catalysts

MDPI

Article

Immobilization of Alcohol Dehydrogenases on Silica-Based Supports and Their Application in Enantioselective Ketone Reductions

Daria Armani [1], Oreste Piccolo [2] and Antonella Petri [1,*]

[1] Department of Chemistry and Industrial Chemistry, University of Pisa, 56126 Pisa, Italy; d.armani@studenti.unipi.it
[2] Studio di Consulenza Scientifica Oreste Piccolo—SCSOP, 23896 Lecco, Italy; orestepiccolo@tin.it
* Correspondence: antonella.petri@unipi.it

Abstract: The use of immobilized alcohol dehydrogenases (ADHs) offers numerous advantages, especially in the reaction conditions required by industrial applications. Looking for more efficient and cost-effective methods of ADH immobilization, in this study we explored silica-based supports as an alternative to the use of functionalized polymeric resins. Three commercially available ADHs were immobilized by adsorption and covalent bond formation. The obtained supported biocatalysts were applied for the bioreduction of acetophenone and some derivatives with good yields and excellent enantioselectivity. The important intermediate (*S*)-1-[3,5-bis(trifluoromethyl)phenyl]ethanol was obtained with a high enantiomeric excess (>99%) by using the highest performing immobilized ADH sample. The reusability of this biocatalyst was investigated in a flow system for five consecutive runs; the experiments showed that the biocatalyst could be recycled without a loss of activity and enantioselectivity. Finally, cross-linking with the glutaraldehyde of the supported biocatalyst was also carried out to prevent the leaching of the enzyme during the catalytic reactions.

Keywords: alcohol dehydrogenase; immobilization; silica-based support; enantioselective biocatalytic reduction; chiral alcohol

Citation: Armani, D.; Piccolo, O.; Petri, A. Immobilization of Alcohol Dehydrogenases on Silica-Based Supports and Their Application in Enantioselective Ketone Reductions. *Catalysts* **2024**, *14*, 148. https://doi.org/10.3390/catal14020148

Academic Editors: Luciana R. B. Gonçalves and Nathália Saraiva Rios

Received: 16 January 2024
Revised: 12 February 2024
Accepted: 15 February 2024
Published: 17 February 2024

1. Introduction

In recent decades, biocatalysis has been established as a green and sustainable technology widely applied in industry, thanks in particular to the biocompatible and biodegradable nature of the enzymes. Aiming at an economic return from the process subjected to sustainable optimization, biocatalysts should be stable over time under different reaction conditions and easily recoverable so as not to add expensive product purification steps from catalyst traces to the process [1]. Enzymatic reactions are commonly performed in mild reaction conditions, but several biocatalysts have shown stability under more extreme conditions, especially in their immobilized form, without evident losses in efficiency and selectivity [2–5]. Biocatalytic processes have therefore become a green alternative to traditional asymmetric catalysis based on metal, with the aim of obtaining the synthesis of compounds and/or intermediates through cost-effective strategies with a lower environmental impact [6]. Therefore, enzymes have found applications in several fields, ranging from the formulation of detergents and polymeric materials to the paper and cellulose production chains, the textile industry, and the synthesis of basic chemicals and pharmaceutical intermediates. Research in this area has shifted towards the optimization of existing processes following the principles of green chemistry and to the development of new strategies [7,8].

Redox reactions represent one of the most studied classes among enzymatic transformations [9,10]. Biocatalysts capable of catalyzing both the reduction of carbonyl compounds and the oxidation of alcohols can be identified in the alcohol dehydrogenase

(ADH) subclass [11]. These enzymes are naturally available or expressed by common host microorganisms and suitable for engineering the proteins that constitute their structure [12].

The catalytic mechanism of ADH-catalyzed reductions involves, at first, the formation of a substrate–cofactor intermediate and, subsequently, the transfer of a hydride from the cofactor to the substrate [12,13]. Commercial nicotinamide cofactors, either phosphorylated or non-phosphorylated, are rather expensive and therefore used in catalytic amounts and regenerated in situ. The recycling of cofactors is possible through using the coupled-enzyme approach or the coupled-substrate approach [9]. In the latter method, an oxidizable co-substrate, such as 2-propanol (IPA), is added, thus allowing the shift of the reversible main reaction towards the formation of the product.

The enantioselective reduction of prochiral ketones catalyzed by ADHs produces chiral alcohols that can be employed as key synthetic intermediates in the fragrance and pharmaceutical industries [14–16] by transforming the hydroxyl groups into different functional groups [17].

