

Article

Immobilization and Catalytic Properties of Limonene-1,2-Epoxyde Hydrolase

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Abstract

In this work, limonene-1,2-epoxide hydrolase (LEH) from *Rhodococcus erythropolis* was immobilized on Immobead 150P and various Purolite[®] methacrylate-based carriers, and subsequently applied in the hydrolysis of *cis*-/*trans*-(+)-limonene-1,2-epoxide. The immobilization efficiency was assessed based on the recovery of activity and the immobilization yield. The best results were observed when LEH was immobilized on Purolite Lifetech[™] ECR8309M modified with glutaraldehyde and 1,4-diaminobutane, yielding a specific activity of 60.5 U·g^{−1}. The optimal temperature for enzymatic reaction increased from 40 °C (free LEH) to 60 °C following immobilization. Thermal inactivation studies revealed that the free LEH followed a simple aggregation mechanism, whereas the immobilized form conformed to a two-step deactivation model, involving an initial reversible equilibrium followed by an irreversible inactivation step. When applied in 10 reaction cycles at 30 °C the immobilized biocatalyst retained 62% of the initial activity in the presence of 10% (*v/v*) acetonitrile and 75% in its absence. At temperatures up to 60 °C, immobilized LEH appeared very stable, retaining more than 80% of activity at 60 °C after 6 reaction cycles. This study improves our understanding of the inactivation mechanism of LEH, and the results highlight covalent immobilization as a promising method for the thermal stabilization of LEH.

Keywords: limonene-1,2-epoxide hydrolase; enzyme immobilization; thermal inactivation



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1. Introduction

Epoxide hydrolases are enzymes that catalyze the hydrolytic opening of the oxirane ring to form the corresponding vicinal diol. Owing to their relatively wide range of substrates, high stereo- and regioselectivity, and ability to catalyze reactions without cofactors, they have found many potential applications in synthetic organic chemistry, as well as in the pharmaceutical and food industries [1].

Limonene-1,2-epoxide hydrolases (EC 3.3.2.8.) (LEHs) represent an atypical group of epoxide hydrolases [2]. Unlike other epoxide hydrolases, LEHs do not contain an α/β -fold, a core domain of eight β -strands connected by α -helices, which is characteristic of α/β -fold hydrolases [3]. The first LEH to be discovered and functionally characterized was derived from the actinomycete bacterium *R. erythropolis*, where it functions in a metabolic pathway that enables the utilization of limonene as the sole carbon and energy source [2,4].

Since then, only a few wild-type LEHs have been identified based on amino acid sequence similarity [5–9].

The LEH from *R. erythropolis* has a relatively narrow substrate spectrum and shows significantly lower activities toward other epoxides in addition to limonene-1,2-epoxide. Its enantioselectivity toward *meso*-epoxides is also almost non-existent [10]. Owing to this fact and the good knowledge of its structure and reaction mechanism, LEH has been used in several studies aimed at improving its enantioselectivity [11–15]. The active site of LEH from *R. erythropolis* was rationally redesigned to enable the synthesis of chiral N- and O-heterocycles via Baldwin cyclization from hydroxy- and amino-substituted epoxides and oxetanes, resulting in high conversions and enantiomeric ratios [16]. Recently, the enantioselectivity of two LEHs toward 12 different racemic oxetanes was enhanced through enzyme engineering, with the aim of preparing optically pure 1,3-diols and oxetanes [9].

Enzyme engineering has significantly enhanced the synthetic utility of LEH; nevertheless, the hydrolysis of its “native” substrate, limonene-1,2-epoxide, remains of considerable practical interest. Currently, both (+)- and (-)-limonene-1,2-epoxide are commercially available as mixtures of their respective *cis*- and *trans*-isomers. Efforts to obtain enantiopure limonene-1,2-epoxide via asymmetric oxidation have yielded satisfactory conversions; however, the resulting epoxide purity has generally been suboptimal. Selective hydrolysis of the individual isomers has also been investigated with improved outcomes, though the reactions typically require stringent physical and chemical conditions [17]. The application of LEHs with complementary stereoselectivity has enabled the biocatalytic resolution of all four enantiomers of limonene-1,2-epoxide from *cis/trans* mixtures of both (+)- and (-)-isomers. This approach also facilitates the production of optically pure limonene-1,2-diol [18]. Notably, (+)-*trans*-limonene-1,2-epoxide serves as a precursor for the synthesis of limonene-based polycarbonates through polymerization with CO₂ or cyclohexene oxide [19–21]. The hydrolysis product, limonene-1,2-diol, is also interesting in terms of practical applications. It is an important intermediate in the synthesis of compounds such as the (1*S*,4*R*)- and (1*S*,4*S*)-isomers of 4-isopropyl-1-methyl-2-cyclohexen-1-ol [22] and diterpenes prevezol B and C [23,24]. Furthermore, limonene-1,2-diol shows promising anti-inflammatory [25], immunosuppressive [26], and antimicrobial activities against the bacterium *Cryptococcus neoformans* [27].

The commercial utilization of LEHs remains limited, primarily due to the high costs associated with their single-use applications, which underscores the importance of their immobilization. Epoxide hydrolases have been immobilized using a wide range of techniques, including adsorption [28,29], entrapment [30,31], encapsulation [32,33], affinity binding [34,35], cross-linking [36,37], and, most prevalently, covalent binding [38–43]. The reported advantages of epoxide hydrolase immobilization include enhanced operational, thermal, and storage stability, as well as improved enantioselectivity [1]. Despite the extensive body of research on epoxide hydrolase immobilization, studies specifically focused on the immobilization of limonene-1,2-epoxide hydrolase are notably absent.

In the present study, LEH was immobilized for the first time. For this purpose, recombinant LEH from *R. erythropolis* was linked to methacrylate carriers that had been functionalized with various reactive groups. The immobilization methods included covalent binding and affinity immobilization via His-tag and divalent metal ions. The hydrolysis of (+)-limonene-1,2-epoxide catalyzed by free and immobilized LEH was investigated. Given that the thermal inactivation of LEH from *R. erythropolis* represents a significant limitation to its practical application [2], this study has mainly focused on the influence of LEH immobilization on its thermal stability.

2. Results and Discussion

2.1. Expression and Purification of LEH

The *limA* gene, encoding LEH from *R. erythropolis*, was overexpressed in *E. coli* with a C-terminal hexa-histidine tag. Cultivation was performed in shake flasks, and the enzyme was isolated from the cell-free extract using Ni^{2+} -affinity chromatography (Table S1). The purification process resulted in a 2.56-fold enrichment, yielding a specific activity of $76.76 \text{ U} \cdot \text{mg}_{\text{prot}}^{-1}$. SDS-PAGE analysis revealed a monomeric molecular weight of about 18 kDa (Figure S1), aligning with the theoretical mass of LEH [2], fused to the hexa-histidine tag [44].

The recombinant enzyme catalyzed the stepwise hydrolysis of both isomers of (+)-limonene-1,2-epoxide, as depicted in the reaction pathway (Figure 1A). The time-course of the reaction is shown in Figure 1B. By adjusting the enzyme-to-substrate ratio (up to 3 mg protein per mmol substrate) and selecting an appropriate reaction time (up to 120 min), selective hydrolysis of the *cis*-isomer was achieved, with the *trans*-isomer remaining largely unconverted in the reaction mixture (Figure 1B).

