



Immobilization and stabilization of alcohol dehydrogenase on polyvinyl alcohol fibre

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ABSTRACT

A polyvinyl alcohol (PVA) fibrous carrier has been chemically modified for the immobilization of yeast alcohol dehydrogenase (ADH) with an aim to increase its stability over a wide pH range, prolong its activity upon storage, and enhance its reusability. The strategy for immobilization involved functionalization of the fibrous carrier with chloropropionyl chloride followed by amination with ethylenediamine. Tethering of the ADH enzyme to the PVA scaffold was achieved with glutaraldehyde. The activity profile of the immobilized enzyme was compared to soluble enzyme as a function of pH, temperature and reusability. The immobilization of ADH on PVA fibrous carrier shifted the optimal reaction pH from 7 to 9, and improved the thermostability at 60 °C. Furthermore, the immobilized enzyme retained 60% of its original activity after eight cycles of reuse. These results demonstrate that PVA based textiles can serve as a flexible, reusable carrier for enzyme immobilization.

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1. Introduction

Alcohol dehydrogenase (ADH) has been widely used as a biocatalyst in synthetic chemistry. ADH catalyzes selective oxidation and reduction reactions and can be used for kinetic resolution, asymmetric synthesis [1–3], and drug preparation [4–6]. Its use in industrial transformations however, is limited by poor stability due to sensitivity to temperature and pH [7–9]. Under acidic conditions, ADH's multimeric subunits dissociate, and under alkaline conditions its tertiary structure gets distorted, both of which result in a loss of enzyme activity [10–12]. Attempts have been made to enhance the stability of ADHs *via* protein engineering [13,14], chemical modification and immobilization [15,16].

When immobilized on solid carriers, enzymes are generally more stable to denaturants and elevated temperature. Immobilized enzymes can be conveniently recovered from a process stream and recycled. This leads to simpler downstream processing and improved economics [17]. Various technologies have been developed for enzyme immobilization which comprise binding to a solid carrier, entrapment (encapsulation) and crosslinking [18,19]. Binding to a carrier can be *via* physical interaction (e.g. van der

Waals and hydrophobic interactions), and ionic and covalent bonding. Covalent immobilization provides a strong linkage between the enzyme and its carrier matrix to minimise enzyme leakage into the product stream, and therefore has been widely adopted by the chemical community [20–22].

ADH immobilization has been facilitated *via* several carriers including cellulose [23], epoxy functionalized nanoparticles [24], amino epoxy Sepabeads [25], agarose beads [26], and agarose activated carriers like MANAE-agarose, PEI-agarose, and glyoxyl agarose [27]. However, many of these protocols required toxic chemicals and/or expensive carrier materials such as synthetic mesoporous particles. Small particulate carriers can also result in slow reaction kinetics, and high back-pressure and blockage when used in packed columns and reactors [28–31].

Fibre and textile carriers have recently drawn considerable attention for enzyme immobilization due to their low price, large specific surface area, and ease of fabrication [32]. To date, common fibres such as cotton [33], silk [34], wool [28], polyester [35,36] and nylon [37–39] have been employed for enzyme immobilization mostly in non-woven form [14]. To the best of our knowledge, however, there are no reports on the use of polyvinyl alcohol (PVA) fibrous materials as carriers for enzyme immobilization despite its widespread utility in its hydrogel form [40,41].

PVA has been previously reported in the literature as a carrier for enzyme immobilization in various formats such as beads

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[1,2], hydrogel particles [3–5], hybrid films [6,7] and electrospun nanofibrous mats [8]. The use of these formats of PVA in continuous flow reactors could adversely affect the flow parameters such as increasing backpressure and ultimately affecting the yield of the reaction. To the best of our knowledge, there are no reports on the use of polyvinyl alcohol (PVA) fabric materials as carriers for enzyme immobilization. The main advantage of fibres, including PVA fibres, is the ease of conversion into a fabric format with different “porosities” using traditional textile fabrication techniques such as weaving and knitting. Changing the porosity of the carrier can overcome flow issues related to back pressure etc. Further, PVA also possesses a functional group i.e. secondary hydroxyl capable of undergoing functionalization prior to enzyme immobilization. All of these features make PVA fabric a good candidate for the construction of a flexible carrier-enzyme construct suitable for continuous flow through processing.

Herein we report the immobilization of ADH onto PVA fibres in the form of knitted fabric discs. Carrier modification was facilitated by acylation of PVA with chloropropionyl chloride followed by the introduction of a spacer diamine. Subsequent reaction with glutaraldehyde and ADH then led to covalent attachment of the enzyme. The immobilized enzyme-carrier constructs were then analysed for reactivity, stability and recyclability and compared in performance to soluble ADH.

2. Materials and methods

2.1. Materials

3-Chloropropionylchloride (CPC-Cl), ethylenediamine (EDA), hexamethylenediamine (HMA), 1,12-dodecadiamine, glutaraldehyde (25 wt.% in water), alcohol dehydrogenase (ADH) from yeast (≥ 300 units/mg), nicotinamide adenine dinucleotide (NAD^+), and Tris buffer were purchased from Sigma-Aldrich. Ethanol and tetrahydrofuran (THF) (analytical grade) were used as supplied from Merck. PVA yarn (250 dtex (i.e. mass in kg/1000 m length of yarn) 100 filaments, Solvron SHC) was purchased from Nitivy, Japan.

2.2. Methods

2.2.1. Characterisation

Infrared spectral analysis was carried out using a Nicolet 6700 ATR-FTIR (Thermo Scientific) in absorbance mode. Solid-state ^{13}C cross-polarization (CP-MAS) NMR spectra were recorded on a Bruker AV500 MAS spectrometer. Morphology analysis was performed on a Scanning Electron Microscope (SEM, Philips XL30). The samples were imaged using a Zeiss Merlin FESEM after being coated with iridium under vacuum. Enzyme activity was measured using a Cary 300 Bio-UV visible spectrophotometer. Knitting was carried out using a flatbed Shima WG-14 (Japan).

