol, erythritol, xylitol and sorbitol as HBDs. These components were chosen based on previous studies of enzyme stabilization in DES. In the case of HBAs for example, [20], [25], and [19] observed good stability of different laccases in betaine-based DES, whereas choline chloride was a good choice for the stabilization of cutinase from *Amycolatopsis mediterannei* [2] and β-glucosidase from *Streptomyces griseus* [33]. Choline acetate and choline dihydrogen citrate were selected based on [34] and [24], respectively. Tetramethylammonium chloride was added to the studied HBAs since it is reported as useful in protein extraction [35]. The HBDs were polyols with different amount of hydroxyl groups. Based on [20], the increasing number of hydroxyl groups could affect laccase stability, thus the same criterion was applied here.

Since these products are solid or very viscous liquids, aqueous solutions were prepared by diluting them in 0.1 M phosphate buffer (20 wt %). The thermostability of HiC cutinase was evaluated by incubating the enzyme in the aqueous solutions of HBAs and HBDs at 90 °C for 30 min and the results are reported in Fig. 2 in terms of residual activity. In the case of HBAs (Fig. 2a), the best results in terms of cutinase thermal stabilization are given by ChDHC where cutinase retained 58% of its initial activity, followed by ChAc where 44% residual activity was measured. Lower values were found in Bet and TMACl, where the residual activity was 9%, and in ChCl, where it was 20%, in all cases lower than the residual activity measured in phosphate buffer.

Comparing HBA having the same $[Ch]^+$ counterion (ChCl, ChAc, ChDHC), the presence of $[Ac]^-$ and $[DHC]^-$ contribute most to the enzyme stabilization, whilst all the $[Cl]^-$ containing HBA determine a drop in cutinase residual activity. Notably, this is in contrast with results reported by others [2] who observed an increase in AmCut stability after its incubation in 14% ChCl at 37 °C. This suggests that the HBA stabilization effect is enzyme sensitive. Indeed, a great activity enhancement of laccase in ChDHC, and an inhibiting effect in ChCl have been observed [24], whilst, in contrast with our results, betaine was found to be a very effective stabilizer for laccases [19,20], with a higher efficiency than ChDHC [20]. In the case of Hic cutinase, considering that ChAc and betaine have similar groups to interact with the enzyme, the higher residual activity obtained with ChAc compared to the betaine could indicate that ions might be a better choice than zwitterion.

According to these results, ChAc and ChDHC were the best HBA components for cutinase stabilization.

In the case of HBD solutions, the residual activity of HiC cutinase after incubation at 90 °C was in almost all cases not significantly higher than that in phosphate buffer, where cutinase retained 29% of its initial activity (Fig. 2b). A lower value was obtained in EtG, where the enzyme preserved 6% of its activity. For further analysis, Xyl was selected as the most promising HBD component.

Computational analysis of cutinase – HBA and - HBD interactions

Small molecule docking calculations were performed to deepen the role of NADES components in the stabilization of cutinase towards thermal degradation. The binding energy spread (kcal mol^{-1}) of the best conformation acquired by cutinase-NADES molecules complex was calculated for every couple of cutinase and NADESs component (Fig. 3). These values were compared with the results from the residual activity measurements at 90 °C (Fig. 2). A correlation between the residual activities and the binding energy was found for all the tested HBAs ($r = 0.725$) and HBDs ($r = 0.857$), where the more positive is the binding energy, the higher is the residual activity.

The localization of NADES components on cutinase structure is displayed in Fig. 4. The binding energies in the best binding configuration for all the HBDs- and HBAs- enzyme couples, along with interacting amino acids and type of interaction, have been identified and reported in the Supplementary materials (Table S1).