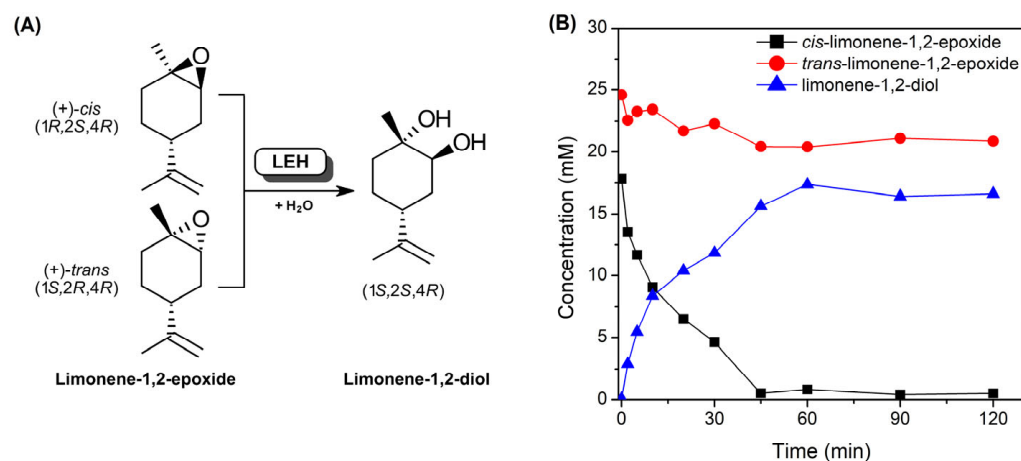


Figure 1. (A) Reaction scheme of hydrolysis of (+)-limonene-1,2-epoxide (mixture of *cis*- and *trans*-isomers) catalyzed by LEH. (B) Hydrolysis of *cis*-limonene-1,2-epoxide catalyzed by LEH (concentration of proteins in the reaction mixture $20 \mu\text{g} \cdot \text{mL}^{-1}$, 40 mM (+)-limonene-1,2-epoxide).

A key consideration in reactions catalyzed by epoxide hydrolases is the inherently low solubility of epoxide substrates in aqueous media. To address this limitation, water-miscible cosolvents are commonly employed to enhance substrate solubility and improve reaction efficiency [7]. Ferrandi et al. demonstrated that LEHs are capable of hydrolyzing limonene-1,2-epoxide at concentrations as high as 2 M when the substrate is introduced into the reaction as a pure compound [18]. In the present study, a substrate concentration of 50 mM was used, substantially exceeding its aqueous solubility of 4.6 mM at 25°C [45]. The addition of 10% (*v/v*) acetonitrile to the reaction mixture slightly increased the solubility of limonene-1,2-epoxide but also resulted in the formation of a stable emulsion that could not be resolved by centrifugation.

2.2. Immobilization of LEH on Different Carriers

Multipoint covalent immobilization has been shown to enhance the structural rigidity of immobilized enzymes [46]. The extent of mobility restriction imposed on enzyme molecules depends largely on the length of the spacer arm used for immobilization. Shorter spacer arms tend to impose greater conformational constraints, while longer spacer arms facilitate the formation of additional enzyme-support interactions, thereby enabling more extensive multipoint covalent binding. Selecting an optimal spacer arm length is thus a

key step in the design of robust immobilized enzyme systems. As such, the evaluation of different spacer arm lengths is a rational strategy for optimizing covalent enzyme immobilization [46–48].

For the immobilization of LEH, four methacrylate-based carriers were employed: two epoxy-functionalized carriers, namely, Purolite Lifetech™ ECR8209M (Purolite Epoxy) and Immobead 150P (Immobead), and two amino-functionalized carriers, namely, Purolite Lifetech™ ECR8309M (Purolite Amino C2) and ECR8409M (Purolite Amino C6). To assess the influence of spacer arm length on activity recovery and immobilization yield, the carriers were modified using combinations of 1,4-diaminobutane (DAB) and glutaraldehyde (GA) (Figures S2 and S3).

All the carriers tested exhibited very good immobilization efficiency when >99% of the protein used for immobilization was bound, except for the Immobead carrier, where the immobilization yield was slightly lower (98.5%) (Table 1). Notably, all carriers modified with extended spacer arms (Immobead-DAB-GA, Purolite Epoxy-DAB-GA, Purolite Epoxy-IDA-Ni²⁺, Purolite Amino C2-GA-DAB-GA, Purolite Amino C6-GA-DAB-GA, Purolite Amino C2-GA-DAB-GA-DAB-GA, and Purolite Amino C6-GA-DAB-GA-DAB-GA) exhibited comparable abilities to bind the enzyme as the same unmodified carriers.

Table 1. Immobilization yield, activity recovery and specific activity of LEH immobilized on modified carriers.

Carrier—Modification	Immobilization Yield (%)	Retained Activity (%)	Immobilized Preparation Activity (U·g ^{−1})
Purolite Epoxy	99.2	0.07	0.7
Immobead	98.5	0.04	0.5
Purolite Epoxy-IDA-Ni ²⁺	99.4	0.79	2.1
Purolite Epoxy-DAB-GA	99.0	2.19	15.0
Immobead-DAB-GA	100.0	0.61	4.2
Purolite Amino C2-GA	99.6	6.02	41.3
Purolite Amino C6-GA	100.0	5.74	45.5
Purolite Amino C2-GA-DAB-GA	99.5	7.57	51.9
Purolite Amino C6-GA-DAB-GA	99.5	8.83	60.5
Purolite Amino C2-GA-DAB-GA-DAB-GA	99.1	6.37	30.6
Purolite Amino C6-GA-DAB-GA-DAB-GA	99.1	6.54	44.7

Initially, the immobilization of LEH was tested using the epoxy carriers Purolite Epoxy and Immobead, where the lowest activity recovery was observed among all the immobilization methods, i.e., 0.07% and 0.04%, respectively. Compared with immobilization on epoxy carriers, immobilization on glutaraldehyde-activated amino carriers (Purolite Amino C2-GA and Purolite Amino C6-GA) resulted in about 100-fold greater activity recovery (6.02% and 5.74% for LEH linked to Purolite Amino C2-GA and to Purolite Amino C6-GA, respectively) (Table 1). This is a similar observation to that in a study of immobilization of mutant epoxide hydrolase from *Vigna radiata* (VnEH2_{M263N}) on amino and epoxy carriers [42] where better results with amino carriers were also reported.

The immobilization of LEH on epoxy carriers modified with 1,4-diaminobutane and glutaraldehyde (Figure S2) was based on a similar strategy employed by Yildirim et al. [49] for the immobilization of epoxide hydrolase from *Aspergillus niger*. According to our results, compared with immobilization on unmodified epoxy carriers, immobilization of LEH on these modified carriers resulted in 15-fold and 31-fold increases in activity retention, respectively (Table 1).

Similar modifications were performed with Purolite Amino C2 and C6 carriers (Figure S3). The first carrier modifications with 1,4-diaminobutane and glutaraldehyde (Purolite Amino C2-GA-DAB-GA and Purolite Amino C6-GA-DAB-GA) resulted in the highest activity retention after immobilization, 7.57% and 8.83%, respectively, and the highest immobilized activities, $51.9 \text{ U} \cdot \text{g}^{-1}$ and $60.5 \text{ U} \cdot \text{g}^{-1}$. However, subsequent extension of the spacer arm through repeated modifications with 1,4-diaminobutane and glutaraldehyde (Purolite Amino C2-GA-DAB-GA-DAB-GA and Purolite Amino C6-GA-DAB-GA-DAB-GA) led to a decrease in activity retention (Table 1).

As shown by the above results, the enzyme activity yield after immobilization was relatively low. The enzyme incubated without a carrier under the same conditions as those used during the immobilization reaction (laboratory temperature, buffer solution, and mixing) for 24 h retained 100% of the initial activity. Therefore, the loss of enzyme activity was not a simple result of enzyme inactivation during immobilization. Since the enzyme is bound inside the particles due to the pore size, one of the probable reasons for the significant decrease in activity is the diffusion limitations in the pores. However, the results in Table 1 show that the decrease in activity after immobilization also depends on the method of carrier activation and immobilization technique, suggesting the possibility of other reasons for the loss of activity, which are most likely steric hindrances and conformational changes in the enzyme molecules.