2.2.2. Fabrication of knitted samples

PVA (Solvron SHC) yarn was knitted into a 2×1 rib with a loop length of 5.5 mm using a Shima WG-14 knitting machine. Discs of 13 mm in diameter were stamped from the knitted fabric, washed with deionised water, ethanol and dried in an oven at 50°C before being subjected to further chemical modification.

2.2.3. PVA modification with chloropropionyl chloride (PVA-Cl)

PVA fabric discs (4.00 g, 0.0918 mol of available OH) were added into mixture of THF (80 mL) and triethylamine (28.8 mL, 0.211 mol) at room temperature in a three neck round bottom flask. 3-Chloropropionyl chloride (21.0 mL, 0.22 mol) was added dropwise at 0°C over 30 min and then the reaction was heated at 60°C for 18 h. The discs were then removed from the reaction mixture and washed with distilled water, ethanol and dried at 50°C .

2.2.4. Spacer inclusion on PVA-Cl carrier

The introduction of a spacer involves two steps (A and B) as shown in Fig. 1.

2.2.4.1. Ethylenediamine modification (PVA-Cl-EDA). The PVA-Cl discs (1 g) were added to an EDA solution (30% (w/v) in ethanol, 10 mL) and heated at 60°C for 5 h. After cooling, the aminated discs were washed with distilled water and then ethanol to remove residual diamine.

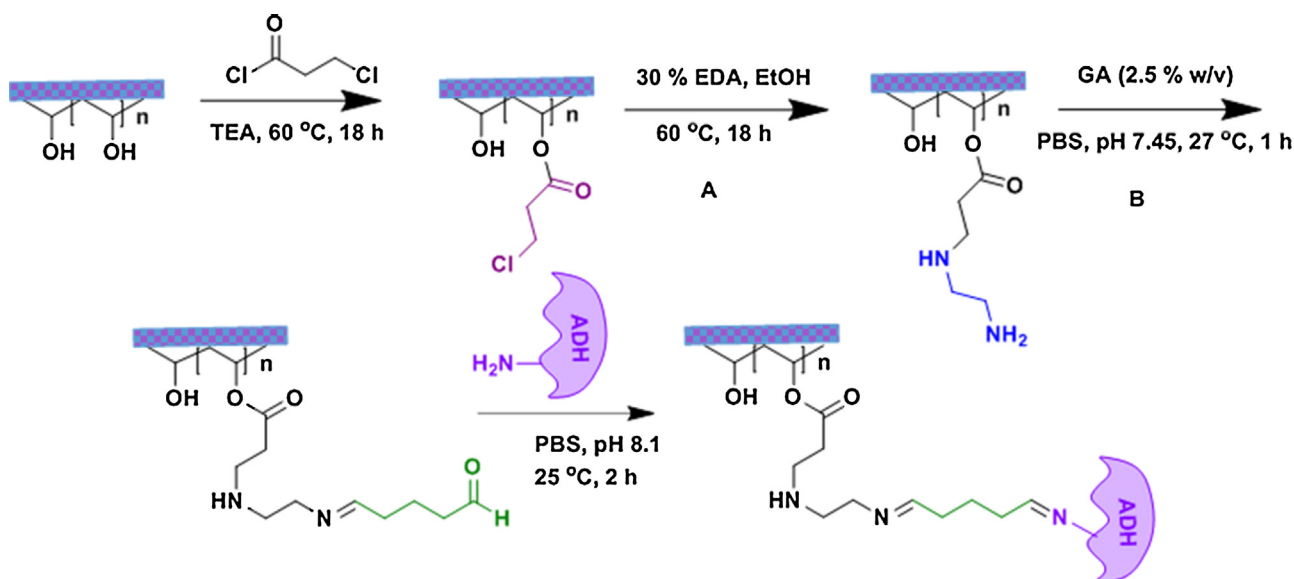


Fig. 1. Schematic representation of covalent immobilization of ADH on modified PVA fibrous carrier.

2.2.4.2. Cross-linking with glutaraldehyde (PVA-Cl-EDA-GA). The PVA-Cl-EDA fabric discs were activated with 2.5% (w/v) GA in phosphate buffer (PBS 0.05 M, pH 7.5) for 1 h at 27 °C in a sealed flask [42]. The carrier discs were thoroughly washed with distilled water over 30 min. to remove excess GA.

2.2.5. ADH immobilization

ADH (0.5 mg/mL) was dissolved in phosphate buffer (0.05 M, pH 8.1). The GA activated fabric discs (PVA-Cl-EDA-GA) were placed in a 12 well plate containing the ADH solution. After incubation for 2 h at 25 °C the carrier discs were washed with phosphate buffer (0.05 M, pH 8.1) four times over 30 min to remove unbound enzyme. PVA fabric without surface modification, PVA-Cl and PVA-Cl-EDA, were treated in an analogous way and subsequently used as controls.

2.2.6. ADH activity assay

ADH catalyses the oxidation of ethanol to acetaldehyde in the presence of nicotinamide adenine dinucleotide (NAD⁺).

The activity of ADH was determined spectrophotometrically by measuring the absorbance of NADH at 340 nm [43]. The activity of ADH immobilized on the carrier was assayed by immersing the carrier discs individually in Tris buffer (100 mM, pH 8.8), ethanol (20 mM), and NAD⁺ (1 mM) at 25 °C in 12 well plate. After shaking for 4 min at 100 rpm on an orbital shaker, 1 mL of the solution was withdrawn for absorbance measurement. The activity measurement experiments were performed in triplicate and the results were presented as averages \pm standard deviation.

