Unravelling Alcohol Dehydrogenase Catalysis in Organic-aqueous Biphasic Systems Combining Experiments and Molecular Dynamics Simulations

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KEYWORDS

Biocatalysis, alcohol dehydrogenases (ADHs), organic-aqueous biphasic systems, experimental analysis, molecular dynamics (MD) simulations

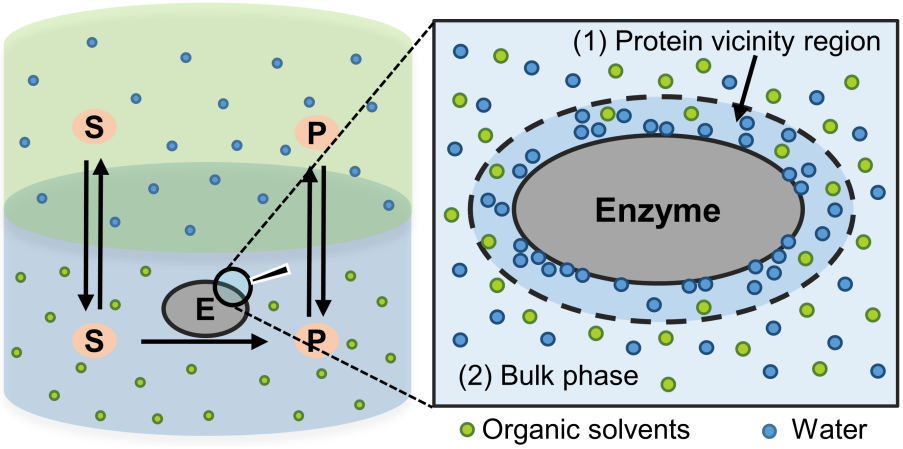
ABSTRACT

The use of oxidoreductases in organic-aqueous biphasic systems is advantageous (effective solvation of reactants, minimization of substrate/product-induced inhibition, improved volumetric productivity, and straightforward downstream processing). This paper explores the effects of organic solvents on enzymes by combining experimental and computational studies. Various organic solvents displaying a broad range of hydrophobicity and functionalities are used, namely ethyl acetate, 2-methyltetrahydrofuran, methyl *tert*-butyl ether, cyclopentyl methyl ether, toluene, cyclohexane, heptane, and dodecane. The catalytic performance of model enzyme horse liver alcohol dehydrogenase concerning its activity, stability, and selectivity is experimentally evaluated. The results are interpreted with molecular dynamics simulations by assessing the: (i) protein location in biphasic media; (ii) organic solvent distribution; and (iii) enzyme conformation. Herein, the stability states the robustness of the enzyme while storing it in biphasic media without catalysis takes place. Overall, different toxicities of the solvent to the enzyme can be pinpointed: 'molecular toxicity', related to the solvent functional groups; and 'interfacial toxicity', related to the position of the enzyme at the interface. Likewise, some solvents are more prone to be located close to the active site of the enzyme, triggering other effects on the enzymatic performance. Thus,

methyl *tert*-butyl ether resulted as an optimal option for the enzyme, whereas other solvents like toluene and 2-methyltetrahydrofuran were detrimental. The combined forces of experiments and simulations have shown to be useful tools to study the effects of reaction media, thus, guiding solvent selection.

INTRODUCTION

Biocatalysis is currently considered an alternative to traditional chemical synthesis to meet the present and future needs for the sustainable manufacturing of chemicals.[1-5] Enzymes have evolved to function optimally in aqueous media, but biocatalytic reactions performed in water pose challenges like the: (i) low solubility and stability of chemicals; (ii) water-induced side reactions; (iii) substrate-/product-inhibition; and (iv) time-/cost-intensive downstream processing.[6] An elegant solution is the introduction of water-immiscible organic solvents to form biphasic systems.[7-9] Herein, the water-soluble biocatalysts remain in the aqueous phase (or at the liquid-liquid interfaces) while the hydrophobic reactants are dissolved in the non-reactive organic phase as substrate reservoirs or product extractors (**Scheme 1**).



Scheme 1. Schematic visualization of the immiscible organic-aqueous biphasic system with distribution of substrate/product (left), and visualization of solvent distribution around an enzyme (right). S: substrate, P: product, and E: enzyme

Given the overall high substrate loadings achieved by the introduction of the organic phase, improved volumetric productivities thus can be envisaged.[10] Besides, the biotransformations that are thermodynamically unfavored in aqueous systems, such as the synthesis of esters, could be feasible in biphasic systems by mitigating hydrolysis.[11] However, biphasic systems are still scarcely used, because enzymes may be often deactivated in the presence of organic solvents.[12] These detrimental effects have been attributed to the 'interfacial toxicity' and 'molecular toxicity' caused by the interaction between protein and interfaces or organic solvent molecules dissolved in the aqueous phase, respectively.[13-16] In other words, the effects of organic solvents on a specific enzyme entirely depend on the solvents' nature. The physicochemical properties of organic solvents (e.g., chemical functionalities and hydrophobicity) play a pivotal role in the catalytic performance and robustness (e.g., activity and stability) of enzymes in biphasic systems.[17] Particularly, the hydrophobicity, defined by the octanol-water partition coefficient ($\log P^{OW}$), has traditionally been one of the significant parameters as it correlates to the capability of stripping

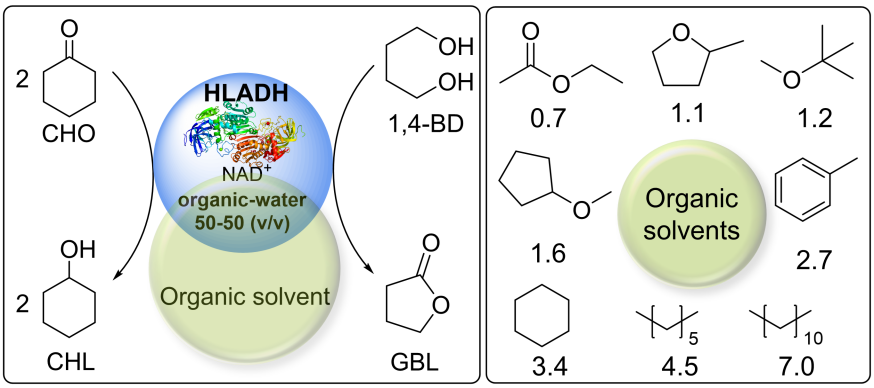
'bound water' from enzymes' surface.[18] In general, hydrophilic solvents with logP^{OW} < 2 can rapidly deactivate enzymes.[19] Conversely, water-immiscible solvents (logP^{OW} ≥ 2) cause negligible enzyme distortion and ensure high enzymatic activity.[17, 20-22] While the logP^{OW} rule can function in some systems, some studies have demonstrated that the logP^{OW} cannot be the only criterion, as high enzymatic activities have been also documented in organic solvents with logP^{OW} < 2, for example methyl *tert*-butyl ether (MTBE, logP^{OW} = 1.2), and diisopropyl ether (DIPE, logP^{OW} = 1.4).[23, 24] Other potential influences, like the molecular structure of solvents (i.e., round shape vs. long chain, bulkiness, and functionalities) need to be considered.[24, 25] Hence, establishing biocatalysis in biphasic systems requires a deep understanding of the different influences that solvents can generate on enzymes.