Importantly, LEH in all the immobilized preparations with amino carriers retained the ability to preferentially hydrolyze the *cis*-isomer of (+)-limonene-1,2-epoxide. The increase in consumption of the *trans*-isomer indicates a different ratio of active enzyme to substrate compared with the reaction with the free enzyme depicted in Figure 2. The epoxide substrate in the reaction mixture was completely converted within 24 h.

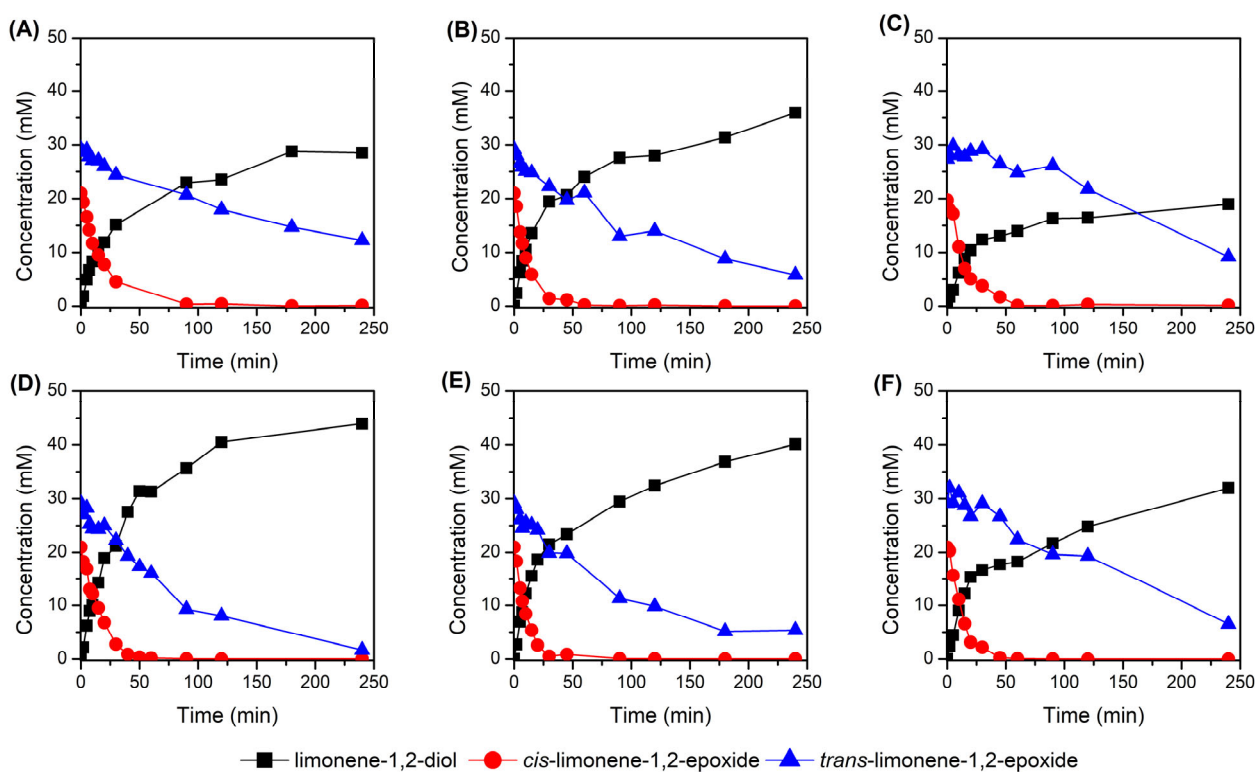


Figure 2. Hydrolysis of the *cis*- and *trans*-isomers of (+)-limonene-1,2-epoxide to (+)-limonene-1,2-diol with (A) LEH@Purolite Amino C2-GA, (B) LEH@Purolite Amino C2-GA-DAB-GA, (C) LEH@Purolite Amino C2-GA-DAB-GA-DAB-GA, (D) LEH@Purolite Amino C6-GA, (E) LEH@Purolite Amino C6-GA-DAB-GA, (F) LEH@Purolite Amino C6-GA-DAB-GA-DAB-GA.

Affinity-based immobilization of LEH was also investigated using an IDA-modified Purolite Epoxy carrier loaded with Ni^{2+} ions. To ensure that immobilization occurred exclusively through affinity interactions with the C-terminal His-tag, residual epoxy groups on the carrier were blocked using ethanolamine (Figure S4). The resulting preparation of LEH immobilized on Purolite Epoxy-IDA- Ni^{2+} (LEH@Purolite Epoxy-IDA- Ni^{2+}), retained only 0.79% of the initial enzymatic activity (Table 1), likely due to suboptimal orientation of the enzyme on the carrier surface. In the LEH structure, the C-terminal region is situated near the entrance of the active site. Although the presence of a C-terminal His-tag does not impair the activity of the free enzyme, its use in immobilization likely results in the active site facing the carrier surface, creating steric hindrances that restricts substrate access. Given that the most favorable immobilization results were achieved using the Purolite Amino C6-GA-DAB-GA carrier, this carrier was chosen for subsequent experiments and characterization of the immobilized LEH.

2.3. Temperature-Activity Profiles of Free and Immobilized LEH

The temperature activity profiles of both free LEH and immobilized on Purolite Amino C6-GA-DAB-GA are presented in Figure 3A. Free LEH exhibited a distinct temperature optimum at 40 °C. Reported values for the temperature optimum of LEH vary widely in the literature, ranging from 30 °C [6] to 50 °C [2]. This variability is likely due to the inherently low thermal stability of LEH [2,50], as well as differences in experimental conditions used to assess enzymatic activity, such as reaction duration, cosolvent presence, and enzyme or substrate concentrations. For LEH@Purolite Amino C6-GA-DAB-GA, the temperature optimum shifted significantly to 60 °C. In general, factors influencing the change in optimum temperature are the type of immobilization (e.g., covalent bonding, adsorption) which can affect the enzyme structure, the carrier material which can influence the enzyme conformation, and conformational changes in enzyme after immobilization that can restrict the enzyme mobility [51]. As a result, often an increase in optimum temperature after the enzyme immobilization can be observed [52–54]. The most likely explanation for the observed upward shift in immobilized LEH lies in multipoint covalent attachment of enzyme molecules, which restricts thermally induced conformational flexibility [55].

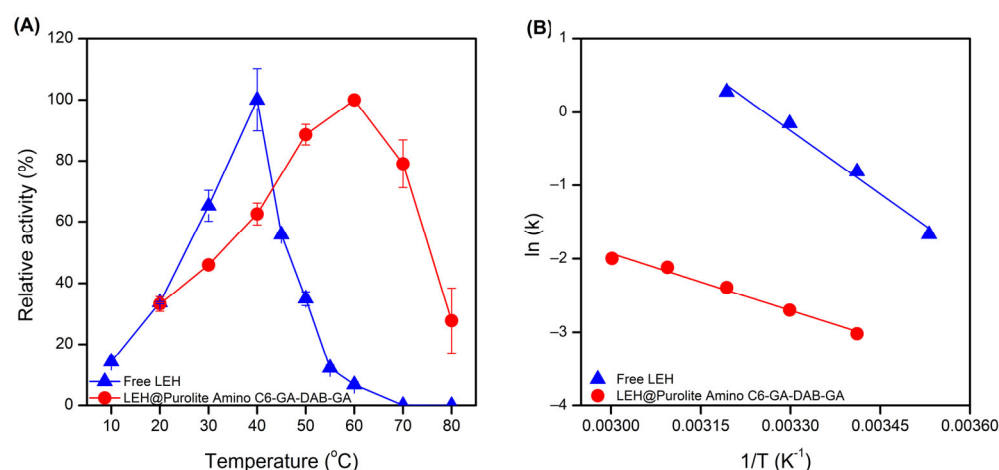


Figure 3. (A) Temperature-activity profile of free and immobilized LEH (LEH@Purolite Amino C6-GA-DAB-GA), (B) Arrhenius plots of free and immobilized LEH.