The effect of pH on enzyme stability was measured in different buffer systems; for pH 6.5–8.0 sodium phosphate buffer (0.05 M)

and for pH 8.5–10.5, sodium carbonate/bicarbonate buffer (0.1 M) were used. The soluble ADH and immobilized ADH were incubated at 40 °C for 2 h in the various pH buffer solutions. The thermostability study was performed by incubating the soluble ADH (0.5 mg/mL) and the immobilized ADH for 2 h in phosphate buffer (0.05 M, pH 8.1) at various temperatures (20 °C, 40 °C, 60 °C, 80 °C) in a water bath. After each incubation, the enzyme was chilled in crushed ice for 5 min. The enzyme was then slowly brought to room temperature and activity was determined as described above.

Reusability of immobilized enzyme was measured by repetitive usage of the immobilized ADH to catalyse ethanol to acetaldehyde. After each use, the discs were washed with phosphate buffer (0.05 M, pH 7.5) in triplicate and the activity was remeasured using a fresh reaction mixture.

3. Results and discussion

3.1. Surface modification of PVA

Covalent attachment of an enzyme to a solid carrier can be achieved by carrier modification to generate reactive groups on the carrier. The electrophilic formyl groups introduced on the carrier will react with nucleophilic sites on the enzyme. In this paper we have used this approach to modify the PVA backbone to achieve enzyme immobilization.

Chloroacetylation [44] and bromoacetylation [45] of powdered PVA with chloroacetic acid and bromopropionyl bromide reagents have been used to synthesize branched polymers (e.g. stars, combs, or dendrigrafts) with higher tensile strength. In this

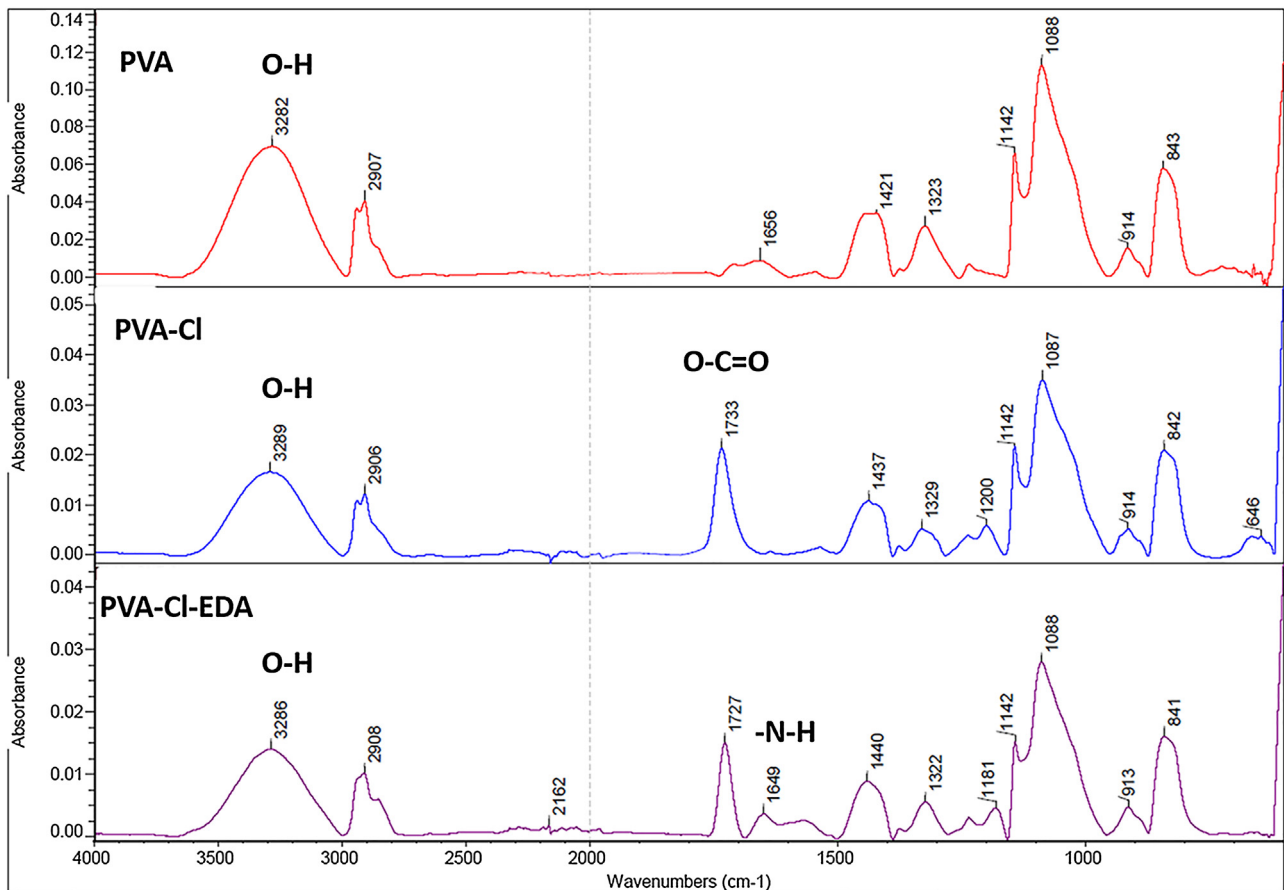


Fig. 2. ATR-FTIR spectra of PVA, PVC-Cl, and PVA-Cl-EDA.

Table 1
ADH activity after immobilization on various modified PVA carriers.

