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Improving laccase thermostability with aqueous natural deep eutectic solvents

Astrid E. Delorme, Jean-Michel Andanson*, Vincent Verney*

Université Clermont Auvergne, CNRS, SIGMA Clermont, ICCF, F-63000 Clermont-Ferrand, France

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ABSTRACT

The wide-spread use of laccases in industry is often limited due to the enzyme inactivation over time at conditions which exceeds the operating conditions of the enzymes, which are neutral pH and ambient temperatures (30–40 °C). Natural Deep Eutectic Solvents (NADESs) have attracted considerable attention as reaction media in biocatalysis due to their promising compatibility with enzymes and sustainable derivation. In this contribution we demonstrate the possibility of applying aqueous NADESs as incubation media to alter the activity and inhibit thermal inactivation of laccase *T. versicolor*. For example we show that by incubating 0.25 g L⁻¹ laccase in an aqueous 25 wt% betaine-xylitol based NADES at 70 °C for 15 min, the measured residual activity of laccase is a near 10 fold greater than the measured residual activity of laccase when incubated without the NADES. Moreover, the comparison of the residual activities of the enzyme in presence betaine, xylitol or NADES is clearly showing the advantage of using a NADES over its individual components. The drastic enhancement of the enzyme thermostability by pre-incubation of laccase in NADES media showcases a facile, cheap and green method of boosting the stability laccase.

1. Introduction

With the pressing need for chemical industries to adopt greener methods, there has been a growing interest in biologically catalyzed reactions as means of selective and environmentally benign syntheses and processes. Biologically catalyzed reactions are processes in which enzymes and whole cells are used as catalysts for conversions of organic or inorganic substrates [1,2]. The attractiveness of using enzymes in green chemistry stems from their highly catalytic and selective nature, their derivation from renewable resources, their inherent biodegradability and their ability to operate at neutral pH and ambient temperature and pressure [1]. A prime example of enzymes which have recently found many applications in the field of environmental remediation and green catalysis are laccases [3]. Laccases are copper-blue polyphenol oxidases that are versatile enzymes used in a wide spectrum of catalysis such as biodegradation of pharmaceuticals and phenols in wastewater [4,5], detoxification of bioderived ethanol [6], chlorine-free bleaching of chemical pulps [7], and in organic synthesis [8,9].

Despite the advantages of enzymatic reactions, the wide-spread use of laccases in industry is often limited due to their poor operational stability and low resistance to the process conditions [3,10,11]. The main bottleneck for the widespread use of laccase is its poor activity and

stability at elevated temperatures and at pHs below or exceeding the range of 6–7 [12]. The ability of laccases to operate at higher temperature is particularly important since elevated temperature often favors high reaction rates in many processes. For example, a study demonstrated that high temperatures (>60 °C) yield high initial rates of phenol oxidations by laccase, but the thermal enzyme inactivation rapidly overcame this benefit and stopped the oxidation [13,14].

Several strategies to improve the activity of laccase as well as impede thermal inactivation of enzymes are currently being investigated [14]. The most common strategies used to improve enzyme stability are chemical modifications and immobilization of enzymes on solid supports. However, these techniques are often synthetically demanding and unsustainable in terms of costs and synthesis route [10,15]. Recently, the introduction of a co-solvent such as polyethylene glycol and even ionic liquids have shown promise in improving the kinetics of reactions mediated by enzymes such as alcohol dehydrogenase and α -chymotrypsin [16–19]. Moreover, deep eutectic solvents (DESS) have emerged as alternative non-toxic solvents for biotechnological applications, due to their biocompatibility with enzymes [20]. In general, DESS are composed of a hydrogen bond acceptor (HBA), e.g. a salt, and a hydrogen bond donor (HBD), such as polyols and sugars [21]. The hydrogen bonds result from the interactions between HBA and the HBD, which typically leads to DESS having a notably low melting temperature compared to those of its individual components. Particularly, attractive properties that have been associated with DESS include their low flammability, low volatility, thermal and chemical stability, wide polarity,

* Corresponding authors.

E-mail addresses: j-michel.andanson@uca.fr (J.-M. Andanson), vincent.verney@uca.fr (V. Verney).

facile synthesis, low vapor pressure and high solvability [22–24]. A subset of DESs, termed natural deep eutectic solvents (NADESs), are prepared solely using raw materials of natural origin (e.g. sugars and amino acids) [21]. Furthermore, considering that many of the NADES components occur in our daily food, for example betaine (HBA) and xylitol (HBD), makes the use of NADES suitable for food-applications, such as in food-packaging [25,26].