The activation energy (E_a) of the LEH reaction was determined from the Arrhenius plot (Figure 3B). The activation energy determined for free and immobilized LEH was 48.06 $\text{kJ}\cdot\text{mol}^{-1}$ and 21.40 $\text{kJ}\cdot\text{mol}^{-1}$, respectively. van der Werf, Overkamp and de Bont [2] reported that the activation energy of free LEH was 51.8 $\text{kJ}\cdot\text{mol}^{-1}$. The slight difference from

our calculated values can be attributed to different reaction conditions (higher substrate concentrations and the use of a cosolvent). A more than twofold decrease in the activation energy for the immobilized enzyme approaching the activation energy of molecular diffusion indicates the presence of diffusional limitations.

From the approximate calculations using the hydrodynamic diameter of LEH (calculated by the HullRad program [56] for PDB entry 1NU3), mean particle size and volume, we determined that the majority of the enzyme was covalently bound inside the pores in the carrier (500 times greater than that on the surface of the carrier at a protein loading of $7.91 \text{ mg}_{\text{prot}} \cdot \text{g}_{\text{carrier}}^{-1}$). This fact also confirms that the observed catalytic behavior of immobilized LEH is partly due to the mass transfer limitations in particle pores.

2.4. Thermal Inactivation of Free and Immobilized LEH

The thermal inactivation of free LEH was examined with respect to both protein concentration and temperature. Enzyme solutions were incubated under defined conditions, and samples were periodically withdrawn to assess residual activity. Figure 4 presents inactivation kinetics at a representative temperature across three different protein concentrations. Notably, higher protein concentrations were associated with more rapid inactivation. This observation deviates from the expectations of a simple unimolecular first-order inactivation model, in which the time-dependent decline in relative enzyme activity—described by Equation (11)—would be independent of concentration and yield overlapping decay curves.

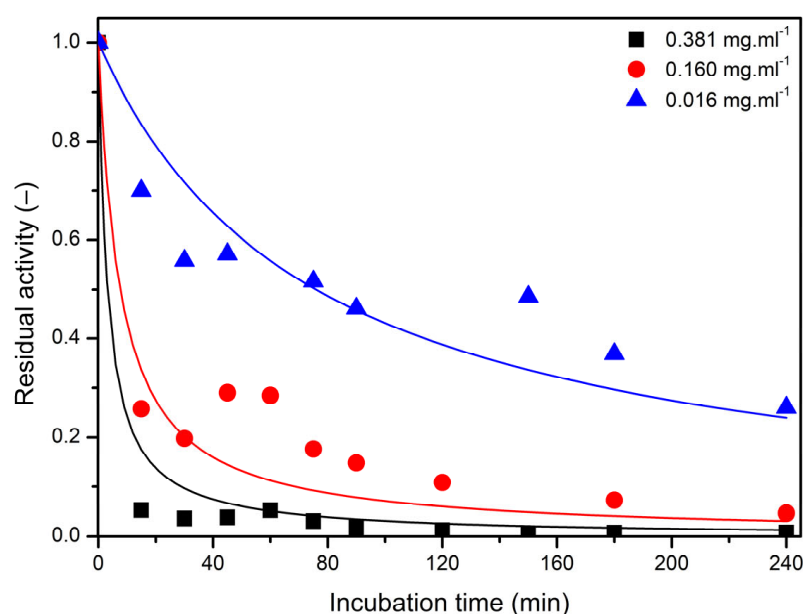


Figure 4. Thermal inactivation of free LEH at 50 °C with different protein concentrations during incubation. Lines represent data calculated from a simple aggregation model (Equation (3)), where the inactivation constant value was $k_d = 0.412 \pm 0.043 \text{ (mg} \cdot \text{mL}^{-1} \cdot \text{min}^{-1})$. The goodness-of-fit parameters are $R^2 = 0.941$ (coefficient of determination), RMSE = 0.0748 (residual mean square error).

Kinetic analysis of the data presented in Figure 4 showed a reasonably good fit with a simplified aggregation-based inactivation model defined by Equation (3). Although the actual mechanism in protein solutions undergoing aggregation is likely more complex—resulting in heterogeneous populations of enzyme molecule aggregates with varying sizes—a more detailed mechanistic investigation would require additional experimental evidence.

The effect of temperature on the thermal inactivation of soluble LEH was further examined across a temperature range of 40 to 60 °C, as illustrated in Figure 5A. While

a temperature of 70 °C was also tested, the inactivation occurred too rapidly to yield reliable kinetic data. The inactivation profiles at each temperature were modeled using the aggregation-based inactivation approach, and the derived inactivation rate constants (k_d) were subsequently used to construct an Arrhenius plot (Figure 5B). The resulting activation energy of $165 \pm 45 \text{ kJ}\cdot\text{mol}^{-1}$ indicates a pronounced temperature sensitivity of LEH in its soluble form.

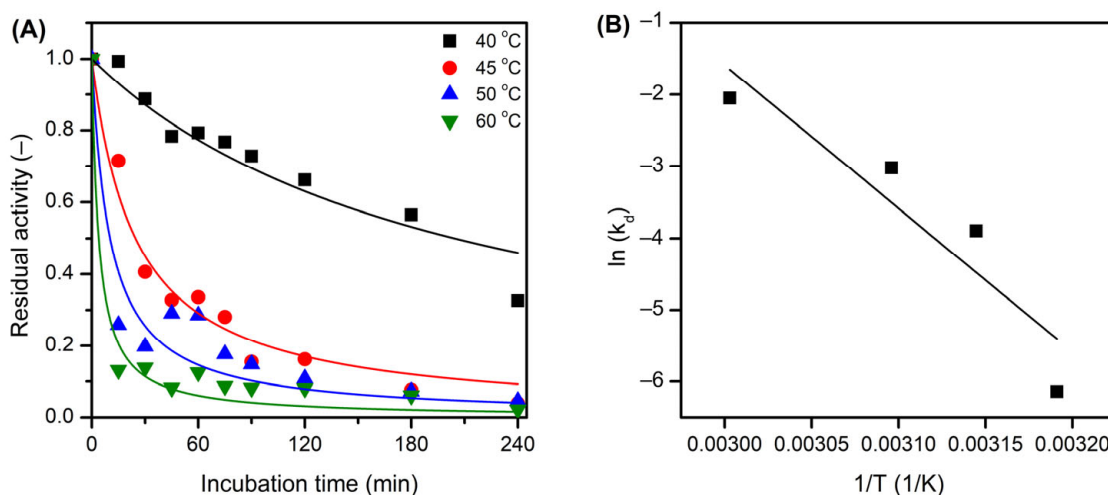


Figure 5. Thermal inactivation of LEH at different temperatures. The protein concentration in the incubation mixture was $0.160 \text{ mg}\cdot\text{mL}^{-1}$. (A) Experimental data (points) and values calculated from the aggregation inactivation model (lines) according to Equation (3); (B) Arrhenius plot for the inactivation constant; the value of the activation energy was $165 \pm 45 \text{ kJ}\cdot\text{mol}^{-1}$. The googles-of-fit parameters for respective temperatures are $R^2 = 0.923$, RMSE = 0.0531 for 40 °C; $R^2 = 0.972$, RMSE = 0.0478 for 45 °C, $R^2 = 0.909$, RMSE = 0.0787 for 50 °C, and $R^2 = 0.977$, RMSE = 0.0417 for 60 °C.