PVA fibrous carriers	Activity (mmol/g min)
PVA-Cl-EDA-GA+ADH	0.765 ± 0.03
PVA-Cl-EDA+ADH	0.210 ± 0.02
PVA-Cl+ADH	0.170 ± 0.02
PVA+ADH	0.105 ± 0.07

study PVA fibres were functionalized by reaction with chloropropionyl chloride. In order to optimise the acylation process, the reaction was performed at different temperatures (20 °C, 40 °C, 60 °C, and 80 °C) and the resulting polymer was analysed by IR (data not shown). Optimal reaction was observed at 60 °C. Further increase in reaction temperature (85 °C) caused rapid disintegration of the PVA fabric resulting in the formation of a gel. In addition, the effect of reaction time (2 h, 6 h, 12 h, 18 h, and 24 h) was assessed and acylation was found to increase from 2 h to 18 h and plateau thereafter (data not shown). Hence, optimised acylation conditions were determined to be reaction at 60 °C for 18 h.

Several studies have reported the effect of spacer length and type of spacer between enzyme and carrier and its effect on the activity of the immobilized enzyme [38,46,47]. Higher activity is observed when a spacer is incorporated between the carrier and enzyme to reduce steric hindrance, promote conformational mobility, and reduce adverse surface interaction [18,19,48]. A spacer was introduced to the PVA-Cl discs by reaction with diamine in ethanol and resultant amine was reacted with glutaraldehyde [49,50].

3.1.1. Characterization of modified PVA carrier

The surface modification of PVA-Cl and PVA-Cl-EDA were confirmed by ATR-FTIR shown in Fig. 2. The spectra for PVA, PVA-Cl, and PVA-Cl-EDA fabrics show the change of chemical structure of the original and modified PVA fibrous carrier respectively. The PVA-Cl spectrum shows a distinct strong peak at 1733 cm^{-1} due to the ester group stretching vibration [44] and a peak at 646 cm^{-1} corresponding to the C—Cl stretching vibration of the PVA-Cl side chain. The PVA-Cl-EDA spectrum showed a new weak peak at 1649 cm^{-1} due to N—H bending vibration resulting from amino group present in PVA-Cl-EDA. Moreover the peak at 646 cm^{-1} disappears in the spectrum of the PVA-Cl-EDA. This supports successful nucleophilic substitution by ethylenediamine on the modified PVA-Cl-EDA construct. The presence of a broad peak at approximately 3286 cm^{-1} supports the presence of O—H and N—H stretching bands. Further characterisation with ^{13}C NMR was reported in Supplementary document (S_1).

The morphologies of PVA fibrous carriers with and without surface modification are shown in Fig. 3. Importantly, the integrity of the modified PVA fibrous carrier structure was maintained throughout the chemical modification process.

3.2. ADH immobilization

The immobilization of an enzyme onto carriers can take place either by adsorption or by the formation of covalent bonds between the nucleophilic amino acids of the enzyme and the functional group of the solid carrier. The binding forces of physical adsorption, such as van der Waals binding, hydrophobic or ionic interactions, are often too weak to keep the adsorbed enzymes

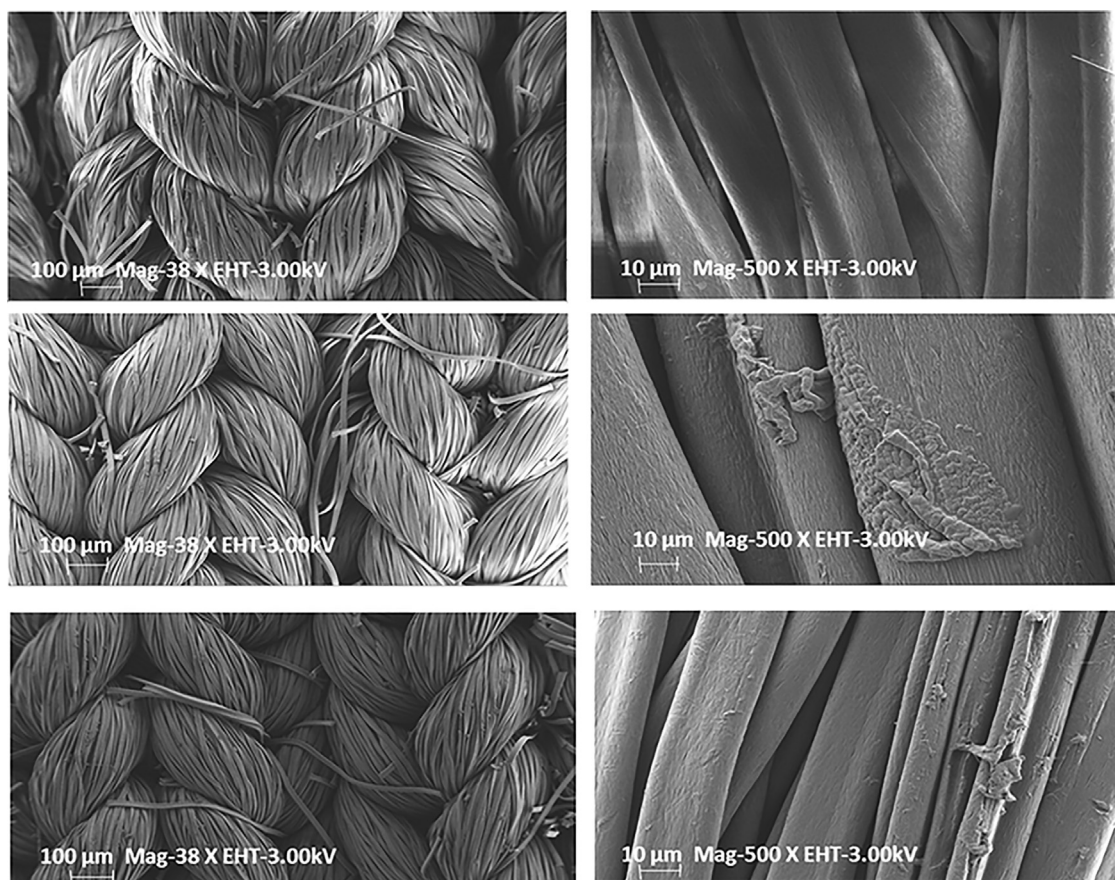


Fig. 3. SEM micrographs of PVA fibre surfaces (100 μm and 10 μm) after different chemical modifications: A) Before treatment; B) After treatment with chloropropionyl chloride; C) After treatment with chloropropionyl chloride, ethylenediamine and glutaraldehyde.

fixed to the carrier during use or washing. However, covalent attachment is strong and enables the enzyme molecules to be anchored to the carrier during use or repeated washing [18,19,51].