In addition to being non-toxic alternative solvents, encouraging results on the NADESs capabilities of maintaining and even enhance the activity of laccase have been reported [27]. Khodaverdian and co-workers showed that by incubating laccase in aqueous betaine based NADES the activity of laccase is drastically increased. An enhancement of activity suggests that higher rates of reactions can be obtained, providing a facile means of boosting the kinetics of a reaction catalyzed by enzymes. They also incubated laccase in three aqueous betaine based NADES at 80 °C and 90 °C for up to 80 min and 30 min, respectively, and demonstrated an enhancement of enzyme thermostability when laccase is pre-treated in the aqueous NADES media [28]. In another study, Toledo and co-workers showed that laccase is twice as active when incubated in aqueous 50 wt% choline dihydrogen citrate based NADES medium compared to when laccase is incubated in the aqueous buffered solution without the NADES. The nature of the NADESs, such as chemical structure, the ratio of each component as well as the concentration of the NADES in aqueous solutions were shown to influence the activity of laccase. In contrast to Khodaverdian and co-workers discoveries, Toledo and co-workers found no significant thermoprotection of laccase as a result of NADES pre-treatment. The enzyme thermostability was, however, tested when laccase was incubated in the aqueous NADES media at 60 °C and stored for up to 20 days where aliquots were taken daily [27,28]. Considering that many enzyme-catalyzed processes only require elevated temperatures for minutes or even seconds the possibility of improving the thermostability of laccase at these timescales is highly attractive for industrial applications [13]. For example the incorporation of enzymes in degradable multi-layer plastics used in food packaging requires enzymes that can withstand high temperatures, ranging from 70 °C to 300 °C during the time lapse of a few minutes (≤ 10 min) [29].

With the goal of producing robust enzyme catalyst, we propose a facile and cost-effective method of significantly improving the thermostability of laccase by pre-incubating laccase in aqueous NADES media. To the best of our knowledge, this work is the first study focusing exclusively on the optimization of the laccase thermostability by incubation in aqueous NADES media and which has the aim of developing thermostable enzyme systems for processes that require elevated temperatures during a timeframe of a few hours or less.

For our initial investigations of aqueous NADES media effect on enzyme activity and thermostability we selected the most promising NADES compositions and concentrations in aqueous solutions that have previously been reported for enzyme activity enhancement [27,28]. Based on our initial results we conducted a screening of aqueous betaine based NADES solutions with varying HBD, aqueous concentrations and HBA:HBD ratio. In total 18 NADES were synthesized, and the laccase activity was measured when laccase was incubated in aqueous solutions containing 25 wt% of the synthesized NADES. We tested enzyme thermostability when laccase was incubated in 12 different based NADES aqueous solutions, at a concentration of 25 wt%, and the reference solution at temperatures of 60 °C, 70 °C and 90 °C for up to 120 min, 60 min and 1 min, respectively (taking aliquots periodically). The influence of the concentration of NADES in the aqueous media was also investigated. We demonstrate that the activity of laccase is influenced by the NADES media consequently also affecting the kinetics of the laccase-mediated reactions. Additionally, the thermostability of laccase *Trametes versicolor* (*T. versicolor*) is significantly increased in a series of different NADES media (see Table 1) when exposed to high temperatures (≥ 60 °C) and for varying incubation times.

2. Materials and method

2.1. Materials

Reagents were obtained from Sigma-Aldrich and TCI Chemicals and were used as received. Laccase from *T. versicolor* (powder, light brown, 0.5 U mg^{-1}) was purchased from Sigma-Aldrich and choline dihydrogen phosphate was purchased from IoLiTec. The preparation of cholinium- and betaine- based NADES was carried out by adding the hydrogen-bond acceptor (Bet, ChDHP and ChDHC) to each hydrogen-bond donor (EG, Gly, PrOH, Xyl, Sor and Ery) in glass vials. For the initial measurements of laccase activity, laccase was incubated in 9 aqueous NADES media, with the following HBA:HBD ratios; 1ChCl:2EG, 1ChCl:2Gly, 1ChCl:3PrOH, 1Bet:3EG, 1Bet:3Gly, 1Bet:Xyl, 1Bet:3PrOH, 2ChDHC:1Xyl and 1ChDHP:2Xyl. For the dependence analyses of the laccase thermostability on the HBD component of the NADES, a 1Bet:3HBD molar ratio of the NADES was used. For the screening of the Bet:Xyl NADES ratios, the following set of ratios were used; 1:3, 1:2, 2:3, 1:1, 3:2, 2:1 and 3:1.

The HBA and HBD mixtures were heated in an oven at 90–100 °C for 10 min and shaken, this was repeated until a homogeneous clear liquid formed, with the exception for the synthesis of 1Bet:3PrOH, no water was added prior to this point. All NADES were stored in the dark in a sealed vial. After the preparation of the NADESs, a solution of aqueous 50 wt% NADES and 50 mM phosphate buffer (pH 7) was prepared for each NADES. A 1 mL laccase incubation solution was then prepared with 500 μL of the aqueous 50 wt% NADES solutions and 500 μL of a 50 mM phosphate buffer (pH 7) solution containing 0.5 g L^{-1} laccase. The final concentration of NADES and laccase in the laccase incubation solution were 25 wt% and 0.25 g L^{-1} , respectively.

2.2. Laccase activity in aqueous NADES media

The activity of laccase was assayed according to the method described by Toledo and co-workers using 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as the substrate [27]. A 1 mL laccase incubation solution was prepared with phosphate buffer (50 mM, pH 7) aqueous solution with 25 wt% NADES, unless otherwise stated, and with a laccase concentration of 0.25 g L^{-1} . Laccase was then incubated in this solution at room temperature for at least 10 min, prior to performing the laccase activity assay. The enzymatic reaction was carried out at 25 °C by adding 100 μL of the laccase incubation solution in 500 μL of 0.4 mM ABTS (in water) and 1400 μL of 50 mM citrate and 100 mM phosphate buffer at pH 4.3 while stirring. The oxidation of ABTS was monitored by the increase in absorbance measured in the kinetic model of a UV-Vis spectrophotometer (Jasco V650 spectrometer) at 417 nm and using a cuvette of 10 mm optical path length. All the measurements of the reaction solutions were stirred and thermostated at 25 °C during the kinetic of the reaction. Results presented in this work are the mean from three experiments of three different laccase incubation solutions.