Studies on understanding biocatalysis in biphasic systems, as well as in organic systems have focused on hydrolases (EC 3) (mainly lipases[26] and a few haloalkane dehalogenases[27, 28]) whereas the study on oxidoreductases (EC 1), serving important roles in organic synthesis facilitating the introduction of chiral motives and selective moieties,[29-31] is still limited.[32] In particular, as a representative subclass of oxidoreductases, alcohol dehydrogenases (ADHs) (EC 1.1.1.1) are of high interest for their applications under biphasic conditions.[33] Earlier, Gröger *et al*. reported the use of ADH in enzyme-compatible biphasic reaction media for practical asymmetric reduction of ketones, benefiting a lot from the enhanced solubility of poorly water-soluble ketone substrates.[8] Kara and Hollmann reported the use of an ADH for reduction reactions in water-deficient organic media. Therein, the diminished activity of the ADH in 'neat substrates' (i.e., solvent-free systems) could be increased by using an organic solvent predominantly, whereby the addition of only 2.5 vol.% of water was sufficient for optimal activity.[23] The solvent screening was based on the activity and stability of the ADH in solvents with different polarities (logP^{OW}, 1.0–5.6), revealing

MTBE ($\log P^{OW}$ = 1.2) as the best reaction media. In the follow-up study, this approach was successfully scaled up to 2-L, yielding 150 g isolated enantiopure (*enantiomeric excess (ee)* ≥ 99% (*S*)) alcohol product.[34] Despite several studies reported for the application of ADHs in organic solvents, micro-aqueous organic media, and neat substrate systems,[9, 23, 35-39] no systematic investigation of the interaction of ADHs with organic solvent molecules regarding their activity, stability, and selectivity has been reported so far. Defining the different toxicities of solvents and spotting amino acid sites that can be genetically designed to generate more robust biocatalysts, would significantly facilitate the implementation of biphasic media under industrial conditions.

To better understand the effects of organic solvents on enzymes, a holistic approach combining experimental work and *in silico* analysis must be established. Experimental techniques can provide structural insights into solvent effects, but atomistic-scale data remain unexplored. Hence, molecular dynamics (MD) simulations are a useful tool kit to empower the in-depth study of solvent effects on enzymes. So far, few successful examples have illustrated the structure-function of enzymes (e.g., lipases, haloalkane dehalogenases) at the atomistic and molecular levels.[17, 40-42] Very recently, we reported a study on the effect of deep eutectic solvents (DESs) on ADHs.[43, 44] Herein, the observed experimental results could be well-interpreted with the help of MD simulations. Accordingly, the correlation between hydration layers, structural flexibility of enzymes, catalytic performance, and robustness could be established. However, most MD simulations studying proteins in organic solvents are for one-phase systems with rather few cases assessing the activation on aqueous-organic interfaces[45] or the denaturation on supercritical CO_2-water interfaces[46] (again, for lipases). In this paper, the effects of a variety of organic solvents on the biocatalytic performance and robustness (e.g., activity, stereo-selectivity, and stability) of a representative ADH, horse liver alcohol dehydrogenase (HLADH) in biphasic media are studied

(**Scheme 2**). The combination of experiments and atomistic-scale simulations firmly supports the exploration and thus will assist the selection of solvents for biocatalysis in non-conventional media.



Scheme 2. HLADH-catalyzed reduction of cyclohexanone (CHO) to cyclohexanol (CHL) coupled with cosubstrate 1,4-butanediol (1,4-BD) for cofactor regeneration in organic-aqueous biphasic systems (left). Various organic solvents with a wide range of hydrophobicity (e.g., logP^{OW}) (right): ethyl acetate (EtOAc, 0.7), 2-methyltetrahydrofuran (2-MeTHF, 1.1), methyl *tert*-butyl ether (MTBE, 1.2), cyclopentyl methyl ether (CPME, 1.6), toluene (2.7), cyclohexane (3.4), heptane (4.5), dodecane (7.0).

RESULTS AND DISCUSSION

To extensively study the effects of organic solvents on enzyme catalysis, eight organic solvents were selected covering a wide range of logP^{OW} values from 0.7 to 7.0 (**Scheme 2**). The choice of solvents for biphasic systems was based on their inertness, immiscibility, and sustainability.[47, 48] The supplier information and physicochemical properties (logP^{OW} values, boiling points, dipole moment, dielectric constant, interfacial tension, solubility of solvent in water, and solubility of water in solvent) are listed in **Table S1** and **Table S2**, respectively. Importantly, solvents were also chosen according to their different chemical structures. Instead of considering merely one

parameter logP^{OW}, the inherent properties of chemical structures may be more relevant for solvent screening.[24] Therefore, ethyl acetate (EtOAc) was used as a representative sample of esters, 2-methyltetrahydrofuran (2-MeTHF) of cyclic ethers, cyclopentyl methyl ether (CPME) and methyl *tert*-butyl ether (MTBE) of simple ethers, toluene of aromatic chemicals, cyclohexane of cyclic alkanes, heptane and dodecane of linear non-branched alkanes. Some of these solvents have already been successfully applied for ADH-catalyzed reactions.[23, 24]

In the first set of experiments, the activity of the purified HLADH (SDS-PAGE, **Figure S1**) was studied with the reduction of cyclohexanone (CHO) promoted by the 'smart cosubstrate' 1,4-butanediol (1,4-BD) in biphasic systems while aqueous media (Tris-HCl buffer, 50 mM, pH 7.5) was used as a reference. The formation of product cyclohexanol (CHL) was analyzed with gas chromatography (GC methodology and representative chromatogram seen in **Table S3** and **Figure S2**). In general, all selected organic solvents imposed detrimental effects on the enzyme leading to lower specific activities and similar or lower product formation compared to the aqueous system (**Figure 1** and **Figure S3**).