The application of ADHs can also be expanded by using immobilization techniques that allow the obtaining of more stable and better performing biocatalysts [5,9,18,19]. Several immobilization methods are already available in the literature for this class of enzyme [20–27], involving various types of supports and thus different physical as well as chemical interactions with the enzyme.

Among the available supports, inorganic materials such as silica gels have several advantages, such as thermal as well as mechanical stability and a competitive cost compared to other commercial carriers, which make them particularly suitable for scale-up processes [5,28]. In addition, their surfaces can be easily modified with different functional groups.

Mesoporous materials are highly suitable for enzymatic immobilization, offering pores with suitable sizes and high surface areas [29,30]. To date, different studies have reported the use of various mesoporous silica for ADH immobilization via physical adsorption or covalent binding [31–37]. Physical adsorption is characterized by simple protocols not requiring the previous functionalization of the carrier. Covalent immobilization is usually achieved through the formation of more stable bonds between the reactive groups of chemically modified silica supports and the enzyme. In most cases, good activities and stabilities of the immobilized biocatalysts were observed; however, only a few applications of silica-supported ADHs for the preparation of enantiopure alcohols were described [34,36].

A major benefit of using immobilized enzymes for biocatalysis is the possibility of using the catalyst in a flow system. Biotransformations in flow conditions offer considerable advantages compared to batch reactions, such as reduced enzyme inhibition, easy product recovery, and simple evaluation of the biocatalyst reusability. To the best of the author's knowledge, silica-supported isolated ADHs have never been used in flow.

In this work, the immobilization of three commercially available ADHs onto silica-based supports was studied for application in the enantioselective reduction of prochiral ketones. Two immobilization techniques were investigated: enzyme adsorption on a commercial non-functionalized silica gel (SiO_2) and covalent binding on a commercial amino-functionalized silica gel (SiO_2-NH_2). The immobilization parameters were investigated to evaluate the effect of the different methods on the activity of the immobilized biocatalysts.

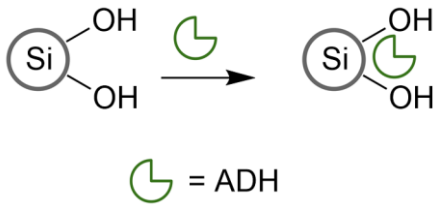
The immobilized ADH samples were applied in the enantioselective reduction of three different substrates following the substrate-to-product conversion as a function of the reaction time and comparing the results with those obtained with the corresponding soluble ADHs. The enantioselectivity of the reactions was evaluated: the obtained chiral alcohols are of interest because they can be used as intermediates for the synthesis of fine chemicals. The best performing supported biocatalyst was also employed in recyclability tests in flow conditions. Finally, a post-immobilization cross-linking step was performed on the supported enzyme to prevent the leaching of the enzyme during the biotransformation.

2. Results and Discussion

2.1. Immobilization of ADHs

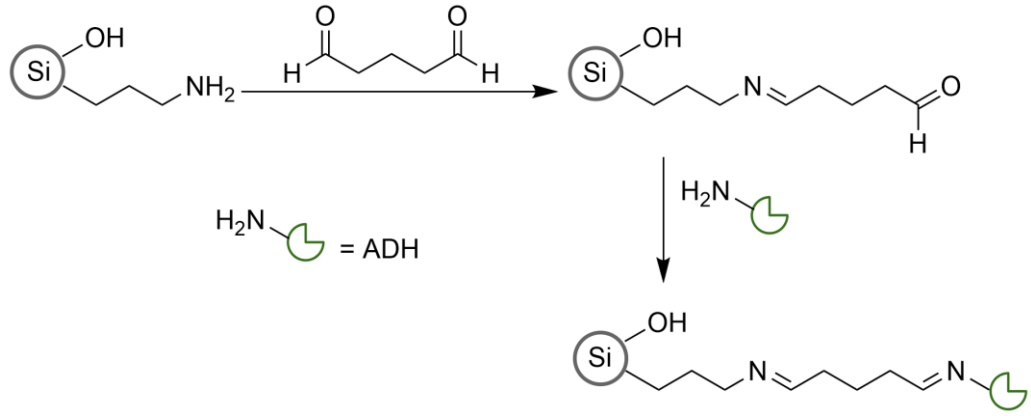
The immobilization of ADHs on silica-based supports was performed using two different strategies: (i) adsorption of the enzyme on the support and (ii) the formation of covalent bonds between the amino groups of the enzyme and the activated groups of a functionalized support.