2.3. Laccase thermostability in aqueous NADES media

Laccase thermostability was evaluated in a phosphate buffer (50 mM, pH 7) aqueous solution containing NADES at concentrations of 25 wt%, unless otherwise stated, with a laccase concentration of 0.25 g L^{-1} . The laccase incubation solution was incubated at room temperature for 10 min, and then magnetically stirred and heated at 60 °C, 70 °C or 90 °C for different intervals of times. For the thermostability tests at 60 °C aliquots for the immunoassays were taken after 1, 5, 10, 20, 30, 60 and 120 min, at 70 °C aliquots were taken after 1, 5, 10, 20, 30 and 60 min and at 90 °C aliquots were taken after 15, 30, 45 and 60 s. After a set amount of time the vial containing the laccase solution was cooled to room temperature in a water bath. An aliquot was then taken from the cooled laccase solution for the laccase activity assay as

Table 1
Composition, chemical structure, abbreviation and molar ratios of NADESs.

Name	Structure	Abbreviations
Hydrogen-bond acceptor		HBA
Choline chloride		ChCl
Choline dihydrogen citrate		ChDHC
Choline dihydrogen phosphate		ChDHP
Betaine		Bet
Hydrogen-bond donor		HBD
1,2-Propanediol		PrOH
Glycerol		Gly
Ethylene glycol		EG
Xylitol		Xyl
Erythritol		Ery
Sorbitol		Sor

described above. The thermostability tests were repeated in triplicates. Experimental data were deposited in the Zenodo data repository (<https://zenodo.org/>) and are publicly available (10.5281/zenodo.3935852).

3. Results and discussion

3.1. Effect of HBA on the thermostability of the laccase

Inspired by the recent work conducted by Toledo and co-workers the choline dihydrogen citrate (ChDHC), choline dihydrogen phosphate (ChDHP) and betaine (Bet) as the HBA of the NADES coupled with xylitol (Xyl) as the HBD were prepared [27], for an initial comparison of laccase activity with betaine based NADES, motivated by the work conducted by Khodaverdian and co-workers [28], and choline chloride (ChCl) based NADES, since these are the most commonly used NADES [30]. One unit (U) of laccase activity is defined as the amount of enzyme able to oxidize 1 μmol of ABTS per minute. The relative laccase activity measured in various solutions of aqueous 25 wt% NADES was assessed by comparing with the activity of laccase in the reference solution (in 50 mM phosphate-phosphate buffer, pH 7), and summarized in Fig. 1. It should be noted that the term NADES aqueous solution was considered since data from the literature shows that NADES maintain their

structure in aqueous solution, although dependent on the water content [23,31].

It is clear from Fig. 1 that the relative laccase activity is significantly lower (< 25%) when laccase is incubated in the three ChCl based NADES. The relative laccase activity is drastically improved when exchanging the ChCl HBA to Bet, which can be seen when comparing 1ChCl:3PrOH (choline chloride with 1,2-propanediol with a 1 to 3 molar ratio) and 1Bet:3PrOH in Fig. 1. These results are in agreement with Toledo and co-workers findings, in which they found that the relative activity of laccase is particularly low in aqueous ChCl based NADES. The presence of the chloride ion is likely to cause enzyme denaturing [27].

From the short review of NADES and laccase relative activity, the HBA; ChDHC, ChDHP and Bet coupled with Xyl as the HBD were used to test the thermostability of laccase at 60 °C. The NADES were prepared in the molar ratios which shown to be most promising in terms of enhancing the laccase activity in our preliminary tests (Fig. 1). Thus, 2ChDHC:1Xyl, 1ChDHP:2Xyl and 1Bet:3Xyl were prepared and with a 25 wt% in an aqueous solution (in 50 mM phosphate-phosphate buffer, pH 7).

The thermostability of laccase in these three aqueous NADES media and in the reference media (in 50 mM phosphate buffer, pH 7, without any NADES), was assessed by comparing the activity of before and after

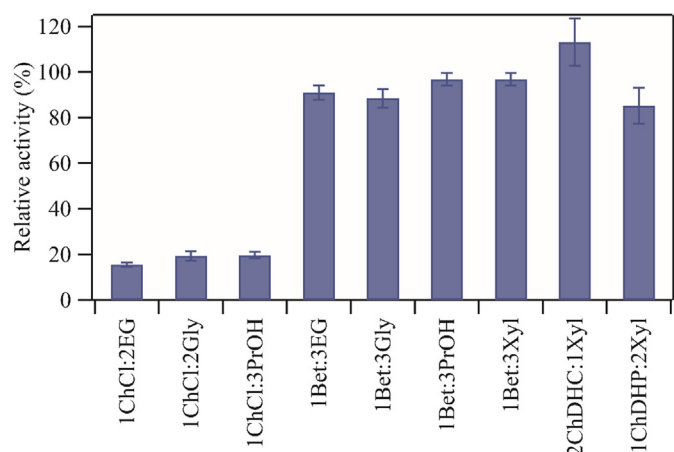


Fig. 1. Comparison of measured relative activity of laccase when incubated in 9 different aqueous NADES media. The laccase activity was measured of incubation solutions containing 0.25 g L^{-1} laccase and 50 mM phosphate buffer (reference) in aqueous $25 \text{ wt} \%$ NADES media. The measured activity of laccase in the aqueous NADES containing incubation solutions are measured relative to the laccase activity of incubation containing no NADES. Three incubation solutions data point were prepared in order to evaluate the uncertainty of the experiments.