The docking binding energies between the ions composing HBAs and cutinase increase in the order $[DHC]^- > Bet > [Ch]^+ > [Ac]^- > [TMA]^+ > Cl^-$ (Fig. 3). In more detail, $[Ch]^+$ and $[Ac]^-$ establish both hydrogen bonds (glutamine and serine) and hydrophobic interactions (glycine, glutamine, leucine, isoleucine) with cutinase. $[DHC]^-$ is the only HBA constituent that establishes a single hydrogen bond with glutamine, while Bet and $[TMA]^+$ are involved in hydrophobic interactions only (serine, threonine leucine, isoleucine, histidine and glycine). Analyzing the positions where NADES components interact, all the tested HBAs localize close to the entrance of the active site. Although being the HBA involved in a single hydrogen bond with GLN136, $[DHC]^-$ shows the highest binding energy from docking simulations. Moreover, GLN136 side chain is involved in hydrogen bond with $[Ch]^+$, along with hydrophobic interactions with other aminoacids of the same region. It is worth noting that this residue is not conserved among the sequences of other known cutinases (see alignment S2), suggesting further mutagenesis to confirm its crucial role in NADES-mediated stabilization. $[Ch]^+$, $[Ac]^-$ and $[TMA]^+$ establish hydrophobic interactions with the

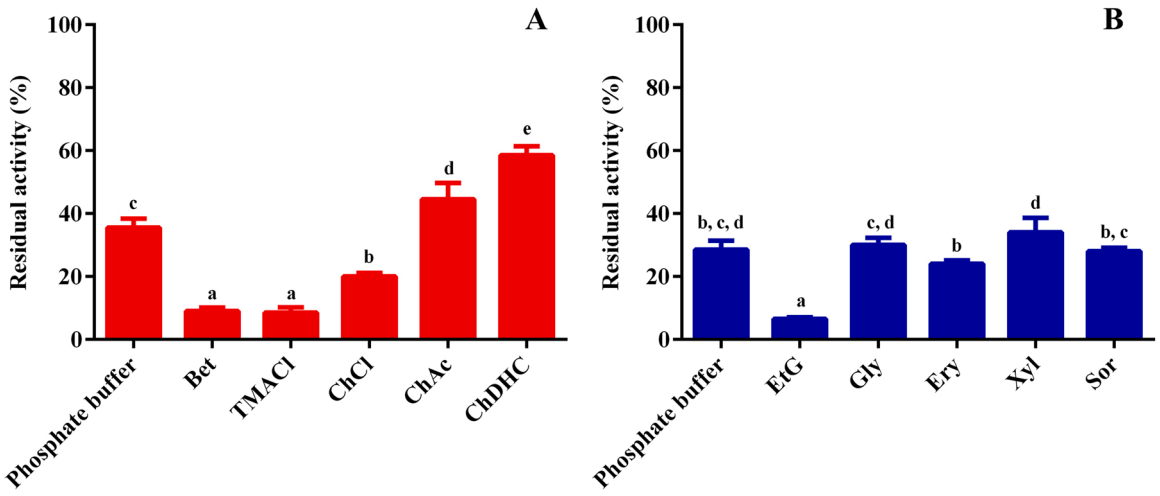


Fig. 2. Comparison of cutinase thermostability after 30 min at 90 °C in 0.1 M phosphate buffer pH 7.8 and in HBA (a) and HBD (b) aqueous solutions (20 wt% in 0.1 M phosphate buffer). The residual activity (%) is obtained by comparing the activity after 30 min with the initial activity in the same solution (control, 100%). (Lowercase letters indicate the result of Tukey's test; means sharing the same letter belongs to the same group, so the differences between those means are not significant).