Enhancing thermal stability is one of the most commonly reported benefits of epoxide hydrolase immobilization [1]. Among various stabilizing effects, immobilization can shield the enzyme from irreversible inactivation associated with the aggregation of partially unfolded protein molecules [46]. To assess this potentially stabilizing effect for LEH, thermal inactivation of the enzyme immobilized on Purolite Amino C6-GA-DAB-GA was studied across a range of temperatures, following a similar approach as used for the soluble enzyme. The resulting kinetic data are presented in Figure 6. A comparative analysis with the inactivation data for the free enzyme (Figure 5) revealed that immobilization conferred the most notable stabilization within the 40–50 °C temperature range. However, at 60 °C, both immobilized and free enzymes exhibited substantially faster inactivation rates, although the inactivation rate of the immobilized enzyme at this temperature is half that of the soluble form. To model the thermal inactivation kinetics, a multi-temperature fitting approach was employed using the aggregation model in combination with the Arrhenius equation to account for the temperature dependence of the inactivation rate constant. Figure 6 shows the experimental data alongside model fits. The dotted lines represent the aggregation model, while the solid lines correspond to the two-step model described by Equation (4). The two-step model describes the experimental data more accurately, as evident from the differences between the trends of the calculated and observed data, and the R^2 and RMSE values.

The presented results indicate, as expected, that the binding of enzyme molecules to the solid surface of carrier particles prevents their aggregation, leading to significantly improved enzyme stability.

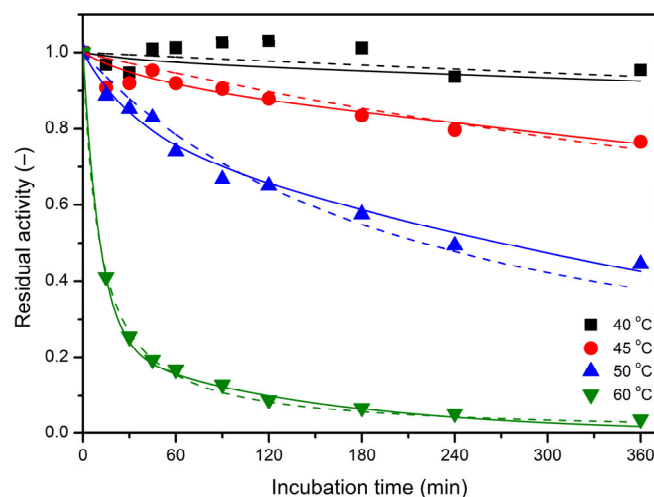


Figure 6. Thermal inactivation of LEH@Amino C6-GA-DAB-GA at different temperatures. The solid lines represent data from the inactivation model according to Equation (4) ($R^2 = 0.994$, RMSE = 0.02669); the dotted lines correspond to the aggregation model (Equation (3)) ($R^2 = 0.992$, RMSE = 0.0299).

2.5. Operational Stability of Immobilized LEH

One of the primary advantages of enzyme immobilization is the potential for catalyst reuse, which can significantly reduce overall process costs, provided the biocatalyst maintains sufficient stability. The operational stability of LEH@Amino C6-GA-DAB-GA was evaluated over ten consecutive batch reaction cycles (Figure 7). Given the use of acetonitrile as a cosolvent, its impact on the performance of the immobilized enzyme was assessed by comparing activity retention in the presence and absence of the solvent at 30 °C. Reactions conducted without acetonitrile followed the same procedure described in Section 3.6, except that the reaction mixture consisted of 500 μ L of potassium phosphate buffer (50 mM, pH 7.0) and 4.1 μ L of (+)-limonene-1,2-epoxide, which was directly added. Under these conditions, the immobilized enzyme retained 75% of its initial activity after 10 cycles. In contrast, when acetonitrile was included in the reaction medium, residual activity declined to 62% after ten cycles. In addition to enzymatic inactivation, the gradual loss in activity was attributed to mechanical damage of the immobilized enzyme particles and cumulative loss of material during repeated handling across cycles.

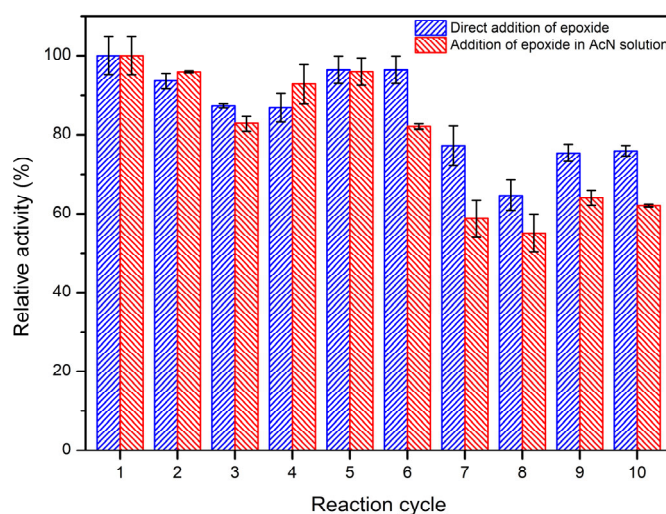


Figure 7. Operational stability of LEH@Amino C6-GA-DAB-GA at 30 °C in the hydrolysis of (+)-limonene-1,2-epoxide added to the reaction mixture directly (blue bars) and dissolved in acetonitrile (AcN) (10% (v/v) of the reaction mixture) (red bars).

The effect of the reaction temperature (30–70 °C) on the repeated use of immobilized LEH was investigated over six 30 min reaction cycles. As can be seen in Figure 8, the immobilized LEH could be reused up to six times without significant loss of activity at reaction temperatures up to 60 °C, while in all reaction cycles that were carried out at temperatures ranging from 30 to 60 °C, the conversion of *cis*-limonene-1,2-epoxide after a 30 min reaction reached >90%. A substantial loss in activity was observed exclusively when the preparation was used at 70 °C, where the relative activity following the sixth reaction cycle was 24.1% (Figure 8). The results obtained demonstrate that immobilized LEH can be reused at a wide range of reaction temperatures (30–60 °C) to enzymatically resolve the *cis*- and *trans*-isomers of (+)-limonene-1,2-epoxide.

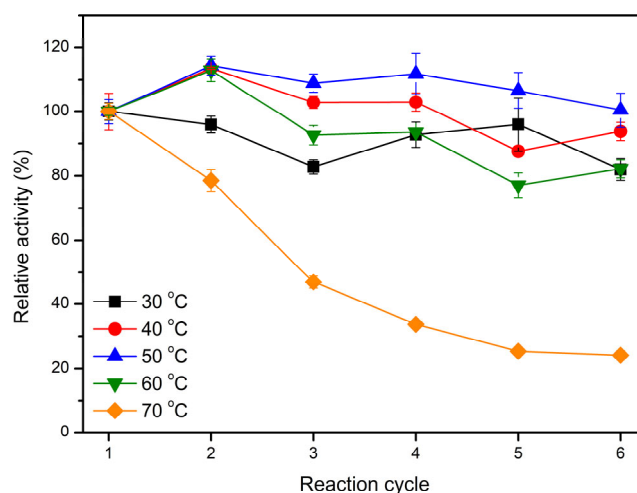


Figure 8. Operational stability of LEH@Amino C6-GA-DAB-GA in the hydrolysis of (+)-limonene-1,2-epoxide at different reaction temperatures (30–70 °C).