the four different laccase solutions had been incubated at $60 \text{ }^\circ\text{C}$ for different length of time. The initial measured activity of laccase in each solution, prior to incubation at $60 \text{ }^\circ\text{C}$, is set as the relative laccase activity (i.e. at time = 0, the residual activity of laccase in each solution = 100%), this is detailed in the Appendix A. Supplementary Data. Vials containing the laccase NADES aqueous solution and the laccase reference solution were then heated to $60 \text{ }^\circ\text{C}$ while continuously stirred to ensure an optimal heat transfer into the sample. After a set amount of time the vials were removed from the heat and cooled to room temperature in a water bath. An aliquot of $100 \text{ }\mu\text{L}$ of the cooled solution was then used for the ABTS enzymatic assay to measure the activity of laccase after being heated. Fig. 2 shows the measured residual activity of laccase in the three aqueous NADES solutions and in the reference solution. With this normalization, the residual activities given here are not disturbed by a possible impact of the presence of the NADES on the activity of the enzyme since the same quantity of NADES is in the reference.

As can be seen from Fig. 2, laccase *T. versicolor* is thermally inactivated when it is incubated at $60 \text{ }^\circ\text{C}$ in the reference solution,

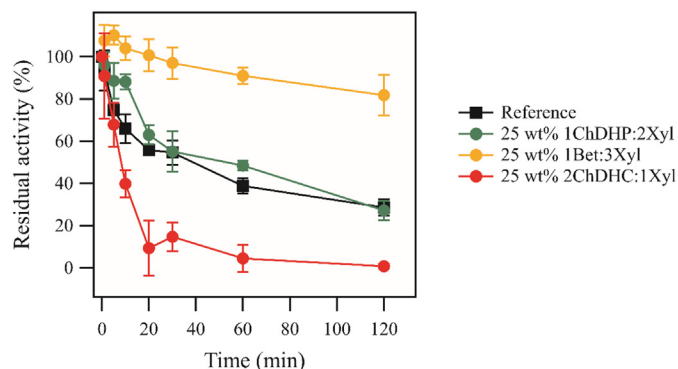


Fig. 2. Comparison of laccase thermostability when incubated in three different aqueous NADES and in the reference solution. Residual activity of 0.25 g L^{-1} laccase was measured when incubated in a phosphate buffer (50 mM , $\text{pH } 7$) aqueous solution in the absence (black squares) and in the presence of aqueous $25 \text{ wt} \%$ 2ChDHC:1Xyl (red dots), $25 \text{ wt} \%$ 1ChDHP:2Xyl (green dots) and $25 \text{ wt} \%$ of 1Bet:2Xyl (orange dots) at $60 \text{ }^\circ\text{C}$ for 1, 5, 10, 20, 30, 60 and 120 min. Three incubation solutions data point were prepared in order to evaluate the uncertainty of the experiments.

aqueous $25 \text{ wt} \%$ 2ChDHC:1Xyl and $25 \text{ wt} \%$ 1ChDHP:2Xyl. The inactivation of laccase at increased temperatures ($\geq 60 \text{ }^\circ\text{C}$) is in agreement with other studies [13,28,32]. On the other hand, when laccase is incubated in aqueous $25 \text{ wt} \%$ 1Bet:3Xyl at $60 \text{ }^\circ\text{C}$ the thermal inactivation of laccase is significantly slower than laccase in the reference solution, demonstrating a remarkable enhancement of the thermostability of laccase compared to the reference solution. It should be noted that the relative activity of laccase when incubated in aqueous $25 \text{ wt} \%$ 1Bet:3Xyl is $96.8 \pm 2.8\%$ (see Fig. 1) and thus the enhancement of thermostability of laccase is not likely to be a consequence of the normalization to initially low laccase activity (at time = 0). At $60 \text{ }^\circ\text{C}$, the half-life of the enzyme is around 30 min in the reference solution without NADES, it has been severely decreased to around 8 min with aqueous $25 \text{ wt} \%$ 2ChDHC:1Xyl while it has been drastically increased to several hours in aqueous $25 \text{ wt} \%$ 1Bet:3Xyl.

The relatively poor thermostability of laccase when incubated in aqueous $25 \text{ wt} \%$ 2ChDHC:1Xyl and aqueous $25 \text{ wt} \%$ 1ChDHP:2Xyl is somewhat surprising considering the previously reported high laccase activities in these two aqueous media (see Fig. 1) [27]. In order to investigate these controversial results, the pH of the laccase incubation solutions as well as the reaction solutions were measured, as it is known that both the laccase stability and the ABTS oxidation reaction are both pH sensible. The pH of the laccase solutions and the reaction solutions (shortly after it was made and after 10 min of stirring) are summarized in Table 2.