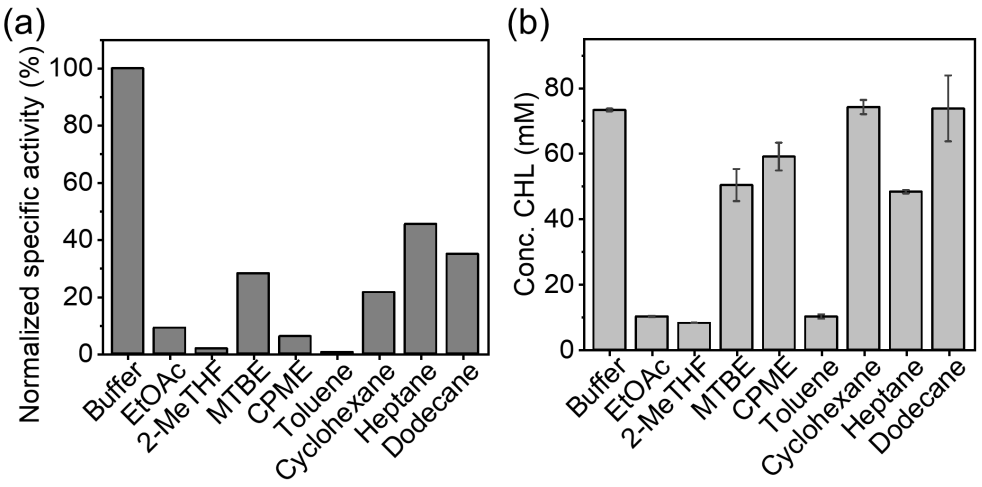


Figure 1. (a) The normalized mass specific activity of HLADH based on the linear approximation of reaction curves at product yields ≤ 10% (initial rates). (b) The final production of cyclohexanol (CHL) after 360 hours in 50 vol.% organic-aqueous biphasic systems while having 100 vol.% buffer (Tris-HCl, 50 mM, pH 7.5) as reference. Reaction conditions: 750 µL Tris-HCl (50 mM, pH 7.5) containing CHO (100 mM), 1,4-BD (50 mM), NAD^+ (1 mM), and HLADH (1 U mL^{-1}) was mixed with 750 µL buffer-saturated organic solvents, which were incubated at 25 °C and 1200 rpm.

Among the screened organic solvents, EtOAc ($\log P^{OW}$ = 0.7), 2-MeTHF ($\log P^{OW}$ = 1.1), and toluene ($\log P^{OW}$ = 2.7) led to the relative inferior catalytic efficiency (both lower specific activity and final product concentration) orderly compared to other solvents and aqueous system (**Figure 1**). Although a lower initial efficiency (rel. specific activity of 6.2%) was observed in CPME ($\log P^{OW}$ = 1.6), a decent final production of a relative 81% compared to the aqueous system was obtained finally. The HLADH remained active for some time in CPME, but it was almost inactivated in the presence of toluene (rel. specific activity of 0.6%, rel. final product yield of

14%). For EtOAc and 2-MeTHF, HLADH also displayed low specific activities (9.1% and 1.8%, respectively) and poor final product yields (14% and 11%, respectively). Despite the potential of using biogenic resources, the catalytic performance of HLADH in 'green' solvents like 2-MeTHF was not satisfactory.[49,50] Conversely, solvents with high logP^{OW} values led to higher product yields (rel. $\geqslant$ 65%), albeit displayed different specific activities. Heptane (logP^{OW} = 4.5) gave rise to the best outcome (rel. specific activity of 45.4%), followed by dodecane (logP^{OW} = 7.0) (35.0%), MTBE (logP^{OW} = 1.2) (28.2%), and cyclohexane (logP^{OW} = 3.44) (21.5%). Apparently, hydrophobic alkane solvents exerted less influence on enzymes, presumably due to their low solubility in water. However, MTBE resulted in an *outlier* as its logP^{OW} is low (1.2), but is an effective medium for the ADH's performance, which is consistent with the literature.[23]

Subsequently, the stability of HLADH in organic-aqueous biphasic systems was studied by measuring the enzyme's half-life time ($t_{1/2}$) (**Figure 2a**). The stability of HLADH in these systems was lower than that in the aqueous buffer and displayed analogous trends as the specific activity (**Figure 1a**). For instance, the enzyme quickly lost its activity in the presence of 2-MeTHF ($t_{1/2}$ = 2.2 h) and toluene ($t_{1/2}$ = 6.6 h) while alkanes resulted in almost identical low stability ($t_{1/2}$ = 30–40 h). In contrast, HLADH in EtOAc ($t_{1/2}$ = 74.4 h) and CPME ($t_{1/2}$ = 70.5 h) biphasic systems showed relatively high stability. Interestingly, the highest stability of HLADH was obtained in MTBE formed biphasic system ($t_{1/2}$ = 338.2 h), which may consequently contribute to the observed high specific activity (**Figure 1a**).