The measured activities for glutaraldehyde activated and non-activated immobilized carriers (i.e. PVA, PVA-Cl, PVA-Cl-EDA and PVA-Cl-EDA-GA) are shown in Table 1. There was a small amount of ADH activity recorded with PVA, PVA-Cl and PVA-Cl-EDA presumably due to nonspecific binding. When ADH was immobilized onto the PVA-Cl-EDA-GA fibrous carrier, a 7.3 fold increase in activity was observed compared to the PVA control (without chemical modification). This increase in activity supports successful ADH immobilization at pH 8.1.

The effect of spacer length on the activity of the immobilized ADH was investigated by incubating PVA-Cl discs in EDA, HMA and 1, 12-dodecadiamine in ethanol (20% w/v) respectively at 60 °C for 5 h (diamine concentrations details were presented in Supporting document S_1). In the second step, the aminated PVA carriers were reacted with GA prior to enzyme attachment. Previous work suggested that branched amines such as PEI and polylysine would provide multiple binding sites for immobilization [52]. In this study this approach was further extended by producing spacer arms of defined length by using linear diamines of increasing carbon length (C2, C6 and C12). The experimental results on the effect of the spacer length on the activity of the immobilized ADH are presented in Table 2 and Supplement document).

Upon ADH immobilization, the highest activity was observed with EDA ($n=2$). When ADH was immobilized with the HMA spacer ($n=6$) a lower activity was recorded, and with the 1, 12-dodecadiamine spacer ($n=12$) activity was greatly diminished. Loss of activity could be either due to aggregation of the saturated amines in the aqueous medium [53] or could also be the fact that longer diamines may be more prone to both ends reacting with the PVA-Cl surface, hence not as many options for ADH to immobilise. The EDA spacer provided the highest activity and hence was used in all subsequent work. The effect of EDA concentration was measured by reacting PVA-Cl fabric discs with EDA concentrations ranging from 10 to 40% (w/v) in ethanol. The EDA modified discs were immobilized with ADH after GA activation. Fig. 4 shows the effect different concentrations of EDA (10 to 30% w/v in ethanol) spacer on ADH activity. The activity of the ADH enzyme increased with increasing the concentration of EDA and the highest activity

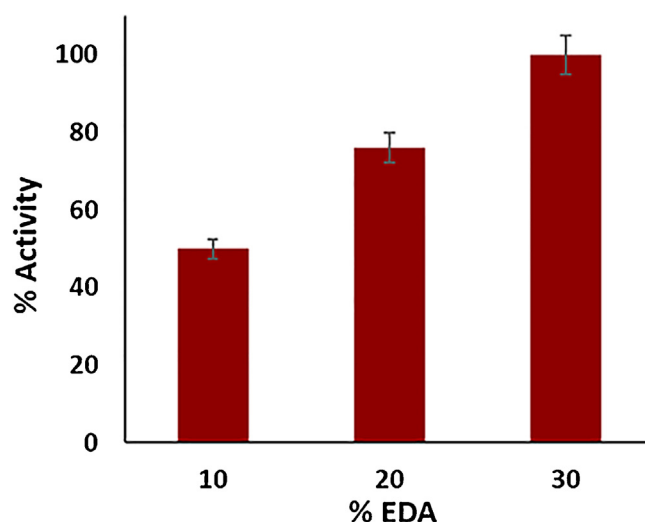


Fig. 4. The effect of EDA concentration on activity of immobilized ADH.

was observed with 30% EDA (0.785 ± 0.05 mmol/min g). Higher concentrations of EDA (above 30%) lead to disintegration of the PVA fabric.

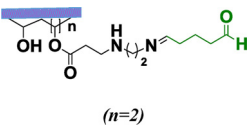
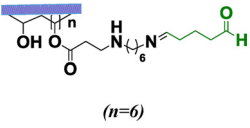
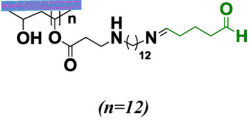
3.3. Stability of immobilized ADH

Immobilized enzymes are more easily recovered and recycled than soluble enzymes and can also possess higher stability over wider physical and chemical conditions [19]. Both of these features are essential for industrial applications where immobilized enzymes are required to sustain wider pH and temperature ranges to achieve optimal process.

3.3.1. pH stability

Enzyme stability is heavily dependent on the pH of the solution. The effect of pH on the enzyme stability was investigated by treating soluble ADH and immobilized ADH at 40 °C for 2 h at different pH values before their activities were measured (Fig. 5). The soluble enzyme showed good stability at pH 5–7. There was a

Table 2
Effect of diamine spacer length on immobilized ADH activity.