It can be seen that the pH of the laccase incubation solutions containing the aqueous 2ChDHC:1Xyl and 1ChDHP:2Xyl NADES media are significantly lower than the pH of the reference incubation solution and the solution containing aqueous $25 \text{ wt} \%$ 1Bet:3Xyl NADES. Furthermore, the measured pH of the reaction mixture after 10 min of mixing (reaction is typically complete after 5 min of stirring) did not change significantly as a result of the reaction. It is well known that laccases are most stable at a neutral pH ($\text{pH } 6\text{--}7$) and when the enzyme is incubated in lower pH ($\text{pH} < 6$) it is inactivated over time [13,32]. Furthermore, Kurniawati and Nicell reported that the rate of inactivation of laccase *T. versicolor* increases as the temperature is raised from 10 to $60 \text{ }^\circ\text{C}$, and that the inactivation is accelerated when laccase is incubated in lower pHs ($\text{pH} < 6$) [13]. The dependency of the laccase activity on the pH is believed to be due to the ionizable side-chains and tertiary structure of laccase that can be denatured at extreme pH [13]. Thus, the relatively poor thermostability of laccase when incubated in the aqueous $25 \text{ wt} \%$ 2ChDHC:1Xyl and aqueous $25 \text{ wt} \%$ 1ChDHP:2Xyl NADES media compared to the aqueous $25 \text{ wt} \%$ 1Bet:3Xyl NADES media could be a result of the much lower pH of the two cholinium based NADES. Interestingly, the thermostability of laccase when incubated in aqueous $25 \text{ wt} \%$ 1ChDHP:2Xyl media is similar and even slightly more stable than laccase when incubated in the reference media, see Fig. 2, which could be a result of the NADES stabilizing the laccases towards both pH and temperature changes. Additionally, the optimum ABTS oxidation occurs at more acidic conditions, pH of about $3\text{--}4$ [33], and therefore the slightly lower pH obtained in the aqueous reaction mixtures containing $25 \text{ wt} \%$ 2ChDHC:1Xyl and $25 \text{ wt} \%$ 1ChDHP:2Xyl could perhaps explain the higher initial activity of laccase that Toledo and co-workers observed, compared to the reference solution [27,33]. These results highlight the importance of considering the effect that the NADES has on the solution pH as it could lead to confusing results.

The betaine based NADES, on the other hand, does not alter the pH of both the incubation and reaction mixture significantly. The enhancement of enzyme thermostability when incubating laccase in aqueous $25 \text{ wt} \%$ 1Bet:3Xyl could be a consequence of potential interactions established with the two NADES components and laccase. For example, in a recent study it was shown that polyols can establish hydrogen bonding with enzyme amino acids and that polyols can be used as chemical modifiers to enhance laccase stability to pH and temperature changes [12]. Since the betaine based NADES showed to be a promising media for improving the thermostability of laccase, the possibility of

Table 2

Measured pH of aqueous laccase (0.25 g L^{-1} in 50 mM phosphate buffer) incubation solutions with added NADES and reactions solutions containing $100 \mu\text{L}$ of the laccase incubation solution.

Incubation media	pH of laccase incubation solution	pH of reaction mixture	pH of reaction mixture after 10 min of stirring
Reference	6.87	4.71	4.61
25 wt% 2ChDHC:1Xyl	3.81	4.40	4.32
25 wt% 1ChDHP:2Xyl	3.76	4.63	4.58
25 wt% 1Bet:3Xyl	6.84	4.74	4.73

optimizing the thermostability of laccase further by varying the polyol of the HBD component of the NADES is investigated in the following section.