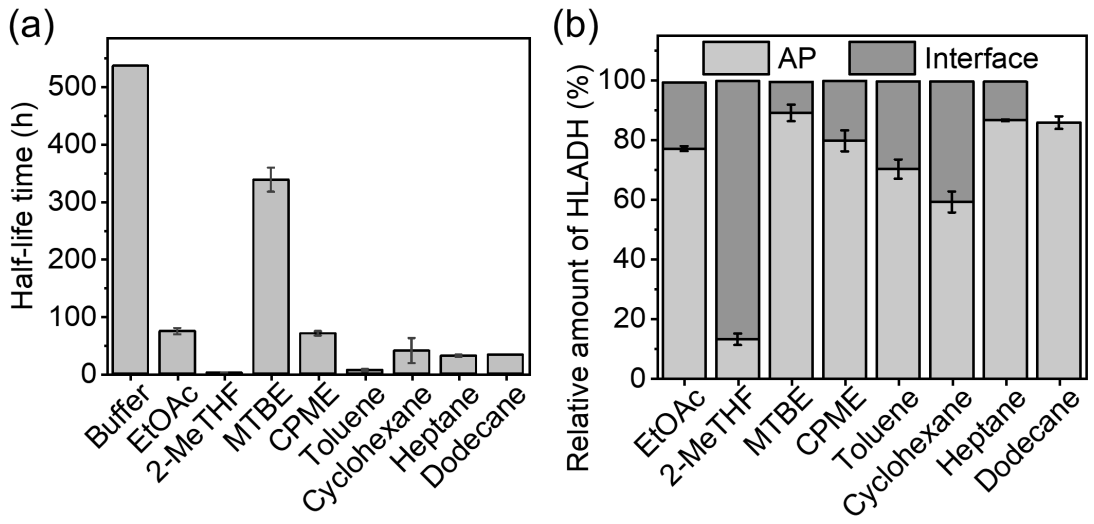


Figure 2. (a) Half-life time ($t_{1/2}$) of HLADH after incubation in 50 vol.% organic-aqueous biphasic systems. (b) The HLADH distribution in the various 50 vol.% organic-aqueous biphasic systems, AP: aqueous phase, organic phase part not shown owing to enzyme's low distribution (≤ 1% rel. amount) while only dodecane's AP was shown due to its high boiling point. Operation conditions: 0.5 mg mL^{-1} purified HLADH in buffer (Tris-HCl, 50 mM, pH 7.5) was incubated in the presence of 50 vol.% of buffer-saturated organic solvents at 26 ± 3 °C. Aliquot samples were taken from aqueous phase after centrifugation at defined intervals for either photocolorimetric assay or Bradford assay to determine the residual activity and protein concentration, respectively.

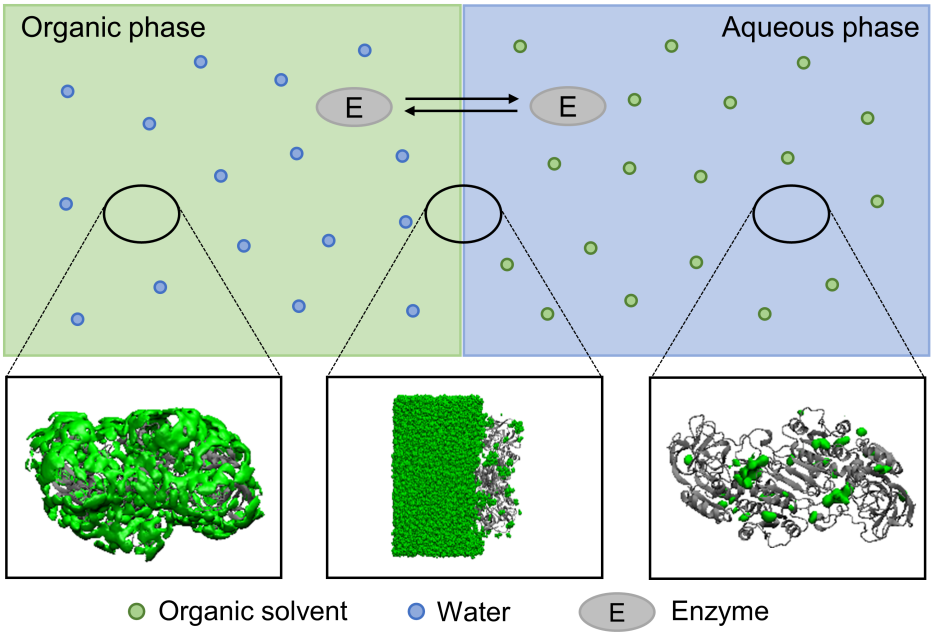
When combing the specific activity (**Figure 1a**), the final product yield of cyclohexanol (CHL) (**Figure 1b**), and the half-life time (**Figure 2a**), we discovered some general correlations. For example, the selected hydrocarbon solvents (cyclohexane, heptane, dodecane) still outperformed most other types of solvents, which is in line with the results reported by Gröger and coworkers[8] as well as other studies.[51] Although resulting in relatively short half-life times, they gave rise to an equal final production as in the aqueous system. Again, MTBE stood out from the crowd probably thanks to its special structure. To well-understand the obtained results, knowing the actual

distribution of the ADH in the biphasic systems might help. Therefore, the protein assay was conducted to quantify the HLADH content in both phases of aqueous-organic biphasic systems. Most HLADH remained in the aqueous phase in the cases of MTBE (89%), and in the hydrophobic solvents, like dodecane (86%), and heptane (86%). In other cases, the presence of the enzyme in the aqueous phase decreased, such in the cases of EtOAc (77%), toluene (70%), cyclohexane (59%), and 2-MeTHF (13%) (**Figure 2b**). Expectedly, less amount of HLADH (≤ 1%) was found in all organic phases. A large portion of HLADH was located on interfaces especially in the case of 2-MeTHF (**Figure S4**), which could cause the deactivation of enzymes and thus result in the observed poor catalytic performance and stability. Overall, these results further demonstrate logP^{OW} cannot be the sole criterion for selecting suitable organic solvents for biocatalysis,[24] which hence calls for a more holistic investigation, whereby other solvent parameters (e.g. dielectric constant, dipole moment) and molecular structures need to be thoroughly considered.

Clearly, we observed the conformity and discrepancy of activity and stability from experiments. Aside from the key factor of logP^{OW} values, the other characteristics of organic solvents were compared to correlate with the obtained biocatalytic results. However, a simple correlation between basic solvent characteristics and their effects on HLADH could not be acquired by limited studies, which was also the same for a previous study on haloalkane dehalogenases.[42] These findings suggest that protein-solvent interactions in different solvent classes are quite specific and difficult to interpret without additional assistances like spectroscopic and computational analyses. Nevertheless, the enantioselectivity of HLADH was further assessed to get a comprehensive understanding of the effects imposed by organic solvents. To this end, the HLADH-catalyzed reduction of ethyl 4-chloro-3-oxobutanoate (COBE) to (*R*)-ethyl 4-chloro-3-hydroxybutanoate (CHBE), a chiral building block for pharmaceutical active compounds, was used.[52] Likewise, the

selectivity was confirmed by the GC equipped with a chiral column (GC methodology and representative chromatogram seen in **Table S4** and **Figure S5**). It turned out that the selectivity of the enzyme was not affected in the screened biphasic systems as ≥ 99% *ee* was achieved for the detectable final product (**Table S5**). To further support this finding, the substrate spectrum was expanded to an aromatic chemical, acetophenone. The reduction of acetophenone was performed in these biphasic systems to produce the enantiopure (*S*)-1-phenylethanol. Likewise, the selectivity was confirmed by GC analysis (methodology and representative chromatogram seen in **Table S6** and **Figure S6**). Again, only one enantiomer was obtained for the detectable product (**Table S7**).