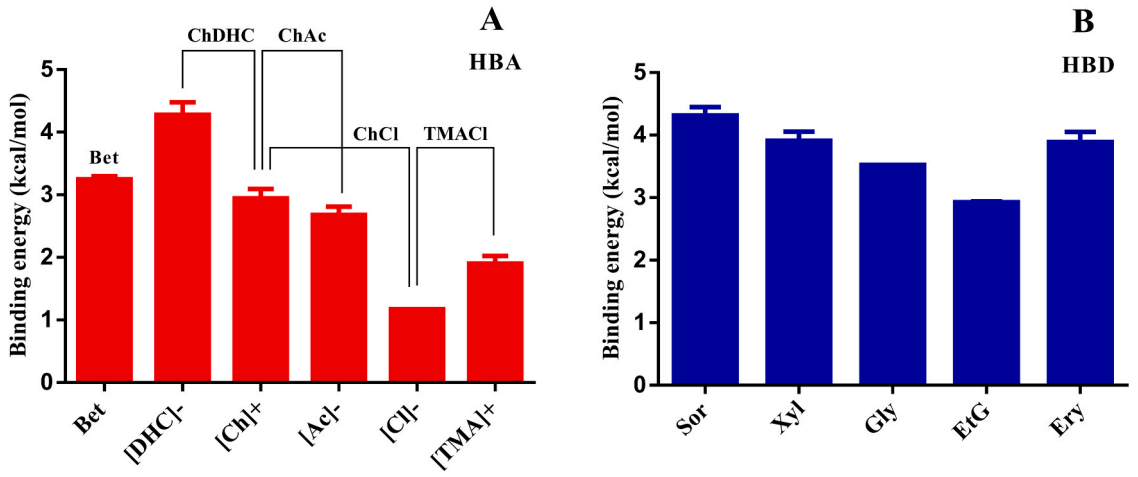


Fig. 3. Binding energies (kcal mol^{-1}) between cutinase and HBA (A) components and HBDs (B) calculated using YASARA structure.

region of the active site entrance with the side chain of LEU167. On the other hand, Bet is the HBA that establishes the greatest number of interactions with the cutinase surface, in particular hydrophobic interactions with ILE169.

The experimental results and the docking simulation suggest that ChAc and ChDHC give the highest contribution to the enzyme stabilization. This is consistent with the highest binding energies observed for $[Ch]^+$, $[DHC]^-$, and $[Ac]^-$. Although Bet also gives a similar energy contribution, this does not correspond to a comparable stabilization effect. The higher cutinase stabilization due to ChDHC and ChAc could be the result of their synergic action on the cutinase conformation. Therefore, further investigations were carried out for choosing between ChDHC and ChAc as HBAs.

All the tested HBDs make hydrogen bonds with amino acids side chains of cutinase, in particular with glycine, serine, threonine, aspartic acid and glutamine. The only exception is Xyl, which establishes only hydrophobic interactions with side chains of glycine and leucine. In addition, Xyl, Sor, and Ery show a similar contribution to the binding energy, which is slightly higher for Sor. Gly contributes to a less extent to the binding energy stabilization, whilst EtG displays the worst stabilizing effect in terms of binding energy. In particular, the values of binding energy of each HBD – cutinase increases in the order EtG < Gly < Ery < Xyl < Sor, in correlation with the increasing number of hydroxyl groups of the polyol. Therefore, it is evident that the stabilizing effect is dependent on the number of hydroxyl groups present in the HBDs, in agreement as previously reported in [24] and [19] using docking simulations between various HBDs and laccases. Furthermore, in agreement with their findings, it seems that there is no correlation between the number of interactions and the predicted stabilizing effect. All the tested HBDs localize on the surface of cutinase. In particular, Xyl, Gly and Ery establish hydrophobic interaction with the region of the active site entrance with the side chain of LEU167, confirming this aminoacid as the most involved in the interactions with NADESs components. Interestingly, as previously observed for GLN136, this residue is not conserved among known cutinases. However, Gly locates in the active site of the cutinase, interacting through a hydrogen bond with SER105 which is one of the aminoacids involved in the enzyme catalytic triad. This could explain how Gly does not have the best stabilizing effect on cutinase in terms of binding energy. Conversely, Sor and EtG locate on the protein surface in a different position than the other HBDs, on the side of the active site pocket. In particular, Sor interacts with GLY32 through two hydrogen bonds and GLY59 and GLY60, through hydrophobic interactions. EtG establishes a hydrogen bond with ASP63 and several hydrophobic interactions with ASP63, ALA65, THR68. Although