Diamine	Spacer	Activity (mmol/g min)
Ethylenediamine (EDA)	 ($n=2$)	0.512 ± 0.05
Hexamethylenediamine (HMA)	 ($n=6$)	0.283 ± 0.01
1,12-dodecadiamine	 ($n=12$)	0.063 ± 0.04

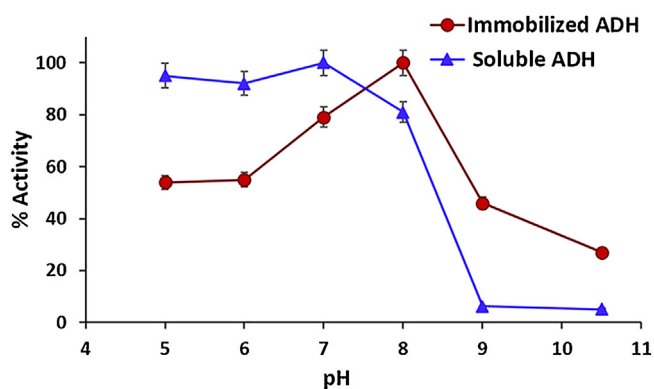


Fig. 5. Stability of soluble and immobilized ADH at different pHs. Both soluble ADH and immobilized ADH were incubated at 40 °C for 2 h in different pH buffered solutions.

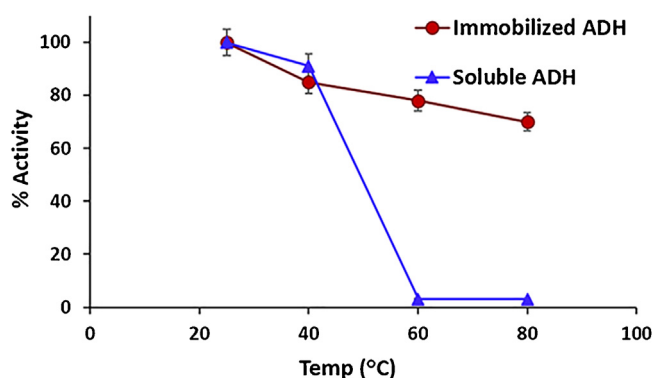


Fig. 6. Thermostability of soluble and immobilized ADH after being heated at different temperatures in phosphate buffer (0.05 M, pH 8.1) for 2 h.

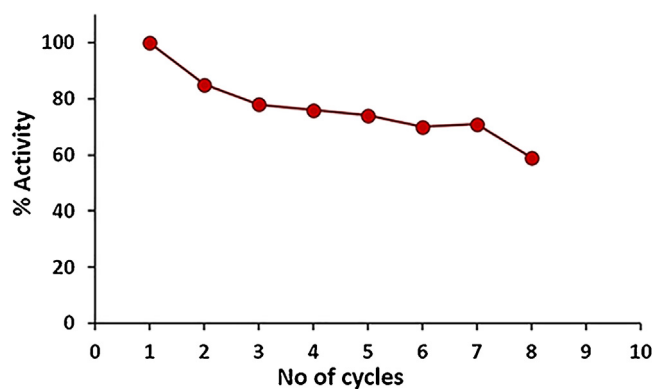


Fig. 7. Reusability of the immobilized ADH.

decrease in stability at pH 8 (with ~80% retention in activity) and complete loss of activity at pH 9 or higher. Immobilization of ADH changed its pH stability profile, with enzyme retaining 46% and 27% of its activity at pH 9 and 10.5, respectively. The optimal stability shifted to pH 8 compared to pH 5–7 for the soluble form. These findings show that covalent immobilization can provide enhanced pH stability with retention of enzyme activity over a broader pH range. Multipoint covalent attachment of the enzyme would reduce the conformational freedom of the enzyme and may be responsible for the enhanced stability profile. A similar

broadening of pH stability has been reported when ADH is immobilized on attapulgite nanofibers [54].

3.3.2. Thermostability

The thermostability of the immobilized and soluble ADH was assessed by treatment at different temperatures (20 °C–80 °C) for 2 h. Fig. 6 shows that there was a rapid thermal inactivation of soluble ADH at 60 °C and higher temperatures. Significantly, the immobilized ADH preserved 80% and 60% of its activity after being heated at 60 °C and 80 °C respectively.

Thermal denaturation of ADH at higher temperature (T_d 63 °C) is thought to arise from irreversible oxidation, aggregation and deamidation of the protein [9]. The observed increased thermal stability of the immobilized enzyme could also be due to multipoint attachment of the enzyme to the carrier (i.e. reduction in intermolecular reaction). Similar improvement in stability was observed when ADH was immobilized on glyoxyl agarose [55], magnetic graphene oxide nanocomposites [56], glass beads [12], and a cyanogen bromide-activated Sepharose system [57].

3.3.3. Reusability

An advantage of immobilized enzymes is their potential for reuse. To evaluate the reusability of the immobilized ADH, carrier discs with immobilized ADH were repeatedly suspended in a fresh reaction mixture for enzyme activity measurements. Between measurements the carrier discs were washed with phosphate buffer (0.05 M, pH 7.5).

Fig. 7 shows that the efficacy of the immobilized ADH declined slightly after each use, probably due to the change in conformation of immobilized enzyme after repeated washings or some enzyme detachment from the carrier (because of imine or ester hydrolysis). After eight consecutive runs, the immobilized ADH retained 60% of its original activity.

4. Conclusions

In summary, PVA textile fibrous carrier has been chemically modified and used for enzyme immobilization. It was found that ADH could be covalently immobilized on the modified PVA fibre to provide an immobilised form of the enzyme possessing good operational stability over a wider pH range and higher thermostability as compared to soluble ADH. Furthermore, the immobilized enzyme retained 60% of its original activity after eight reaction cycles making it attractive for industrial applications. These results indicate that PVA fibrous material can be used as an effective carrier material for enzyme immobilization.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2018.e00260>.