3. Materials and Methods

3.1. Materials

Purolite Lifetech™ ECR8309M, ECR8409M, and ECR8209M carriers were obtained from Purolite Ltd. (Compton, UK). Immobead 150P, (+)-limonene-1,2-epoxide (>97%, combined *cis/trans* isomers in a 21:29 ratio), (1S,2S,4R)-(+)-limonene-1,2-diol (>97% GC purity), *n*-decane (≥99.5%), ethyl acetate (ACS reagent, ≥99.5%), ethanolamine, iminodiacetic acid, 1,4-diaminobutane, glutaraldehyde solution (50 wt% in H₂O), and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). *n*-Hexadecane (≥99%) was sourced from Alfa Aesar (Karlsruhe, Germany), and acetonitrile (HPLC grade, ≥99.8%) was obtained from Fisher Scientific (Loughborough, UK). All other reagents used were of analytical grade and procured from standard commercial suppliers.

3.2. Cloning

The codon-optimized gene of limonene-1,2-epoxide hydrolase (*R. erythropolis* DCL14) [57] was cloned and inserted into a pET28b vector system, and the resulting plasmid was subsequently transformed into *E. coli* BL21 (DE3) cells.

3.3. Production and Purification of Limonene-1,2-Epoxide Hydrolase

Flask cultivation of *E. coli* BL21(DE3) expressing limonene-1,2-epoxide hydrolase was carried out in LB medium following the protocol described by [58]. After 6 h of cultivation, cells were harvested by centrifugation at 20,130 × *g* for 15 min at 4 °C. The resulting cell pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.0) to a final biomass concentration of 9 mg·mL^{−1}. Cell disruption and purification of the His-tagged protein

were performed as previously reported [58], using 400 mM imidazole for elution. Protein-containing fractions were analyzed by SDS-PAGE (Mini-PROTEAN® Tetra Cell, Bio-Rad, Hercules, CA, USA), and those exhibiting a band corresponding to the expected molecular weight of 17 kDa were pooled. The pooled fractions were subsequently concentrated 40-fold and desalted by three sequential buffer exchanges using 0.1 M potassium phosphate buffer (pH 7.0), employing Amicon® Ultra centrifugal filter units (15 mL, 10 kDa MWCO; Merck Millipore Ltd., Carrigtwohill, Ireland).

The protein concentration in the purified enzyme sample was determined by the Bradford method [59] using bovine serum albumin as a protein standard.

3.4. Modification of Carriers

Four commercially available carriers and their modified versions (Table 2) were used for LEH immobilization. Purolite carriers (ECR8209M, ECR8309M, and ECR8409M) had particle sizes of 300–710 µm and pore diameters of 600–1200 Å (according to the manufacturer's specifications). The size of the Immobead 150P particles was 150–500 µm, and their average pore diameter was 100–200 Å [60].

Table 2. Carriers and their modifications used in this study.

Carrier	Carrier Type	Modification/Activation	Designation
Purolite Lifetech™ ECR8209M	Epoxy Methacrylate	- *	Purolite Epoxy
		DAB-GA	Purolite Epoxy-DAB-GA
		IDA-Ni ²⁺	Purolite Epoxy-IDA-Ni ²⁺
Immobead 150P	Epoxy Methacrylate	-	Immobead
		DAB-GA	Immobead-DAB-GA
Purolite Lifetech™ ECR8309M	Amino C2 Methacrylate	GA	Purolite Amino C2-GA
		GA-DAB-GA	Purolite Amino C2-GA-DAB-GA
		GA-DAB-GA-DAB-GA	Purolite Amino C2-GA-DAB-GA-DAB-GA
Purolite Lifetech™ ECR8409M	Amino C6 Methacrylate	GA	Purolite Amino C6-GA
		GA-DAB-GA	Purolite Amino C6-GA-DAB-GA
		GA-DAB-GA-DAB-GA	Purolite Amino C6-GA-DAB-GA-DAB-GA

* Carrier was used directly without modification or preactivation. GA = glutaraldehyde, DAB = 1,4-diaminobutane, IDA = iminodiacetic acid.

3.4.1. Modification of Epoxy Carriers with 1,4-Diaminobutane and Glutaraldehyde

The modification of the epoxy carriers Purolite Lifetech™ ECR8209M and Immobead 150P was performed in two steps. The first step was performed by reacting the carrier with 1,4-diaminobutane (DAB) to introduce alkylamino groups onto the carrier. The carrier was washed with deionized water, suction-dried on a sintered glass filter, transferred to a sealed dark vial, and mixed with four times the volume (*w/v*) of a 2% (*w/v*) DAB water solution. The mixture was incubated for 24 h at room temperature under moderate agitation (250–300 rpm) on an orbital shaker (GFL 3005, Burgwedel, Germany). After incubation, the carrier was thoroughly washed with deionized water. In the second step, the carrier was mixed with four volumes (*w/v*) of 2% (*w/v*) glutaraldehyde (GA) in water and incubated for 1 h at room temperature under mild agitation on an orbital shaker. Finally, the carrier was washed several times with deionized water and 50 mM potassium phosphate buffer, pH 7.0, on a sintered glass filter and immediately used for immobilization.

3.4.2. Modification of Epoxy Carriers with IDA and Ni^{2+}

The epoxy-functionalized carrier Purolite Lifetech™ ECR8209M was modified in three sequential steps, following an adapted protocol based on the method described by Varga et al. [61]. In the first step, the IDA-borate solution (2 M IDA, 0.1 M H_3BO_3 , pH adjusted to 8.5 with 4 M NaOH) was mixed with the carrier at a ratio of 2 mL of solution per 100 mg of carrier. The mixture was incubated in sealed vials, protected from light, for 2 h at room temperature under mild agitation (250–300 rpm) on an orbital shaker. Following incubation, the carrier was washed with deionized water and subjected to a second step in which unreacted epoxy groups were blocked using ethanolamine (EA). A 0.2 M EA solution was added at 0.4 mL per 100 mg of carrier, and the mixture was incubated for additional 2 h at room temperature under the same agitation conditions. The carrier was then washed with deionized water. In the final step, the IDA-functionalized carrier was charged with Ni^{2+} ions by adding a 0.1 M NiCl_2 solution prepared in potassium phosphate buffer (50 mM, pH 7.0) supplemented with 1 M NaCl, at a ratio of 2 mL per 100 mg of carrier. The suspension was incubated for 2 h at room temperature with gentle agitation (250–300 rpm). Afterward, the carrier was washed with excess of deionized water and potassium phosphate buffer (50 mM, pH 7.0) and used immediately for enzyme immobilization.

3.4.3. Activation of Amino Carriers with Glutaraldehyde

Purolite Lifetech™ ECR8309M (Purolite Amino C2) and Purolite Lifetech™ ECR8409M (Purolite Amino C6) carriers were preactivated with glutaraldehyde. The carrier was mixed with four volumes (w/v) of 2% glutaraldehyde solution and incubated for 1 h at room temperature under mild agitation (250–300 rpm) on an orbital shaker. After incubation, the carrier was washed with deionized water and potassium phosphate buffer (50 mM, pH 7.0) and immediately used for immobilization.