To understand the experimental results, which may lead to a comprehensive (generalizable) categorization of the effects of organic solvents, MD simulations at atomistic resolution were carried out for the best performing solvent (MTBE) and for the two of the most detrimental ones (2-MeTHF and toluene). To this end, we conducted MD simulations of one HLADH molecule in the three regions of the biphasic system (**Scheme 3**): (i) the organic phase saturated with water, (ii) a biphasic system with the interfacial layer, and (iii) the aqueous phase saturated with the respective organic solvent. For comparison purpose, MD simulations of HLADH in the pure aqueous environment without the presence of organic solvents were performed. In combination with the experimentally determined enzyme distribution (**Figure 2b**), this can shed light on the molecular processes in these aqueous-organic biphasic systems.



Scheme 3. Schematic visualization of the MD simulation set-up for the investigation of HLADH in aqueous-organic biphasic systems. HLADH can have three different states within the system: solvation in the organic phase saturated with water (left), at the interfacial region (middle), and in the aqueous phase saturated with organic solvent (right). Example snapshots of the protein in each of these regions including the regions in proximity of HLADH (grey structure), that are occupied by the organic solvent (green), are given below. The software VMD[53] has been used for this visualization.

As most of the enzyme molecules are located in the aqueous phase (**Figure 2b**), we first conducted MD simulations of HLADH in the aqueous systems that are saturated with the respective organic solvents. The number of organic solvent molecules in direct contact with HLADH – the so-called solvation layer (**Scheme 1**) – was determined from the MD simulations of the saturated aqueous systems. Around 42 MTBE molecules were in direct contact with the enzyme (within 6 Å), whereas the solvation layer of 2-MeTHF was 2-fold larger (89 molecules within 6 Å). Because of the limited solubility of toluene in the water phase only around 2 molecules

of toluene were in direct contact with HLADH in the aqueous phase. To further understand these solvation effects, spatial distribution functions (SDFs) of the organic molecules around HLADH were calculated from the MD trajectories using GROmaqs.[54] This allows the identification of preferred interactions sites of the organic solvent molecules in the proximity of HLADH. **Figure 3** shows the resulting three-dimensional density distributions of the organic solvents ((a) toluene, (b) MTBE, and (c) 2-MeTHF) in green around the enzyme structure (gray, cartoon). The empty space around the enzyme is occupied by water molecules. In the case of toluene (**Figure 3a**), only two interaction sites at the entrance of the substrate-binding tunnel (next to residue IDs 112 (leucine) and 318 (isoleucine)) and at the cofactor domain (next to reside ID 194 (threonine), 218 (arginine), and 258 (serine)) were found to occur.[55] Conversely, MTBE interacts much more frequently (**Figure 3b**) and its interaction sites are widely distributed over the HLADH surface. Notably, 2-MeTHF populates the entry of the substrate-binding tunnel (**Figure 3c**) and shows a much higher affinity to diffuse into and potentially block the substrate-binding pocket. This could further affect the volume and geometry of enzymes' active sites by enlarging or constricting the catalytic cavities, thus resulting in denaturation without conformational changes in the presence of a lower amount of organic solvents, which was revealed by previous studies.[42] Moreover, many interaction sites of 2-MeTHF and MTBE on the outer surface of HLADH match. Besides solvation effects, structural factors of HLADH were studied in the MD simulations as well. Root mean square deviations (RMSD) of the C_α-atoms of HLADH with respect to the crystallographic data (PDB entry 1HEU[56]) showed no signs of deformation or unfolding of the enzyme structure caused by the analyzed organic solvents. Nevertheless, it must be noted that protein denaturation may still occur as the MD simulations are only capable of sampling a short amount of time (in the nanosecond to microsecond scale). Also, the flexibility of HLADH was monitored using root mean

square fluctuations (RMSF) and showed no significant changes in the simulations of aqueous phases saturated with the three organic solvents when compared to the flexibility of HLADH in pure water (**Figure S7**).

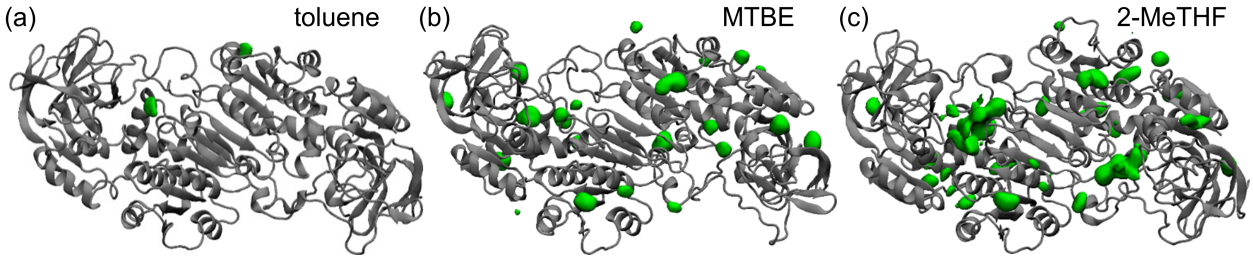


Figure 3. Spatial distribution functions (SDFs) of (a) toluene, (b) MTBE, and (c) 2-MeTHF in aqueous mixtures saturated with the organic solvent within 5 Å of HLADH in the MD simulations. The SDFs have been calculated using GROmaps[54] and are shown as green iso-surface with a relative spatial density of 0.08. HLADH is represented as the gray cartoon. The software VMD[53] has been used for this visualization.