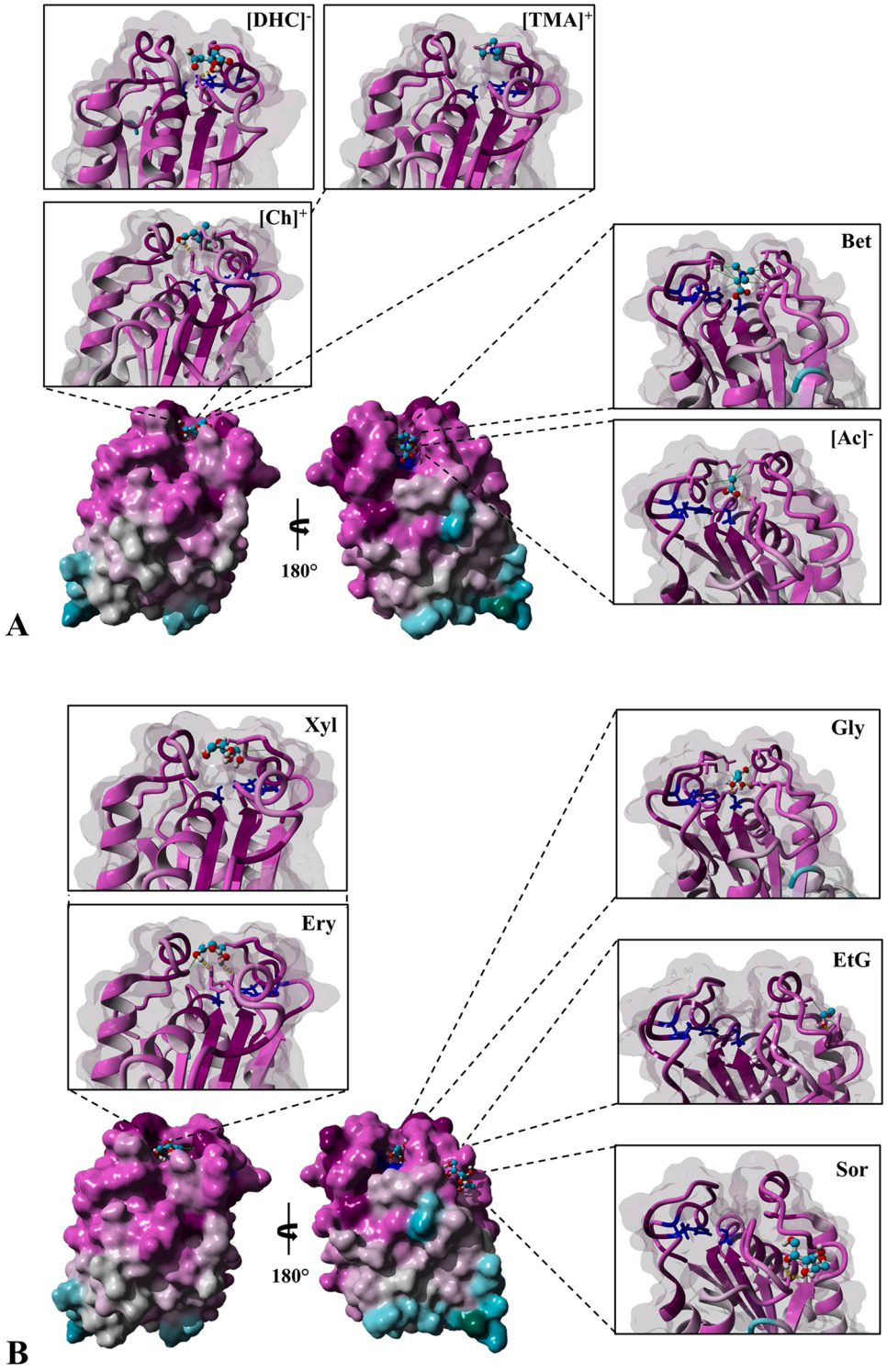


Fig. 4. Docking poses of the highest binding energies between cutinase and HBA components (A) and HBDs (B). Surface of cutinase is shown using ConSurf color-code [42]; conserved aminoacids are bordeaux, average conserved residues are white, variable aminoacids are turquoise.

located in the same region of the enzyme, the interaction of the two HBDs results in a completely different stabilizing effect; Sor has the highest binding energy whilst EtG the lowest one, confirming that the stabilization effect is not dependent on the number of interaction but rather on the molecule orientation.

Unlike the data achieved with HBAs, the calculated binding energies and the measured residual activities with all the HBDs display small differences, except for EtG. For this reason, to choose the HBD for further investigation, the results from experimental and computational data were integrated, thus confirming Xyl as the best candidate for NADES formulation.

Effect of HBA and HBD molar ratio on cutinase thermostability

Considering that ChAc and ChDHC gave both good results in terms of thermostability of cutinase, they were combined with Xyl (the best HBD)

in 1:1 molar ratio, and tested at 20 wt% concentration in phosphate buffer. The thermostability of cutinase was again evaluated by incubating it at 90 °C for 30 min, and the residual activity was measured. The ChDHC:Xyl NADES gave the best thermostability to HiC cutinase, which retained 59% of its activity (Fig. 5A), whereas the ChAc:Xyl NADES had the same effect of phosphate buffer alone, and cutinase preserved only 34% of its initial activity.