References

- [1] J.C. Moore, et al., Advances in the enzymatic reduction of ketones, *Acc. Chem. Res.* 40 (12) (2007) 1412–1419.
- [2] S.M.A. De Wildeman, et al., Biocatalytic reductions: from lab curiosity to "first choice", *Acc. Chem. Res.* 40 (12) (2007) 1260–1266.
- [3] T. Matsuda, R. Yamanaka, K. Nakamura, Recent progress in biocatalysis for asymmetric oxidation and reduction, *Tetrahedron: Asymmetry* 20 (5) (2009) 513–557.
- [4] Y. Huang, et al., Dehydrogenases/reductases for the synthesis of chiral pharmaceutical intermediates, *Curr. Org. Chem.* 14 (14) (2010) 1447–1460.
- [5] R.d.S. Pereira, The use of baker's yeast in the generation of asymmetric centers to produce chiral drugs and other compounds, *Crit. Rev. Biotechnol.* 18 (1) (1998) 25–64.
- [6] M. Breuer, et al., Industrial methods for the production of optically active intermediates, *Angew. Chem. Int. Ed.* 43 (7) (2004) 788–824.
- [7] V. Leskovic, S. Trivić, D. Peričin, The three zinc-containing alcohol dehydrogenases from baker's yeast, *Saccharomyces cerevisiae*, *FEMS Yeast Res.* 2 (4) (2002) 481–494.
- [8] S.B. Raj, S. Ramaswamy, B.V. Plapp, Yeast alcohol dehydrogenase structure and catalysis, *Biochemistry* 53 (36) (2014) 5791–5803.
- [9] K.A. Markossian, et al., Mechanism of thermal aggregation of yeast alcohol dehydrogenase I: role of intramolecular chaperone, *Biochim. Biophys. Acta (BBA)—Proteins Proteom.* 1784 (9) (2008) 1286–1293.
- [10] J.M. Bolivar, et al., Stabilization of a highly active but unstable alcohol dehydrogenase from yeast using immobilization and post-immobilization techniques, *Process Biochem.* 47 (5) (2012) 679–686.
- [11] O. De Smidt, J.C. Du Preez, J. Albertyn, The alcohol dehydrogenases of *Saccharomyces cerevisiae*: a comprehensive review, *FEMS Yeast Res.* 8 (7) (2008) 967–978.
- [12] Y. Yang, R. Chen, H.M. Zhou, Comparison of inactivation and conformational changes of native and apo yeast alcohol dehydrogenase during thermal denaturation, *Biochem. Mol. Biol. Int.* 45 (3) (1998) 475–487.
- [13] K.M. Polizzi, et al., Stability of biocatalysts, *Curr. Opin. Chem. Biol.* 11 (2) (2007) 220–225.
- [14] S.W. Chenevert, et al., Amino acids important in enzyme activity and dimer stability for *Drosophila* alcohol dehydrogenase, *Biochem. J.* 308 (Pt. 2) (1995) 419–423.
- [15] V. Stepankova, et al., Strategies for stabilization of enzymes in organic solvents, *ACS Catal.* 3 (12) (2013) 2823–2836.
- [16] Y. Zhang, J. Ge, Z. Liu, Enhanced activity of immobilized or chemically modified enzymes, *ACS Catal.* 5 (8) (2015) 4503–4513.
- [17] R.K. Singh, et al., From protein engineering to immobilization: promising strategies for the upgrade of industrial enzymes, *Int. J. Mol. Sci.* 14 (1) (2013) 1232–1277.
- [18] B. Brena, P. González-Pombo, F. Batista-Viera, Immobilization of enzymes: a literature survey, in: J.M. Guisán (Ed.), *Immobilization of Enzymes and Cells*, Humana Press, 2013, pp. 15–31.
- [19] S. Datta, L.R. Christena, Y.R.S. Rajaram, Enzyme immobilization: an overview on techniques and support materials, *3 Biotech* 3 (1) (2013) 1–9.
- [20] I. Es, J.D. Goncalves Vieira, A.C. Amaral, Principles, techniques, and applications of biocatalyst immobilization for industrial application, *Appl. Microbiol. Biotechnol.* 99 (5) (2015) 2065–2082.
- [21] R.A. Sheldon, S. van Pelt, Enzyme immobilization in biocatalysis: why, what and how, *Chem. Soc. Rev.* 42 (15) (2013) 6223–6235.
- [22] M.G. Roig, et al., Biotechnology and applied biology section: methods for immobilizing enzymes, *Biochem. Educ.* 14 (4) (1986) 180–185.
- [23] K.A. Pithawala, A. Bahadur, Studies on the immobilization of alcohol dehydrogenase and alkaline phosphatase onto cellulosic supports, *Cell. Chem. Technol.* 36 (3–4) (2002) 265–273.
- [24] X.-P. Jiang, et al., Immobilization of dehydrogenase onto epoxy-functionalized nanoparticles for synthesis of (*R*)-mandelic acid, *Int. J. Biol. Macromol.* 88 (2016) 9–17.
- [25] C. Mateo, et al., Epoxy-amino groups: a new tool for improved immobilization of proteins by the epoxy method, *Biomacromolecules* 4 (3) (2003) 772–777.
- [26] J.M. Guisán, Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes, *Enzyme Microb. Technol.* 10 (6) (1988) 375–382.
- [27] C. Mateo, et al., Glyoxyl agarose: a fully inert and hydrophilic support for immobilization and high stabilization of proteins, *Enzyme Microb. Technol.* 39 (2) (2006) 274–280.
- [28] X.D. Feng, et al., Enabling the utilization of wool as an enzyme support: enhancing the activity and stability of lipase immobilized onto woolen cloth, *Colloids Surf. B: Biointerfaces* 102 (2013) 526–533.
- [29] P. Zucca, E. Sanjust, Inorganic materials as supports for covalent enzyme immobilization: methods and mechanisms, *Mol. Cell. Probes* 19 (9) (2014) 14139.