A non-functionalized SiO_2 was used as a carrier for physical adsorption (Scheme 1).



Scheme 1. Enzyme immobilization by adsorption on silica gel.

Immobilization through covalent bonds was performed by using 3-aminopropyl-functionalized silica gel. To provide a suitable linker, the support needs to be activated with glutaraldehyde. The aldehyde groups subsequently react with the amino groups of the enzyme, resulting in the formation of Schiff bases (Scheme 2) [28,38].



Scheme 2. Glutaraldehyde activation of functionalized silica gel and immobilization via covalent bond formation.

The immobilization processes were evaluated by the determination of the immobilization parameters (Table 1). The total activity of all samples was calculated in a standard assay (see Section 3), and the best results are highlighted in bold.

Table 1. Specific activity, binding efficiency, and activity recovery of soluble and immobilized ADHs on solid supports.

ADHs	Support	Specific Activity of Immobilized Enzyme [a] (U/g Support)	Binding Efficiency [b] (%)	Activity Recovery [c] (%)
EMIN001	**SiO_2**	**3.1 ± 0.3**	**98**	**36**
	SiO_2-NH_2	0.3 ± 0.1	45	3
EMIN028	**SiO_2**	**0.7 ± 0.5**	**81**	**29**
	SiO_2-NH_2	1.9 ± 0.2	20	8

Table 1. *Cont.*

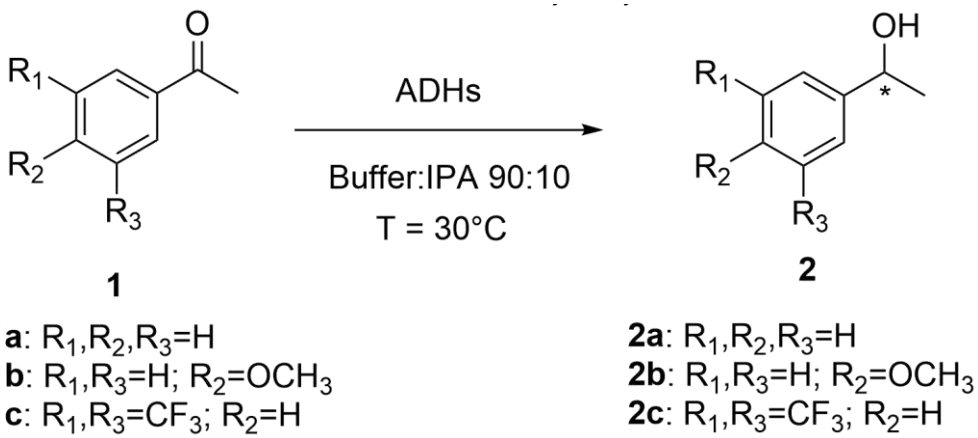
ADHs	Support	Specific Activity of Immobilized Enzyme [a] (U/g Support)	Binding Efficiency [b] (%)	Activity Recovery [c] (%)
ADH105	**SiO_2**	**3.6 ± 0.4**	**86**	**12**
	SiO_2-NH_2	0.6 ± 0.1	60	2

[a] Specific activity is the observed activity of the immobilized enzyme per g of support (specific activity of the immobilized samples). [b] Binding efficiency is the percentage ratio between the total amount of immobilized enzyme (protein amount in the starting solution minus protein amount in the supernatant) and the total protein amount initially applied with the starting solution. [c] Activity recovery is the percentage ratio between the observed activity of the immobilized samples (in units) and the activity initially applied for the immobilization (in units).

As shown in Table 1, covalent immobilization on amino-functionalized silica resulted in low specific activity and activity recovery. A higher activity and high binding efficiency (> 80%) were observed for ADH samples immobilized via adsorption on non-functionalized silica, such that this method was highly promising for application in enantioselective reductions.

*2.2. Enantioselective Reduction of Ketones **1a–1c**: Soluble and Immobilized Biocatalysts*

Three aromatic prochiral ketones were chosen as substrates for studying the performances of the immobilized ADHs in enantioselective reduction (Scheme 3).



Scheme 3. Enantioselective reduction of prochiral ketones **1a–c** catalyzed by ADHs.

Acetophenone (**1a**) has been extensively used as a model substrate for the reduction of aromatic ketones and as a reference in ADH activity assays [10,27].