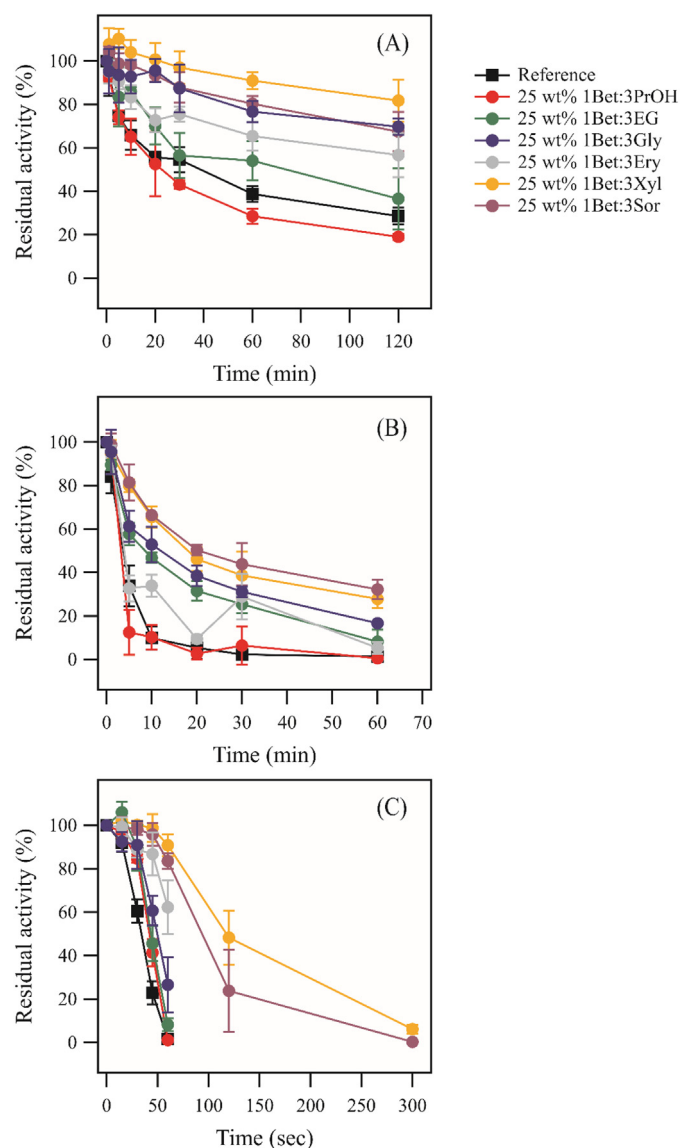


Fig. 3. Comparison of enzyme thermostability when laccase is incubated in various betaine based NADES aqueous solutions and in three different temperatures. Residual activity of 0.25 g L^{-1} laccase when incubated in a phosphate buffer (50 mM , $\text{pH } 7$) aqueous solution in the absence (black squares) and in the presence of aqueous 25 wt% 1Bet:3PrOH (red dots), 25 wt% 1Bet:3EG (green dots), 25 wt% 1Bet:3Gly (blue dots), 25 wt% 1Bet:3Ery (grey dots), 25 wt% 1Bet:3Xyl (orange dots) and 25 wt% 1Bet:3Sor (purple dots), at $60 \text{ }^\circ\text{C}$ (A), $70 \text{ }^\circ\text{C}$ (B) and $90 \text{ }^\circ\text{C}$ (C). The measurements were done in triplicates. The residual activity refers to the comparison of the activity measured at a set amount of time and time 0 (i.e. incubation at room temperature = 100%).

3.2. Laccase thermostability in betaine based NADES with varying HBD

The betaine based NADES were prepared using Bet:HBD molar ratio of 1:3 and with a 25 wt% in an aqueous solution (in 50 mM phosphate-phosphate buffer, $\text{pH } 7$). The thermostability of laccase in the reference solution and in six betaine based NADES aqueous media with polyols of varying amount of hydroxyl groups, namely, sorbitol (Sor), xylitol (Xyl), erythritol (Ery), glycerol (Gly), ethylene glycol (EG) and 1,2-propanediol (PrOH) was assessed by comparing the initial activity of laccase prior to heating (i.e. at time = 0) and the residual activity of laccase after being incubated at $60 \text{ }^\circ\text{C}$, $70 \text{ }^\circ\text{C}$ or $90 \text{ }^\circ\text{C}$ after a set amount of time as shown in Fig. 3. It should be noted that the pH of the laccase incubation solutions and the aqueous reaction solutions with the NADES were measured and showed little deviation from the pH measured in the reference solutions (pH measurements are summarized in Table S2 in the Appendix A. Supplementary Data).

From Fig. 3 it is clear that the laccase thermostability is dependent on the polyol. It should be noted, however, that the NADES crystallized out of the aqueous solution containing 25 wt% 1Bet:3Ery, when left at room temperature for a couple of hours and had to be heated to re-dissolve the NADES into solution, which could potentially have an effect on the observed data. In order to be consistent in the screening of the thermostability of laccase in aqueous 25 wt% 1Bet:3HBD, no other molar ratio of Bet:Ery based NADES were tested, however other molar ratios of Bet:Ery could remain liquid at lower temperature in aqueous solutions. With the exception of the incubation solution containing 1Bet:3PrOH, all the other incubation solutions containing NADES demonstrate an enhancement of the thermostability of laccase compared to the reference solution. In general, laccase is most thermally stable when incubated in aqueous 25 wt% 1Bet:3Xyl, followed closely by aqueous 25 wt% 1Bet:3Sor. For example, after 2 h of incubating laccase in aqueous 25 wt% in 1Bet:3Xyl at $60 \text{ }^\circ\text{C}$, laccase still retained $81.8 \pm 9.6\%$ of its initial activity compared to $28.6 \pm 3.9\%$ without NADES. Similarly at higher temperature, after 1 min at $90 \text{ }^\circ\text{C}$ laccase still retained $90.8 \pm 5.0\%$ of its initial activity while almost no activity ($1.6 \pm 0.9\%$) could be observed in absence of NADES. These results are very promising since a higher thermal stability of enzyme implies the possibility of performing industrially relevant reactions at higher temperatures.

In general, the thermostability of laccase seems to roughly increase with the increasing number of hydroxyl groups in the polyol HBD in the aqueous NADES solution in which the laccase was incubated in. Interestingly, Toledo and co-workers demonstrated through molecular docking experiments that the docking affinity energies for laccase with polyols increase with increasing number of hydroxyl groups in the polyol, which they suggested could be result of the polyols ability to hydrogen bond with the amino acids in the laccase structure [27]. Thus it could be hypothesized that the observed trend in Fig. 3 that the thermostability of laccase is dependent on the number of hydroxyl groups present in the HBD and their ability to bind with the amino acids in the laccase structure.