- [30] N.T.S. Phan, D.H. Brown, P. Styring, A facile method for catalyst immobilisation on silica: nickel-catalysed Kumada reactions in mini-continuous flow and batch reactors, *Green Chem.* 6 (10) (2004) 526–532.
- [31] S.J. Haswell, et al., The application of micro reactors to synthetic chemistry, *Chem. Commun.* (5) (2001) 391–398.
- [32] K. Kiehl, et al., Strategies for permanent immobilization of enzymes on textile carriers, *Eng. Life Sci.* 15 (6) (2015) 622–626.
- [33] J.V. Edwards, et al., Covalent attachment of lysozyme to cotton/cellulose materials: protein versus solid support activation, *Cellulose* 18 (5) (2011) 1239–1249.
- [34] S. Chatterjee, et al., Silk-fiber immobilized lipase-catalyzed hydrolysis of emulsified sunflower oil, *Appl. Biochem. Biotechnol.* 157 (3) (2009) 593–600.
- [35] Y. Gao, et al., Bioremediation of pesticide contaminated water using an organophosphate degrading enzyme immobilized on nonwoven polyester textiles, *Enzyme Microb. Technol.* 54 (2014) 38–44.
- [36] K. Opwis, D. Knittel, E. Schollmeyer, Functionalization of catalase for a photochemical immobilization on poly(ethylene terephthalate), *Biotechnol. J.* 2 (3) (2007) 347–352.
- [37] A. Freeman, M. Sokolovsky, L. Goldstein, Chemically modified nylons as supports for enzyme immobilization, *J. Solid-Phase Biochem.* 1 (4) (1976) 261–274.
- [38] F.H. Isgrove, et al., Enzyme immobilization on nylon-optimization and the steps used to prevent enzyme leakage from the support, *Enzyme Microb. Technol.* 28 (2–3) (2001) 225–232.
- [39] S. Pahuji, et al., Glutaraldehyde activation of polymer Nylon-6 for lipase immobilization: enzyme characteristics and stability, *Bioresour. Technol.* 99 (7) (2008) 2566–2570.
- [40] M. Cerretti, et al., Immobilisation of pectinases into PVA gel for fruit juice application, *Int. J. Food Sci. Technol.* 52 (2) (2017) 531–539.
- [41] M. Rebroč, et al., Hydrolysis of sucrose by invertase entrapped in polyvinyl alcohol hydrogel capsules, *Food Chem.* 102 (3) (2007) 784–787.
- [42] J.C. Santos, et al., *Pseudomonas fluorescens* lipase immobilization on polysiloxane-polyvinyl alcohol composite chemically modified with epichlorohydrin, *J. Mol. Catal. B: Enzym.* 52–53 (0) (2008) 49–57.
- [43] J.R.L. Walker, Spectrophotometric determination of enzyme activity: alcohol dehydrogenase (ADH), *Biochem. Educ.* 20 (1) (1992) 42–43.
- [44] B. Jin, et al., Synthesis, characterization, thermal stability and sensitivity properties of the new energetic polymer through the azidoacetylation of poly(vinyl alcohol), *Polym. Degrad. Stab.* 97 (4) (2012) 473–480.
- [45] J. Bernard, et al., Synthesis of poly(vinyl alcohol) combs via MADIX/RAFT polymerization, *Polymer* 47 (4) (2006) 1073–1080.
- [46] T. Cao, et al., Investigation of spacer length effect on immobilized *Escherichia coli* pili-antibody molecular recognition by AFM, *Biotechnol. Bioeng.* 98 (6) (2007) 1109–1122.
- [47] A. De Maio, et al., Influence of the spacer length on the activity of enzymes immobilised on nylon/polyGMA membranes: part 1. Isothermal conditions, *J. Mol. Catal. B: Enzym.* 21 (4–6) (2003) 239–252.
- [48] E.J. Shim, et al., Development of an enzyme-immobilized support using a polyester woven fabric, *Text. Res. J.* 87 (1) (2017) 3–14.
- [49] I. Migneault, et al., Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking, *Biotechniques* 37 (5) (2004) 790–802.
- [50] K.-J. Kim, S.-B. Lee, N.-W. Han, Kinetics of crosslinking reaction of PVA membrane with glutaraldehyde, *Korean J. Chem. Eng.* 11 (1) (1994) 41–47.
- [51] G. Peng, et al., Stabilized enzyme immobilization on micron-size PSt-GMA microspheres: different methods to improve the carriers' surface biocompatibility, *RSC Adv.* 6 (94) (2016) 91431–91439.
- [52] D. Kim, A.E. Herr, Protein immobilization techniques for microfluidic assays, *Biomicrofluidics* 7 (4) (2013) 041501.
- [53] B.S. Mahesh, *Biotechnology-3: including molecular biology biophysics*, *Biotechnology* 3 (2003) 227.
- [54] Q. Zhao, et al., Characterization of alcohol dehydrogenase from permeabilized brewer's yeast cells immobilized on the derived attapulgite nanofibers, *Appl. Biochem. Biotechnol.* 160 (8) (2010) 2287–2299.
- [55] J.M. Bolivar, et al., Improvement of the stability of alcohol dehydrogenase by covalent immobilization on glyoxyl-agarose, *J. Biotechnol.* 125 (1) (2006) 85–94.
- [56] M.-H. Liao, D.-H. Chen, Immobilization of yeast alcohol dehydrogenase on magnetic nanoparticles for improving its stability, *Biotechnol. Lett.* 23 (20) (2001) 1723–1727.
- [57] H. Görisch, M. Schneider, Stabilization of soluble and immobilized horse liver alcohol dehydrogenase by adenosine 5'-monophosphate, *Biotechnol. Bioeng.* 26 (8) (1984) 998–1002.