The chiral alcohol products obtained from the enantioselective reduction of the chosen substrates are important building blocks for the fine chemical industry, mainly designed to undergo the interconversion of the secondary hydroxyl group located on the stereogenic center [17].

In particular, **2a** over the years has asserted its share in the market of fragrances. In fact, both enantiomers are currently used in pharmacokinetic studies as they are ubiquitously present as a natural rose oil fragrance in personal care products such as perfumes, soaps, and lotions [39,40]. Alcohol **2b** is commonly employed in the natural fragrance industry, but it also shows great potential in the synthesis of active pharmaceutical ingredients (APIs) containing ester and carbonyl groups. In addition, it is currently under investigation among the substances that can be obtained from lignin fractions of waste biomasses [41,42]. Alcohol **2c** is an important chiral intermediate for the preparation of biologically active compounds that have shown effects in treating or preventing immunodisease and are used nowadays for the prevention of nausea associated with chemotherapy treatments in cancer patients [43–45].

Among the methods used to recycle the expensive cofactor necessary for the enzymes to carry out their catalytic activity in the reaction of interest, 2-propanol was chosen as

a co-substrate of prochiral ketones. It is known that the oxidation of IPA to acetone, i.e., a volatile product, allows the equilibrium to be pushed towards the regeneration of the reduced form of the nicotinamide cofactor [11].

2.2.1. Enantioselective Reduction of Acetophenone **1a**

The biocatalytic reduction of **1a** to **2a** involved the introduction of a stereocenter, resulting in the formation of a chiral molecule. The choice of reaction conditions played a pivotal role in ensuring the desired enzymatic activity and enantioselectivity during the transformation of the substrate of interest. Mild reaction conditions were employed in batch reactions performed at 30 °C in a mixture consisting of 90% (*v*/*v*) buffer solution and 10% (*v*/*v*) IPA. IPA was used both as a co-substrate for NAD^+ cofactor recycling and as a solvent for the ketone solubilization. These conditions are commonly used in similar biocatalytic reactions [10,12,13].

The conversion data obtained after 1, 4, and 24 h of reaction are shown in Table 2.

Table 2. Bioreduction of **1a** with soluble and immobilized ADHs after 24 h.

ADH	Support	Conversion (%) [a]			Absolute Configuration [b]	ee (%) [c]
		1 h	4 h	24 h		
EMIN001	None	86	88	89	(*R*)	>99
	SiO_2	74	82	92		>99
	SiO_2-NH_2	12	28	76		>99
EMIN028	None	15	32	78	(*S*)	>99
	SiO_2	10	30	67		>99
	SiO_2-NH_2	9	22	50		>99
ADH105	None	64	83	86	(*S*)	>99
	SiO_2	0	1	2		-
	SiO_2-NH_2	0	3	4		-

[a] The conversion of the substrate was determined via HPLC of samples withdrawn at different reaction times. [b] The absolute configuration was assigned via comparison of the observed elution orders with those of authentic samples of known configurations. [c] The enantiomeric excess of the product was determined via HPLC on the chiral stationary phase.

As expected, all of the enzymes in their soluble forms were more active towards the substrate with respect to their immobilized forms; however, comparable data in terms of conversions were obtained for EMIN001 and EMIN028 adsorbed on silica gel. Lower conversions were obtained with the same enzymes covalently bonded to functionalized silica gel. Very low conversion of the substrate was observed when ADH105 samples immobilized on both SiO_2 and on SiO_2-NH_2 were employed.

As regards the stereochemical course of the reaction, the product was obtained with an enantiomeric excess higher than 99%: EMIN001 allowed the obtaining of (*R*)-**2a**, while EMIN028 and ADH105 led to (*S*)-**2a**.

2.2.2. Enantioselective Reduction of p-Methoxy-acetophenone **1b**

The **1b** substrate was reduced under the same reaction conditions used for **1a**. The results obtained after 1, 4, and 24 h are reported in Table 3.

As observed with **1a**, the soluble enzymes were more active towards the substrate with respect to their immobilized forms, and complete or almost complete conversions were observed after 24 h with all enzymes. As regards the immobilized samples, the best results were obtained with EMIN001 adsorbed on silica gel, which showed a similar behavior compared to the soluble sample of the same enzyme.

In all entries, high enantioselectivities were obtained for the produced **2b**. The absolute configuration was (*R*) for EMIN001 and (*S*) for EMIN028 as well as ADH105, as reported above for **1b**.

Table 3. Bioreduction of **1b** with soluble and immobilized ADHs after 24 h.