From these results, the NADES based on ChDHC was chosen to continue the screening of different parameters. In particular, the molar ratio between ChDHC and Xyl was modified to observe if the excess of one of the two components could have an impact on the enzyme thermostability. The following ChDHC:Xyl molar ratios were screened: 1:1, 2:1, 3:1, 1:2 and 1:3 keeping NADES concentration at 20 wt%. The highest effect on enzyme stabilization was displayed in ChDHC:Xyl 1:1, 3:1 and 1:2 ratios where cutinase retained about 54–61% of initial activity (Fig. 5B).

Regarding ChDHC:Xyl 1:3, the residual activity was lower (43%), and in ChDHC:Xyl 2:1 cutinase preserved the same activity as in phosphate buffer (34%). The excess of ChDHC or Xyl did not show a linear trend, as already reported by [34], where no clear relationship was observed between activity/stability data of *Penicillium expansum* lipase and different HBA/HBD molar ratio in choline acetate-based DES solutions. Moreover, it needs to be considered that the HBA/HBD ratio can have an impact on the different interactions not only within the NADES itself, but also between the NADES components and the enzyme [20].

Since the molar ratio did not have a great impact on the thermostability of cutinase, it was considered not necessary using an excess of HBA or HBD, and we chose to pursue the study using a ChDHC:Xyl molar ratio of 1:1.

Effect of NADES concentration on cutinase thermostability

Another parameter that can have an impact on enzyme thermostability in NADES is their concentration in the reference solution, in this case phosphate buffer 0.1 M. Indeed, although NADESs can be ideal media for enzymatic reactions, they can hardly be utilized on industrial scale because of their high viscosity, and a co-solvent is often necessary [36,37]. In the present study, a 20 wt% concentration was chosen to start the screening. Next, the presence of NADES in the buffer was increased up to 30 and 40 wt% to observe the effect on cutinase thermostability. It has been demonstrated that the DES structure and properties are preserved when water is present as co-solvent up to 20 wt%. From 20–50 wt% water content, DES is present as clusters dispersed in an aqueous phase as co-solvent, and with water amount above 50 wt%, the aqueous solution appears as an electrolyte mixture of DES components [38]. For this set of experiments, since the water amount is always