3.4.4. Extension of Spacer Arms with 1,4-Diaminobutane and Glutaraldehyde

Freshly prepared glutaraldehyde-activated carriers Purolite Lifetech™ ECR8309M (Purolite Amino C2) and Purolite Lifetech™ ECR8409M (Purolite Amino C6) were used for the preparation of DAB-GA modified supports. The carriers were first treated with a 2% (w/v) aqueous solution of 1,4-diaminobutane at a volume ratio of 4:1 (solution:carrier, w/v) and incubated for 2 h at room temperature under gentle agitation (250–300 rpm) using an orbital shaker. After incubation, the carriers were thoroughly washed with deionized water. In the next step, the pre-treated carriers were reacted with a 2% (w/v) glutaraldehyde solution, again using a 4:1 (solution:carrier, w/v) ratio, and incubated for 1 h at room temperature under mild agitation. The resulting carriers (Carrier-GA-DAB-GA) were then washed with deionized water and potassium phosphate buffer (50 mM, pH 7.0) and used immediately for enzyme immobilization.

For the preparation of double diaminobutane-modified carriers (carriers with the group GA-DAB-GA-DAB-GA), the sequential treatment steps with 1,4-diaminobutane and glutaraldehyde were repeated on the previously modified carriers with the GA-DAB-GA group.

3.5. Immobilization of LEH

To immobilize LEH, the same immobilization protocol was used for all the carriers. Before immobilization, the carrier was washed with potassium phosphate buffer (50 mM, pH 7.0) and then transferred into a vial in which the immobilization reaction was carried out. The enzyme solution ($3.8 \text{ mg}_{\text{prot}} \cdot \text{mL}^{-1}$) was mixed with the carrier in a dark vial at an initial protein-to-carrier ratio of $15 \text{ mg}_{\text{prot}} \cdot \text{g}_{\text{carrier}}^{-1}$, and the mixture was agitated on an orbital shaker for 24 h at 250–300 rpm. After immobilization, the immobilized enzyme was

washed with potassium phosphate buffer (50 mM, pH 7.0) supplemented with 0.5 M NaCl. LEH immobilized on IDA-Ni²⁺-modified epoxy carriers was washed with only potassium phosphate buffer. The immobilized enzyme was stored in potassium phosphate buffer (50 mM, pH 7.0) at 4 °C.

The protein concentration in the filtrates was determined using a Bradford protein assay [59]. The immobilization yield was calculated according to the following equation:

$$\text{Immobilization yield (\%)} = \frac{m_{p0} - m_{p1}}{m_{p0}} 100\% \quad (1)$$

where m_{p0} is the mass of proteins used for immobilization, and m_{p1} is the mass of unbound proteins.

The activity recovery after immobilization was calculated as the ratio of the activity of the immobilized enzyme to the initial activity of the enzyme used for immobilization, multiplied by 100%.

3.6. Enzyme Assay

The hydrolytic activity of both free and immobilized LEH was assessed by measuring the rate of hydrolysis of (+)-limonene-1,2-epoxide. Reactions were conducted in 1.5 mL and 2 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany) sealed with Parafilm M to minimize substrate evaporation. For assays involving free LEH, the total reaction volume was 200 µL and reaction solution consisted of enzyme (5–20 µg·mL^{−1}) in 50 mM potassium phosphate buffer (pH 7.0), and 20 µL of a 500 mM stock solution of the epoxide in acetonitrile. Reactions with immobilized LEH were performed using 450 µL of buffer, 50 µL of the same epoxide stock solution, and 10 mg of immobilized enzyme particles. Reaction mixtures (excluding epoxide stock solution) were equilibrated at the desired reaction temperature for 5 min before initiating the reaction by adding the epoxide stock solution. All reactions were conducted at 30 °C with agitation at 1000 rpm using a ThermoMixer C (Eppendorf, Stevenage, UK). Each tube represented an individual time point.

At predefined intervals, reactions were quenched by adding ethyl acetate (1:1 *v/v*) containing n-hexadecane (1 mg·mL^{−1}) as the internal standard. Samples were vigorously mixed for 5 min, centrifuged at 14,000 rpm (MiniSpin Centrifuge, Eppendorf, Hamburg, Germany) for 30 s, and the organic phase was collected for diol analysis by gas chromatography (GC).

To monitor the hydrolysis of epoxide isomers, a parallel quenching procedure was employed using cyclohexane (1:1 *v/v*) containing n-decane (1 mg·mL^{−1}) as the internal standard. After 5 min of shaking and centrifugation under the same conditions, the organic layer was analyzed by GC to determine concentrations of the *cis*- and *trans*-isomers. Subsequently, 100 µL of the aqueous phase was transferred to a new tube and extracted with ethyl acetate containing the internal standard, as described above, to quantify the resulting diol.

One unit (U) of enzymatic activity is defined as the amount of free/immobilized enzyme that catalyzes the formation of 1 µmol of diol per minute under the assay reaction conditions. The enzyme activity was corrected for non-enzymatic hydrolysis of the substrate.

3.7. Effect of Temperature on Free and Immobilized LEH

The temperature-activity profiles of free and immobilized LEH were measured at various temperatures (10–80 °C for free LEH, 20–80 °C for LEH@Purolite Amino C6-GA-DAB-GA) in potassium phosphate buffer (50 mM, pH 7.0). Non-enzymatic hydrolysis of epoxide was considered in all the experiments.

The activation energy (E_a) for free and immobilized LEH was calculated from the Arrhenius equation, using its linearized form:

$$\ln(k) = \ln(A) - \frac{E_a}{R} \left(\frac{1}{T} \right) \quad (2)$$

where T is the thermodynamic temperature in K, A is the preexponential factor, and R is the gas constant ($8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). The rate constant k was calculated from the initial activities of LEH at the corresponding temperatures and pH 7.0.

3.8. Thermal Stability of Free and Immobilized LEH

To investigate the thermal stability of free LEH, the enzyme solution ($0.016\text{--}0.381 \text{ mg}_{\text{prot}} \cdot \text{mL}^{-1}$ in 50 mM potassium phosphate buffer of pH 7.0) was incubated at a given temperature ($40\text{--}60 \text{ }^\circ\text{C}$) under shaking (500 rpm) on a ThermoMixer C (Eppendorf, Stevernage, UK). Samples of the enzyme solution were periodically withdrawn and cooled for 5 min on ice, and the residual activity was measured at $30 \text{ }^\circ\text{C}$ and pH 7.0 as described above in Section 3.6.

The immobilized LEH@Purolite Amino C6-GA-DAB-GA (protein loading = $7.91 \text{ mg}_{\text{prot}} \cdot \text{g}_{\text{carrier}}^{-1}$) in potassium phosphate buffer (50 mM, pH 7.0) was incubated at different temperatures with gentle shaking (500 rpm) on a ThermoMixer C. Samples from the incubated immobilized enzyme were periodically withdrawn (0–360 min) and cooled for 5 min on ice, and the residual activity was measured at $30 \text{ }^\circ\text{C}$ and pH 7.0 as described above in Section 3.6.

3.9. Operational Stability of Immobilized LEH

The immobilized LEH@Purolite Amino C6-GA-DAB-GA was reused in ten cycles of hydrolysis of (+)-limonene-1,2-epoxide. The reactions were performed as described above in Section 3.6, except hydrolysis was discontinued by pipetting $200 \text{ } \mu\text{L}$ of the reaction mixture without immobilized enzyme particles and ethyl acetate (volume ratio of 1:1) supplemented with n-hexadecane ($1 \text{ mg} \cdot \text{mL}^{-1}$) into a new tube. The samples were then extracted and analyzed as described above. After each reaction cycle, the preparations of immobilized enzyme were washed thoroughly with potassium phosphate buffer (50 mM, pH 7.0) and used in the next cycle or stored at $4 \text{ }^\circ\text{C}$ until use in the next cycle. The results are expressed in the form of relative activity calculated as the ratio of residual activity to initial activity. Similarly, the operational stability of LEH@Purolite Amino C6-GA-DAB-GA at different reaction temperatures was determined as described above. Thirty-minute reactions were performed at different temperatures ($30\text{--}70 \text{ }^\circ\text{C}$).