ADH	Carrier	Conversion (%) [a]			Absolute Configuration [b]	ee (%) [c]
		1 h	4 h	24 h		
EMIN001	None	40	73	100	(*R*)	>99
	SiO_2	30	60	90		>99
	SiO_2-NH_2	5	7	11		>99
EMIN028	None	10	30	90	(*S*)	>99
	SiO_2	5	12	45		>99
	SiO_2-NH_2	0	2	5		>99
ADH105	None	90	100	100	(*S*)	>99
	SiO_2	3	5	7		>99
	SiO_2-NH_2	1	2	4		-

[a] The conversion of the substrate was determined via HPLC of samples withdrawn at different reaction times. [b] The absolute configuration was assigned via comparison of the observed elution orders with those of authentic samples of known configurations. [c] The enantiomeric excess of the product was determined via HPLC on the chiral stationary phase.

2.2.3. Enantioselective Reduction of 3′,5′-Bis-(trifluoromethyl)acetophenone **1c**

The biocatalytic reduction of **1c** to **2c** was performed under the same reaction conditions used for **1a** and **1b**. The results obtained are reported below in Table 4.

Table 4. Bioreduction of **1c** with soluble and immobilized ADHs after 24 h.

ADH	Support	Conversion (%) [a]			Absolute Configuration [b]	ee (%) [c]
		1 h	4 h	24 h		
EMIN001	None	11	23	40	(*R*)	>99
	SiO_2	10	21	34		>99
	SiO_2-NH_2	0	1	1		>99
EMIN028	None	25	45	70	(*S*)	>99
	SiO_2	54	93	100		>99
	SiO_2-NH_2	35	63	100		>99
ADH105	None	40	86	100	(*S*)	>99
	SiO_2	-	-	-		-
	SiO_2-NH_2	1	1	2		-

[a] The conversion of the substrate was determined via HPLC of samples withdrawn at different reaction times. [b] The absolute configuration was assigned via comparison of the observed elution orders with those of authentic samples of known configurations. [c] The enantiomeric excess of the product was determined via HPLC on the chiral stationary phase.

As can be observed in Table 4, immobilized EMIN028 showed a higher catalytic activity towards the substrate of interest compared to the soluble enzyme and the other immobilized biocatalysts. Complete conversion of the substrate was obtained with both immobilized samples of this enzyme.

ADH105, in the immobilized form, also showed no catalytic activity with this substrate. Thus, it can be concluded that silica-based supports do not appear to be suitable carriers for the immobilization of this enzyme. EMIN001 allowed the obtaining of (*R*)-**2c** with high enantioselectivities, while EMIN028 led to the formation of (*S*)-**2c**, confirming the stereochemical behavior already observed with the other two substrates, **1a** and **1b**.

2.3. Optimization of the Enantioselective Reduction of ***1c***

Among the reactions carried out on substrates substituted on the aromatic ring, the best results were obtained with EMIN028 adsorbed on a silica gel sample (EMIN28@SiO_2) in the enantioselective reduction of **1c** to (*S*)-**2c**. Therefore, **1c** was chosen as an appropriate candidate to undergo the process of the optimization of the reaction conditions. This process was carried out to synthetize (*S*)-**2c**, an already-established important building block employed in the industrial production of APIs [43–45].

Recently, we have reported the immobilization of a commercial ADH on a set of organic supports with different functional groups [46]. The immobilized biocatalyst proved to be extremely efficient in the asymmetric synthesis of (*S*)-**2c** in a 90:10 (*v*/*v*) 2-propanol (IPA): water solvent system and 30 °C. These conditions, besides increasing the solubility of the substrate, allowed an easy recovery of the product as a white crystalline solid via simple solvent evaporation.

Given these results, we aimed at the optimization of the enantioselective reduction process by investigating the effect of a mainly organic medium on the performance of the biocatalyst immobilized onto silica-based supports.

The results obtained in the bioreduction of **1c** in two solvent mixtures, namely 90:10 buffer/IPA and 90:10 IPA:H_2O, are shown in Figure 1.