3.3. Thermostability of laccase in various molar ratio and weight percentage of aqueous 25 wt% Bet:Xyl NADES media

As shown previously in Fig. 3, the most promising aqueous NADES incubation media in improving laccase thermostability is aqueous

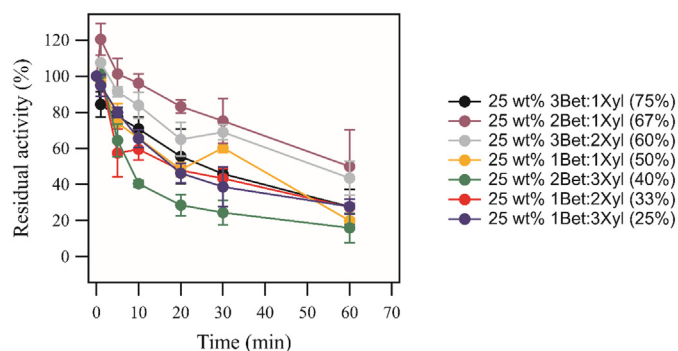


Fig. 4. Impact of the HBA:HBD ratio on the thermostability of laccase (0.25 g L^{-1}), when incubated in a phosphate buffer (50 mM, pH 7) aqueous solution in the presence of 25 wt% of 3Bet:1Xyl (black dots), 25 wt% 2Bet:1Xyl (purple dots), 25 wt% 3Bet:2Xyl (grey dots), 25 wt% 1Bet:1Xyl (yellow dots), 25 wt% 2Bet:3Xyl (green dots), 25 wt% 1Bet:2Xyl (red dots) and 25 wt% 1Bet:3Xyl (blue dots) at 70°C , the molar ratio of Bet: Xyl are given in percentage in parentheses. The residual activity is measured vs. incubation time and measurements were done in triplicates. The residual activity refers to the comparison of the activity measured at a set amount of time and time 0 (i.e. incubation at room temperature = 100%).

25 wt% 1Bet:3Xyl. Thus, in order to find the optimum NADES composition for improving the thermostability of laccase, thermostability tests of laccase were carried out by incubating laccase in aqueous 25 wt% of betaine and xylitol based NADES with varying molar ratios of betaine and xylitol. Fig. 4 shows the measured residual activities of laccase when incubated in various betaine-xylitol based NADES, with varying molar ratios, at 70°C and for varying length of time.

From Fig. 4, it is clear that the thermostability of laccase is dependent on the composition of the betaine-xylitol NADES in which it is incubated in. The thermostability of laccase is at its maximum when laccase was incubated in an aqueous solution containing the betaine-xylitol NADES with the ratio 2Bet:1Xyl. It should then be noted that the best ratio is far from being the maximal amount of betaine even as the single compound betaine is a better stabilizer than xylitol. The dependence of the NADES component ratio could be a result of a range of different interactions between the NADES itself and between the NADES components and the enzyme.

Thermostability tests were then carried out by incubating laccase in three concentrations of 2Bet:1Xyl: 10 wt%, 25 wt% and 50 wt% in

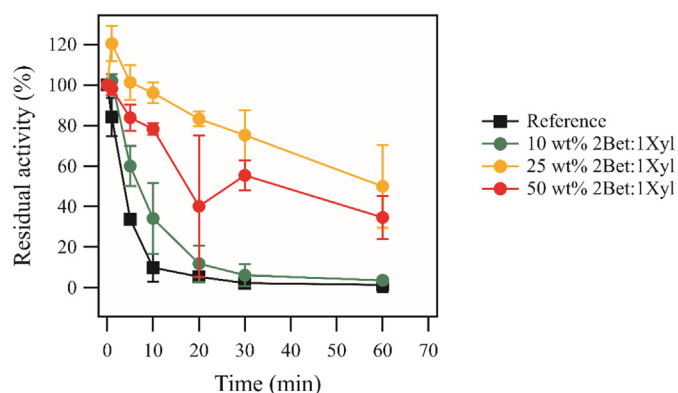


Fig. 5. Comparison of laccase thermostability when incubated in varying weight percentage of 2Bet:1Xyl NADES in aqueous solutions. Residual activity of 0.25 g L^{-1} laccase was measured when laccase was incubated in a phosphate buffer (black squares) aqueous solution in the presence of 10 wt% 2Bet:1Xyl (green dots), 25 wt% 1Bet:2Xyl (yellow dots) and 50 wt% 1Bet:1Xyl (red dots) at 70°C . The measurements were done in triplicates. The residual activity refers to the comparison of the activity measured at a set amount of time and time 0 (i.e. incubation at room temperature = 100%).

aqueous solutions. Fig. 5 shows the measured residual activities of laccase when incubated in various concentrations of 2Bet:1Xyl NADES in aqueous solutions, at 70°C and for varying length of time.

As observed from Fig. 5, the overall best performing concentration of the 2Bet:1Xyl in terms of improving laccase thermostability is 25 wt% in aqueous solution. As an example, after 30 min at 70°C without NADES or with only 10 wt% of NADES in aqueous solution, the activity of the laccase decreased by a 50 fold and 17 fold, respectively while with 25 wt%, most of the activity is conserved ($75.2 \pm 12.4\%$). Another way to illustrate this stabilization is the half-life of the enzyme at 70°C , it is increasing from 4 to 60 min when adding 25 wt% of NADES to the reference solution.

Polyols and betaine have shown potential of acting as additives to improve the thermal stability of laccase [12,34]. Betaine on its own displayed general stabilizing effects on macromolecules and counteracts perturbations of the active enzyme structure during temperature changes. In fact, betaine is a naturally found osmolyte known to prevent protein folding [34]. Kang and co-workers used circular dichroism and fluorescence spectra to demonstrate that polyols can change the conformation of laccase by increasing the hydrogen bonds between the laccase molecules and prevent the adverse change in active secondary structure of laccase due to the increase of temperature [12]. Hence, this raises the question whether the observed enhancement of thermostability of laccase is in fact due to the individual components of the NADES or due to a synergetic effect of both components of the NADES. In the following section we investigate whether solutions of the individual components of 2Bet:1Xyl, xylitol and betaine, are responsible for the observed enhanced thermostability of laccase or if it is the NADES itself which is responsible for improved thermostability.