3.10. GC Analysis

Quantitative analysis of the substrate and the reaction product was performed using a 7890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-5 column ($30.0 \text{ m} \times 250 \text{ } \mu\text{m} \times 0.25 \text{ } \mu\text{m}$) (Agilent Technologies, Santa Clara, CA, USA) and a flame ionization detector, with H_2 as the carrier gas (flow rate of $1 \text{ mL} \cdot \text{min}^{-1}$). The detector and injector temperatures were $250 \text{ }^\circ\text{C}$.

To quantify the total concentration of epoxide and diol in the reaction mixture, $1 \text{ } \mu\text{L}$ of ethyl acetate extracted sample was injected into the GC. The column was heated to $100 \text{ }^\circ\text{C}$ for 30 s, then the temperature was increased to $160 \text{ }^\circ\text{C}$ at $50 \text{ }^\circ\text{C}/\text{min}$, held for 3 min at $160 \text{ }^\circ\text{C}$, increased to $175 \text{ }^\circ\text{C}$ at $50 \text{ }^\circ\text{C}/\text{min}$, and held for 3 min at $175 \text{ }^\circ\text{C}$. The retention times of the analyzed substances were 3.1 min for (+)-limonene-1,2-epoxide, 4.4 min for (+)-limonene-1,2-diol, and 7.2 min for n-hexadecane (Figure S5).

To quantify the concentrations of the *cis*- and *trans*-isomers of limonene-1,2-epoxide, 1 µL of the extracted cyclohexane sample was injected into the GC. The initial oven temperature was set at 115 °C, which was held for 6 min, then increased to 150 °C with a ramp rate of 50 °C/min and held for 2 min at 150 °C. The retention times of the analyzed substances were 3.1 min for n-decane, which was used as an internal standard, 4.9 min for *cis*-(+)-limonene-1,2-epoxide, and 5.0 min for *trans*-(+)-limonene-1,2-epoxide (Figure S6).

3.11. Kinetic Analysis of the Enzyme Inactivation Experiments

Depending on the enzyme form (free or immobilized), two kinetic mechanisms were used to describe the enzyme inactivation kinetics. In the first mechanism the aggregation of enzyme molecules according to the following reaction was assumed:



Another mechanism based on a two-step reaction scheme was used to describe the inactivation kinetics of the immobilized enzyme:



In the equations presented above, the symbols E, I, and D represent the active enzyme, the inactive intermediate, and the denatured form, respectively. Although several alternative inactivation models were evaluated, they are omitted here for brevity; the two models discussed were found to be the simplest and most suitable for describing the experimental data. The reaction progression corresponding to Equation (3), with respect to the active enzyme concentration (E), was determined by solving the associated rate equation.

$$\frac{dc_E}{dt} = -2k_d c_E^2 \quad (5)$$

The rate constant k_d in Equation (5) is also referred to as the inactivation constant. As shown below, it is suitable to transform Equation (5) in terms of the dimensionless enzyme concentration $c'_E = c_E/c_{E0}$, where c_{E0} is the initial enzyme concentration in the inactivation experiment [62]:

$$\frac{dc'_E}{dt} = -2k'_d c'^2_E \quad (6)$$

where $k'_d = k_d c_{E0}$. Similarly, for the kinetic model corresponding to Equation (4), the following rate equations can be written using symbols of rate constants according to Equation (4):

$$\frac{dc_E}{dt} = -k_1 c_E + k_{-1} c_I \quad (7)$$

$$\frac{dc_I}{dt} = k_1 c_E - k_{-1} c_I - k_2 c_I \quad (8)$$

which, after the introduction of the dimensionless concentration $c'_I = c_I/c_{E0}$, become

$$\frac{dc'_E}{dt} = -k_1 c'_E + k_{-1} c'_I \quad (9)$$

$$\frac{dc'_I}{dt} = k_1 c'_E - k_{-1} c'_I - k_2 c'_I \quad (10)$$

The initial dimensionless enzyme concentration in Equations (6), (9), and (10) is 1, and the concentrations of the other forms are assumed to be zero. The practical reason for introducing dimensionless concentrations is the nature of the experimental data that were

measured in the form of relative enzyme activity, which is defined as a ratio of the actual (A) and initial (A_0) enzyme activities:

$$a = \frac{A}{A_0} \quad (11)$$

The enzyme activity under the studied experimental conditions can be defined as a product of the catalytic constant k_{cat} and the enzyme concentration:

$$A = k_{cat}c_E \quad (12)$$

Considering that the value of the catalytic constant is the same for the enzyme during the whole experiment, after combining Equation (11) with Equation (12), the relative activity is as follows:

$$a = \frac{c_E}{c_{E0}} \quad (13)$$

This value is identical to the dimensionless enzyme concentration c'_E .

The inactivation models in the form of the described systems of differential equations were solved using the ode15s procedure in MATLAB R2022a (MathWorks, Inc., Natick, MA, USA), and kinetic parameters were estimated from experimental data by Levenberg–Marquardt non-linear regression using the lsqnonlin procedure.

4. Conclusions

The results presented in this paper show that LEH from *R. erythropolis* produced by recombinant *E. coli* can be successfully immobilized on various methacrylate carriers; however, the method of binding the enzyme to the support has a strong influence on the preservation of the activity of the immobilized enzyme. Immobilization via aldehyde reactive groups resulted in the most active immobilized preparations, resulting in activity retention of two orders of magnitude higher compared to immobilization via epoxy reactive groups. Curiously, affinity immobilization of LEH via its C-terminal His-tag proved to be ineffective, resulting in substantial activity loss. Among all immobilization supports, the highest specific activity was observed with the Purolite Amino C6-GA-DAB-GA showing the positive effect of a spacer arm. The temperature optimum of free LEH was 40 °C, whereas LEH linked to Purolite Amino C6-GA-DAB-GA demonstrated an elevated optimum at 60 °C. This shift is attributed to enhanced stabilization provided by covalent multipoint attachment, particularly effective within the 40–50 °C range, where it mitigates enzyme aggregation. The investigation of inactivation kinetics proved substantial improvement in the thermal stability of LEH by immobilization, particularly by preventing the aggregation of enzyme molecules.

Overall, this study reports about the first immobilization of limonene 1,2-epoxide hydrolase via covalent binding to solid commercial supports and the obtained results represent a pilot study for future research aimed at studying the influence of the immobilization method, conditions, and support properties on the activity yield, and may be interesting and useful for other authors who wish to start their own research in this area.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/catal15100986/s1>. Table S1. Purification steps of the LEH. Figure S1. SDS-PAGE electrophoresis of *E. coli* BL21(DE3) expressing LEH and fractions from LEH purification. ST—molecular weight marker, Line 1—*E. coli* cells before IPTG induction, 2—*E. coli* cells after 6 h IPTG induction, 3—cell free extract, 4—*E. coli* cell debris (sediment from cell disruption), 5—purified LEH fractions (pooled and desalted). Figure S2. Modification of epoxy carriers Purolite ECR8209M and Immobead 150P with DAB and GA and immobilization of LEH. Figure S3. Modification of

amino functionalized carriers (n = 2—Purolite ECR8309M (Amino C2), n = 6—Purolite ECR8409M (Amino C6)) and immobilization of LEH. Figure S4. Modification of Purolite ECR8209M Epoxy carrier with IDA, EA and NiCl₂, and affinity immobilization of LEH. Figure S5. Chromatogram of reaction mixture extracted with ethyl acetate. Figure S6. Chromatogram of reaction mixture extracted with cyclohexane.

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