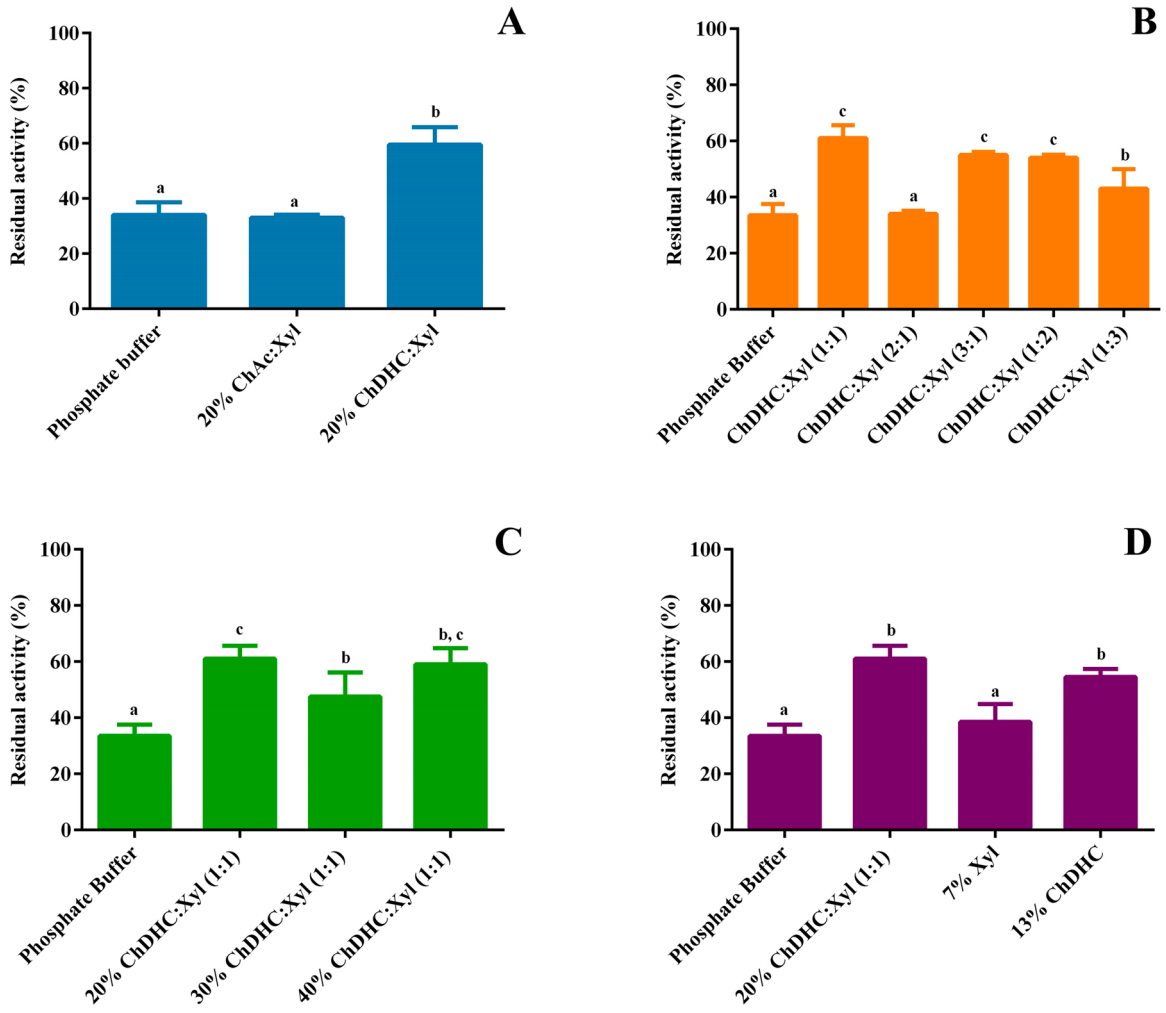


Fig. 5. Comparison of cutinase thermostability after 30 min at 90 °C in 0.1 M phosphate buffer pH 7.8 and in: A) ChAc:Xyl (1:1) and ChDHC:Xyl (1:1) aqueous NADES (20 wt% in 0.1 M phosphate buffer); B) ChAc:Xyl aqueous NADES (20 wt% in 0.1 M phosphate buffer) with different molar ratios; C) ChDHC:Xyl (1:1) aqueous NADES with different weight percentage in 0.1 M phosphate buffer (20, 30 and 40 wt%); D) NADES single components. The residual activity (%) is obtained by comparing the activity after 30 min with the initial activity in the same solution (control, 100%). (Lowercase letters indicate the result of Tukey's test; means sharing the same letter belongs to the same group, so the differences between those means are not significant).

above 50 wt%, the NADES is supposed to behave as an aqueous electrolyte solution of choline dihydrogen citrate and xylitol. Data in Fig. 5C revealed that the amount of NADES used had no significant impact on cutinase thermostability, with an average value of about 50% residual activity retained after 30 min incubation at 90 °C.

The results showed that despite the amount of NADES, the thermostability is always improved compared to buffer solution, being the effect quite similar for all the tested concentrations. As already aforementioned, NADES should behave as an electrolyte aqueous solution in all the three cases tested in this study. Uhoraningoga screened different NADES percentages (10–60% v/v) on the thermal stability of β-glucosidase from *Streptomyces griseus* and observed the best effect with 40 wt% NADES [33]. The authors reported that at such concentration, the individual DES components are largely dissociated, suggesting that the enzyme stabilization could be due to a direct interaction between DES components and the enzyme surface, thus preventing enzyme aggregation and ultimately enhancing the thermostability. Some researchers have referred to this stabilization in DES as a type of coating or "supramolecular net" that protects the enzyme from unfavourable ionic interactions [33,39].