In analogy to the aqueous phase, we conducted MD simulations in the respective water-saturated organic phases. In these simulations a large fraction of the enzyme surface is exposed to the organic solvents. Although less than 1% of HLADH is located in the respective organic phases (**Figure 2b**), this marks the extreme case in which the influence of the organic solvent on HLADH properties is largest. Nevertheless, parts of the HLADH surface are in direct contact with water molecules. **Figure S8** shows the interactions sites of organic solvent molecules (green region) around HLADH while the empty area shows high affinity for water molecules. To assess whether the contact with organic molecules resulted in protein denaturation, here the simulation time has been prolonged to 500 – 750 ns while root mean square deviations (RMSDs) and the radius of

gyration of the C_α-atoms of HLADH, have been monitored (**Figure S9**). The MD simulations in all three organic solvents resulted in a converged enzyme structure within the simulation time indicated by the RMSDs (**Figure S9a**). While the enzyme conformation in MTBE and 2-MeTHF have been reached relatively fast, more than 500 ns were necessary in case of toluene. The converged RMSD value for toluene is in the same range as water. Especially, the radius of gyration does not indicate any unfolding of the enzyme (**Figure S9b**). Furthermore, the solvent accessible surface area has remained constant during the simulations and only a slightly higher hydrophobic surface was found compared to the simulations in water.[57] This indicates that no major structural changes of the enzyme occur in the organic phase. Similar RMSD values do not guarantee a similar structure, therefore the structure of HLADH in organic solvents were compared with the structure at the end of the aqueous phase simulation (**Figure S10**). Although the structure of HLADH in organic solvents, in particular the substrate binding domain (residues 1 – 175 and 319 – 374), deviates from the active structure in aqueous solution (**Figure S10**), the enzyme structure remained in a folded state within the investigated simulation time (500 – 750 ns). However, the conformational changes could affect the cavity formation of the active site and, hence, contribute to enzyme deactivation without protein unfolding.[42] In general chemical denaturation was found to happen within a few ns.[58, 59] Although no denaturation was observed in these time scales for the studied systems, we cannot rule out that conformational changes could also occur over a larger time scale (seconds – minutes), which is unfortunately out of reach for MD simulations.

The enzyme distribution experiments unraveled that the enzyme can also be located at the organic solvent-water interface. To identify the enzyme location/orientation at the interface, we performed MD simulations of HLADH for the three selected biphasic systems. Employing periodic boundary conditions in the biphasic simulations in combination with removing the center

of mass translational velocity of the system, this resulted in stable two-phase regions and, hence, two organic-aqueous interfaces. To rule out a protein location dependence, two simulations with the enzyme structure being initially positioned in the middle of the aqueous or organic phase, respectively, were conducted for each system. Therefore, the enzyme location has been restrained in the respective phase during the equilibration before being released in the sampling stage. Due to the absence of global protein denaturation in the organic phase simulations and the enzyme being dissolved in the aqueous buffer in the experiments, the simulations started from the folded state (PDB entry 1HET[56]). Furthermore, after the equilibrium stages at least the distribution of the solvent accessible surface area of the enzyme starting in the organic phase showed no difference to the structures in the simulations of the organic phase. To verify the used force field, the solubility of water in the organic phase was calculated and compared to experimental literature data (**Table S8**).[58, 60, 61] Overall the solubility in the MD simulations is in qualitative agreement with the experiments, yet with some slight deviations. The solubility of water in MTBE is overestimated by a factor of two in the MD simulations,[61] while its solubility in toluene is reduced.[58] In the case of 2-MeTHF, the organic solvent phase is well represented, whereas the solubility of 2-MeTHF in water appears underestimated by a factor of three.[60] In any case, the differences are in an acceptable order of magnitude given the small absolute differences.

Independent of the starting position, HLADH reached the aqueous phase within the first 20 ns and stayed in this phase in all biphasic simulations. Also, the distribution of the solvent accessible surface area showed no difference after a few nanoseconds in the aqueous phase and therefore indicates that all structural changes observed in the organic phase may be reversible in the investigated timeframes. The time-averaged density distributions for the last 40 ns of the trajectory of organic solvent (black), water (blue) and enzyme (green) along the z coordinate in the MD

simulations, which is perpendicular to the interface, are shown in **Figure 4**. This allows identifying the location and orientation of HLADH with respect to the organic solvent-water interface from the MD simulations. In addition, **Figure S11** shows representative snapshots of the two-phase MD simulations. For a better understanding of the density profiles (**Figure 4**), for one example the snapshot is combined with its respective density profiles in **Figure S12**. Therein, it can be clearly seen that the crossing of the water and organic solvent lines marks the interface regions. Due to periodic boundary conditions in all directions of space, in each MD simulation two phase boundaries can be found. Owing to the amount of water in the simulations, the location of the protein is also visible in the water density profiles, as they show local minima at the positions of the protein peaks (**Figure S12** and **Figure 4**). In the case of toluene (**Figure 4b, e**), HLADH showed a higher affinity towards the biphasic interface compared to the experimental measurements (**Figure 2b**; ~30% interface and ~70% aqueous phase) and in both simulations it was located on the interface region. In the simulations of 2-MeTHF (**Figure 4a, d**), HLADH was located in the interface region in one simulation, while it was located in the aqueous phase for the other. In the experiments more than 87% of the enzyme was in contact with the interface and the rest of HLADH remained in the aqueous phase. Contrary to 2-MeTHF and toluene, HLADH was not located in the interface region in the simulations in MTBE (**Figure 4c, f**), which is in line with a larger portion of HLADH located in the aqueous phase in the experiments (**Figure 2b**). It has also to be stated, that the MD simulations do not exactly reproduce the phase behavior in an experiment, as the interface-to-volume ratio of the phases is drastically increased for the MD simulations compared to the experiments. Therefore, the interface impact may be overrepresented in the MD simulations. To get quantitative representative distributions of the enzyme in the respective phases, free energy calculations would be suitable,[62, 63] but are not part of this work.

Nevertheless, our results demonstrate a preferred interaction of HLADH with the aqueous phase, as even for HLADH, which is attached to the interface, the main volumetric portion of the enzyme was located in the aqueous phase.