3.4. Thermostability of laccase in aqueous solution of NADES components

To determine if the behavior of laccase is due to the individual components of the NADES or because of the NADES itself, determined amounts of the betaine and xylitol were added individually into the aqueous buffer solutions to form solutions having the same concentration of betaine and xylitol as in the aqueous 25 wt% 2Bet:1Xyl solution. It should be noted that by using aqueous 15 wt% betaine and 10 wt% xylitol correspond to the same number of mole and molar concentration of betaine and xylitol present in the aqueous 25 wt% 2Bet:1Xyl aqueous. The thermostability of laccase in these aqueous solutions are compared to the NADES containing solution and the reference solution in Fig. 6.

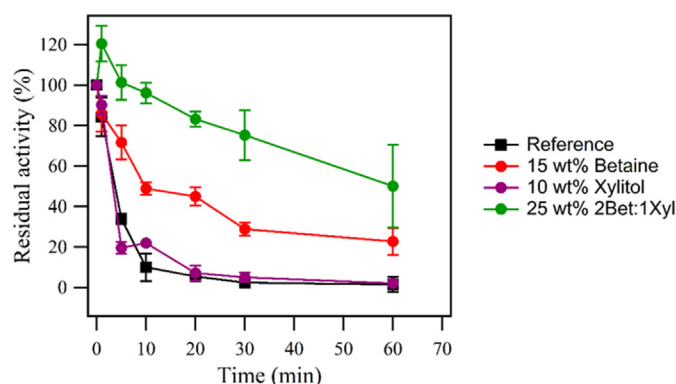


Fig. 6. Comparison of the thermostability of laccase when incubated in the aqueous solutions of the individual components of a NADES, the NADES itself and the reference solution. Residual activity of 0.25 g L^{-1} laccase was measured when laccase was incubated in a phosphate buffer (50 mM, pH 7) aqueous solution in the absence (black squares) and in the presence of aqueous 15 wt% betaine (red dots), 10 wt% xylitol (purple dots) and 25 wt% of 2Bet:1Xyl (green dots) at 70°C . The measurements were done in triplicates. The residual activity refers to the comparison of the activity measured at a set amount of time and time 0 (i.e. incubation at room temperature = 100%).

As shown in Fig. 6, the thermostability of laccase is improved when incubated in aqueous 15 wt% of betaine compared to the reference solution, whereas there is no improvement in the thermostability when laccase is incubated in aqueous 10 wt% xylitol. An even greater enhancement of the thermostability of laccase is achieved when incubating laccase in the aqueous 25 wt% 2Bet:1Xyl solution. As an example after 30 min of laccase incubation at 70 °C, while the laccase retained almost no activity in absence of additive or with xylitol, the presence of betaine leads to 30% of activity while with the NADES around 80% of the activity of preserved. These results are the first demonstration of a cooperative effect of HBD and HBA of NADES on enhancing the thermostability of laccase.

4. Conclusion

We have demonstrated that by the appropriate choice of the constituents, the molar ratio and the concentration of the NADES in the incubation media, the thermostability of laccase could be greatly enhanced. For example, we showed that the thermostability of laccase is dependent on the HBD of the aqueous NADES incubation media and seemingly its number of hydroxyl groups. This could be a result of possible hydrogen bonding interactions between the hydroxyl groups of the polyols and the laccase amino acids. Furthermore, the highest thermostability of laccase was found for the NADES over the individual components in an aqueous solution, demonstrating a cooperative effect of coupling the HBA and HBD components into the NADES in order to act as a thermostabilizing media for laccase. Considering that NADES can easily be tuned to meet specific needs, by for example tuning the interactions between enzyme and NADES components, there is great versatility offered by NADES composition. There is therefore a promising scope of using NADES to improve the thermostability of other enzymes with seemingly different structure and composition than laccase *T. versicolor*. Indeed, initial studies carried out in our group have demonstrated the use of NADES to improve the thermostability of a more thermostable enzyme than laccase *T. versicolor*.

Furthermore, the remarkable enhancement of thermostability of laccase when incubated in betaine based NADES, in particular in aqueous 25 wt% 2Bet:1Xyl, could unlock a range of potential industrial applications for laccases, for example in smart-enzyme multi-layer plastics for food packaging. In these plastics, enzymes are incorporated through extrusion or lamination processes, which are processes that will expose the enzymes to high temperatures (≥ 70 °C) for a few minutes, which results in thermal inactivation of unprotected enzyme. The use of non-toxic and naturally derived NADES to improve the thermostability of enzymes could therefore be a cost-friendly solution to this challenge and would be particularly suitable to use in food packaging [29].

CRedit authorship contribution statement

Astrid E. Delorme: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Jean-Michel Andanson:** Supervision, Conceptualization, Methodology, Writing - review & editing. **Vincent Verney:** Supervision, Conceptualization, Methodology, Writing - review & editing.

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.

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Appendix A. Supplementary data

Method for UV–Vis measurements and additional experimental details. Supplementary data to this article can be found online at doi: <https://doi.org/10.1016/j.ijbiomac.2020.07.022>.

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