These findings can be explained considering that all the molecules involved in protein interaction (HBA, HBD and water) could saturate the enzyme surface even at the lowest NADES concentration or that any additional interaction occurring at the highest concentration could not further affect its stability. From an applicative point of view, this result can turn out into an advantage, being the enzyme stability not sensible to fluctuation in DES concentration, thus widening the range of operative conditions in which this enzyme may work, depending on the catalyzed reaction and/or type and concentration of substrates. However, for the purpose of this work, further investigation was carried out using a NADES concentration of 20 wt%, since a higher dilution is preferable in terms of costs.

Effect of NADES components on cutinase thermostability

In order to evaluate whether the thermostability enhancement in 20 wt% ChDHC:Xyl 1:1 was due to the single components or to the NADES itself, ChDHC and Xyl solutions were prepared at 7 wt% Xyl and 13 wt% ChDHC, corresponding to the same molar concentration of the two components in the NADES. The residual activity of cutinase after incubation at 90 °C in these solutions is shown in Fig. 5D. Compared to the residual activity in phosphate buffer, no improvement was observed in Xyl aqueous solution (39% residual activity), whereas the enzyme thermostability was improved when incubated in ChDHC solution (55% residual activity).

It is worth noting that the residual activity ChDHC is not significantly different from the one observed in the NADES aqueous solution. A similar behavior is reported by [2] where the stability of cutinase from *Amycolatopsis mediterannei* was studied in choline chloride-based DES. Indeed, by keeping the same concentration of ChCl in the HBA solution and DES solution, the enhancement of activity is not significantly different in the choline chloride solution and the choline chloride-based DES at the same concentration, confirming that the greatest contribution to cutinase stability in NADES does not come from the HBD, but from the HBA component. Although the result was not predictable a priori, it can be explained looking more deeply at the docking data. The latter highlight that both Xyl and ChDHC interact in the same protein region, in particular establishing hydrophobic interactions with LEU167. Thus, it seems that the bonds formed by ChDHC contribute the most to the stabilizing effect on the enzyme and/or that the competition towards the same binding site is in favour of ChDHC. This would imply as a future step, the evaluation of other HBD molecules by docking analysis, targeting different regions of the protein. On the other hand, the observed thermostabilization in NADES solution would be beneficial in exploiting cutinase reaction towards difficult to solubilize substrates and/or in innovative applications such as PET-depolymerization [40].

Half-life of cutinase in NADES solution at high temperature

The half-life of cutinase at 90 °C in 0.1 M phosphate buffer with and without the NADES (20 wt% ChDHC:Xyl 1:1) was evaluated. The presence of NADES increases the enzyme half-life almost three times, going from 22 up to 62 min (Suppl. Fig. S2). The increase in half-life after incubation in NADES has already been reported for other enzymes: for β-glucosidase from *Streptomyces griseus* the $t_{1/2}$ increased 2 times at 60 °C and almost 3 times at 80 °C [33], whilst in [20] and [19] the increase of different laccase half-life was observed when incubated in NADES at 70 °C. In this study, cutinase was incubated even at higher temperature, paving the way to its application at industrial scale, where harsh conditions are needed.

Conclusions

A combination of computer aided approach and experimental thermostability studies was applied to select NADES components for HiC cutinase thermal stabilization at 90 °C. Xyl and ChDHC were selected as best hydrogen bond donor and acceptor molecules and the effects of their different ratio and aqueous dilution were evaluated, leading to a three time increase in $t_{1/2}$ at 90 °C when a 20% ChDHC: Xyl (1:1) was used.

The results of the effect of the single HBA and HBD molecules combined with the docking simulations, indicate that the major contribution to cutinase thermostability in NADES solution came from the HBA component. The final outcome of the paper workflow indicates that beside by the binding energy, the choice of the NADES component should be carefully guided by the analysis of the interaction site. As a future perspective, this may imply testing other HBD molecules by molecular docking looking for different sites of interaction.

This study allowed the conditions promoting cutinase thermostabilization using different NADESs to be explored, thus paving the way to their use in a wider range of biocatalytic processes, including new emerging ones, such as their exploitation as additive in polymer formulation to obtain new materials degradable on-demand [13,41].

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Localization of HBA and HBD interactions (Table S1). ConSurf color-coded multiple protein sequence alignment (Fig. S1). Kinetics of thermal inactivation of cutinase at 90 °C in 0.1 M phosphate buffer pH 7.8 and ChDHC:Xyl (1:1) NADES (Suppl. Fig. S2). Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.05.006.

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