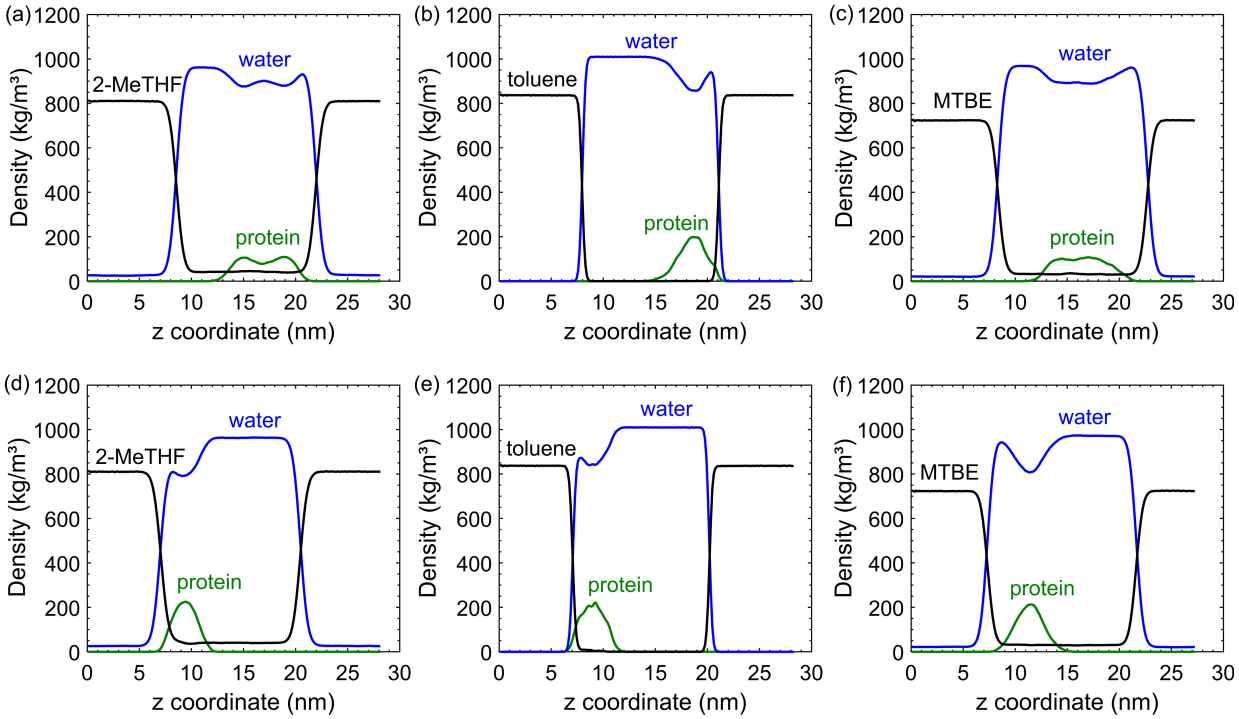


Figure 4. Density distribution in dependency of the coordinate z, that is perpendicular to the interface, in the MD simulations of the biphasic systems. In green the density of the protein, in blue the density of water, and in black the density of the organic solvent for the simulations with 2-MeTHF (a, d), toluene (b, e) and MTBE (c, f). Due to periodic boundary conditions in the MD simulations, two organic solvent-water interfaces exist at $z \approx 8$ nm and $z \approx 21$ nm. The initial position of the protein was in the aqueous phase (a, b, c) and in the organic solvent phase (d, e, f). For one example a combination of these distributions with a system snapshot is shown in **Figure S12**.

Interestingly, the orientation of HLADH on the interface was the same in the three simulations that showed interface contact. That is, the same enzyme region (the entry of substrate-binding tunnels) was in contact with the organic solvent phase. As can be seen from the PDB structure of HLADH in **Figure S13a**, the front side of the enzyme including the substrate-binding tunnel consists of less acidic or basic amino acids compared to the back region (**Figure S13b**). In particular, the entry of the substrate-binding pocket (**Figure S13c**) contains many non-polar residues, which could explain the preferred interaction of these regions with the organic solvent phase. Similar to the one-phase simulations we also monitored the solvation layer of HLADH in the biphasic systems. The number of organic solvent molecules in direct contact with HLADH expectedly increased for all two-phase simulations in which the enzyme is in contact with the interface and is independent of the MD starting position of HLADH (either in organic or aqueous phase). For cases where the enzyme is in the aqueous phase, similar results for the solvation layer can be found compared to the single-phase simulations.

In analogy to the single-phase simulations, spatial distribution functions (SDFs) of the organic solvent molecules around the enzyme structure for the two-phase simulations in 2-MeTHF and toluene are shown for both replica simulations with different starting positions in **Figure 5**. While the contact area of HLADH with the interface is clearly visible in toluene (**Figure 5a, b**), in the case of 2-MeTHF, HLADH seems to be only loosely bound to the interface (**Figure 5c**) or not to be in contact with the interface at all (**Figure 5d**). In both cases, the interaction sites of organic solvent molecules on HLADH are drastically increased for the interface contact cases. Due to the preferred orientation of the HLADH structure with the active center entries in direction of the organic solvent phase, toluene and 2-MeTHF are more likely to diffuse into the substrate-binding tunnel, which could distort the active site cavity and thus the catalytic reaction.[55] For the

simulations in which the enzyme is in contact with the interface over the whole time, we can investigate the structural changes caused by the interface. For the other systems restraining of the protein to the interface would be necessary, which is challenging to do without introducing an unphysical bias into the simulations. While the simulations in 2-MeTHF did not show any evidence of enzyme denaturation, the contact of HLADH with the toluene surface showed deformation of the enzyme structure in one of the two simulations. As shown in **Figure 5a**, the α-helix and the loop connected to this helix (residues 238–260) deviate strongly from the initial configuration and fold towards the toluene phase. This could be an indication that the toluene surface influences the enzyme configuration and therefore the catalytic capabilities of HLADH in toluene-water mixtures. To further investigate the configurational changes, we extended the simulation of the biphasic systems from 100 ns to 400 ns. However, no significant changes of the enzyme can be observed in the following 300 ns. Additionally, the RMSD of the enzyme was in the same region in the biphasic simulation as in the simulations in the aqueous phase.

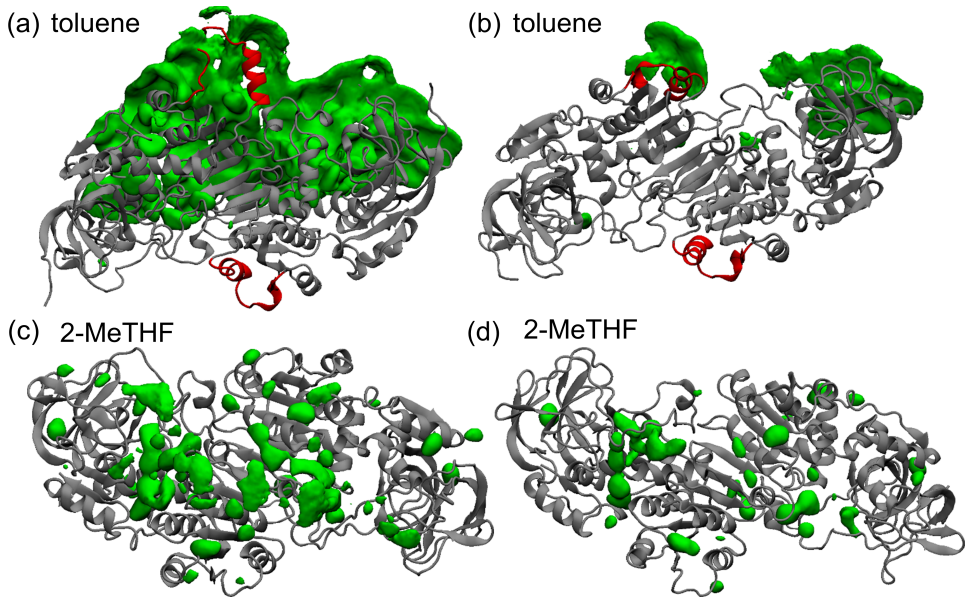


Figure 5. Spatial distribution functions (SDFs) of organic solvent molecules around HLADH in biphasic MD simulations with toluene ((a) HLADH starting position in organic phase and (b) starting position in aqueous phase) and 2-MeTHF ((c) HLADH starting position in organic phase and (d) starting position in aqueous phase) within 5 Å calculated using GROmaps.[54] The enzyme is shown as gray cartoon and the SDFs are shown as iso-surface with a value of 0.08. In the case of toluene, the residues 238 to 260, which are deformed on the toluene surface in one of the simulations, are highlighted in red. The software VMD[53] has been used for this visualization.

The experimental findings of activity, stability, and distribution of HLADH in biphasic organic solvent–water systems showed a detrimental HLADH performance in biphasic media. This raises the question of whether this decreased performance should be attributed to the 'molecular toxicity' of the organic solvent or the 'interfacial toxicity' of the organic solvent-water interface. MD simulations support the argument that the slightly reduced enzyme performance in biphasic

MTBE-water solutions can be solely attributed to the molecular effect of MTBE solvating HLADH in the aqueous phase, while there is no evidence of interfacial toxicity. Strikingly, our results emphasize that, although a significant amount of MTBE is in contact with HLADH, this has a low negative impact on the catalytic capabilities. Conversely, in the case of toluene-water mixtures, molecular toxicity of toluene in the aqueous phase is very unlikely to occur, due to the small number of toluene molecules populating the enzyme surface. On the other hand, our MD simulations indicate a strong influence of the toluene interface and, hence, a 'interfacial toxicity' that could deactivate HLADH. Despite for 2-MeTHF more than 87% of HLADH molecules are located at the interface in the experiments, the MD simulations showed no evidence of enzyme degradation. In the biphasic simulations in 2-MeTHF the binding tunnel is predominantly oriented to the organic phase, which could be considered as 'interfacial toxicity'. In the simulations of the aqueous phase (saturated with 2-MeTHF), 2-MeTHF molecules are located at the substrate binding tunnel of HLADH, which might block the path for substrate molecules and influence HLADH's catalytic capabilities. Thus, in case of 2-MeTHF we consider the deactivation as 'molecular toxicity'. In general, the MD simulations could qualitatively show the distribution of HLADH in the analyzed biphasic systems and help understand the effects of various organic solvent molecules on HLADH.

The obtained experimental and computational results distinguished these selected organic solvents from a biocatalytic point of view and reveal the best choice of MTBE. MTBE has been reported to be beneficial for biocatalysis[36] while also being applied for extraction[64] due to its several advantageous features. However, for solvent selection guidelines to be developed in green chemistry, additional evaluation factors regarding waste, environment, health, and safety must be considered. According to GSK's solvent sustainability guidelines,[65,66] MTBE has safety concerns

of flammability and explosion due to its lower volatility (**Table S9**). Therefore, despite its beneficial effect on ADH catalysis, MTBE needs to be used with caution. The "green solvent" CPME has been explored in other studies, demonstrating its practicality.[36] In this case, it can be an alternative when considering final product yields despite low specific activity and stability. Although EtOAc classified as having 'few known issues' is recommended, the overall catalytic performance is not satisfactory. Overall, given other solvents are listed as 'some known issues', the lab-scale media engineering thus here is more from a biocatalytic perspective with sustainability in mind in response to the EU Green Deal policy. Screening or designing novel enzymes that can perform reactions properly in these recommended solvents is clearly a potential lead for future research in biocatalysis.

CONCLUSIONS

The catalytic performance of a model alcohol dehydrogenase, HLADH, in biphasic systems (50:50 water: organic solvent vol./vol.) has been explored with combined experimental and computational expertise. Eight widely used organic solvents with diverse chemical structures and physicochemical characteristics have been investigated. Depending on the organic solvent, HLADH displayed different activities and stabilities in those systems. The study has confirmed the potential of MTBE for alcohol dehydrogenase catalysis and the general outperformance of hydrocarbon solvents. For the first time, the deactivation mechanism of ADH in these organic solvents was interpreted with the support of MD simulations. Both the 'molecular toxicity' and 'interfacial toxicity' caused changes to the conformational structures, solvation layers, and can even sterically block the substrate-binding tunnel, thus partially or completely reducing the activity of the enzyme. This study further proved the enormous potential of combining experiments and MD simulations for the study of solvent effects on enzymes. It can be foreseen that the joint forces

not only benefit ADHs but also other classes of enzymes when it comes to 'solvent engineering'. The identification of the rationale behind enzyme deactivation in organic solvents may give rise to options to develop new variants with higher robustness to be applied in industrially-sound reactions in non-conventional media.

ASSOCIATED CONTENT

Supporting Information. Materials and experimental details (enzyme preparation, catalysis procedure, half-life time measurement and calculation equation, enzyme distribution experiment); details of GC methodology and GC representative chromatograms; MD simulation methods and analysis data (PDF). The Supporting Information is available free of charge on the ACS Publications website.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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ABBREVIATIONS

ADHs, alcohol dehydrogenase; HLADH, horse live alcohol dehydrogenase; MD, molecular dynamics; CHO, cyclohexanone; CHL, cyclohexanol; 1,4-BD, 1,4-butanediol; EtOAc, ethyl acetate; 2-MeTHF, 2-methyltetrahydrofuran; MTBE, methyl *tert*-butyl ether; CPME, cyclopentyl methyl ether; GC, gas chromatography; SDFs, spatial distribution functions; RMSD, root mean square deviation.

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