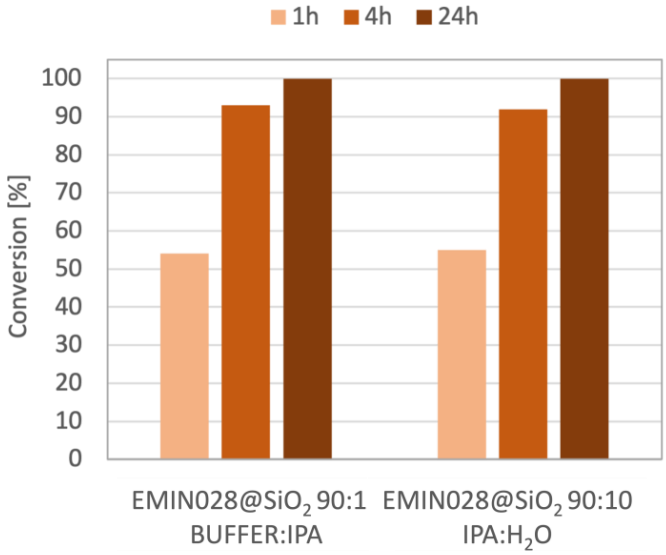


Figure 1. Enantioselective reduction of **1c** to (*S*)-**2c** catalyzed by EMIN028@SiO_2 in two different solvent mixtures at 30 °C.

As can be observed from the graph, no significant difference in the conversion rate of **1c** to **2c** was observed. In addition, the enantiomeric excess of the product was higher than 99% for the (*S*)-enantiomer of **2c**. Therefore, it was demonstrated that the catalytic performance of EMIN028@SiO_2 was retained without depending on aqueous buffer conditions, which are generally preferred for enzymatic reactions.

Having in hand the good results obtained in terms of both activity and enantioselectivity, the optimization of the reaction in terms of the recycling of the enzyme under flow conditions was studied. Indeed, one of the main advantages of using immobilized enzymes is the easy recovery of the enzyme at the end of the reaction via simple filtration and its reuse in successive reactions.

Thus, a PEEK column filled with EMIN028@SiO_2 was fed with a substrate solution in 90:10 IPA:H_2O containing the cofactor NAD^+. The reaction mixture was circulated within the reactor by using a suitable pump for 24 h. Both the PEEK column and the reaction mixture were thermostated at a temperature of 30 °C. The progress of the reaction was monitored by withdrawing aliquots of the reaction mixture.

Conversion and ee data were determined as already described for batch tests. When complete conversion was reached and no substrate was detected, the column was washed with the reaction solvent mixture until no product was detected via HPLC. The column was then reused for five consecutive reaction cycles. The results are shown Figure 2.

The bioreduction of **1c** catalyzed by immobilized EMIN028 and performed under the above-mentioned flow conditions furnished the product (*S*)-**2c** in high conversions (100%) and excellent enantioselectivities (>99%) in each reaction cycle. In addition, thanks to the mainly organic reaction medium, the enantiopure alcohol was recovered from the reaction

mixture via simple solvent evaporation avoiding the filtration and extraction steps, usually necessary as downstream procedures.

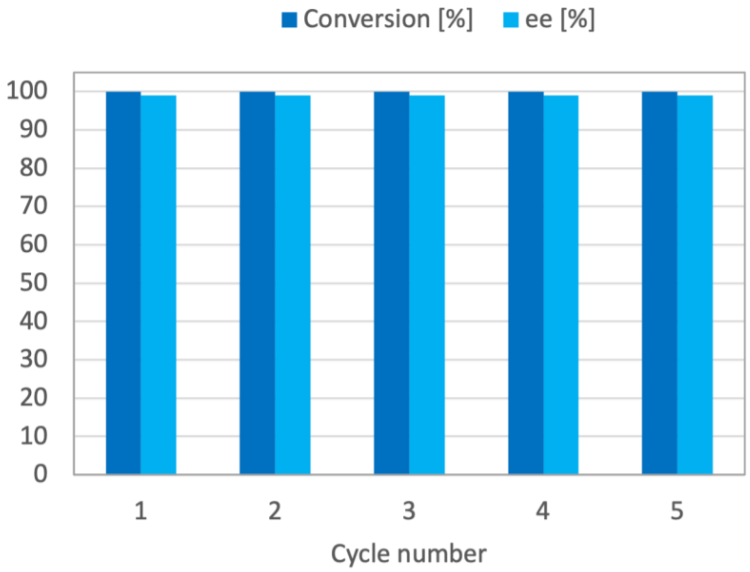


Figure 2. Reuse of EMIN028@SiO_2 in the enantioselective reduction of **1c** to (*S*)-**2c**.

As a further improvement of our process, the cross-linking of the immobilized biocatalyst was investigated. This post-immobilization step usually prevents protein leaching from the support during the repeated use without compromising the biocatalyst activity in terms of conversion and enantioselectivity [47]. One of the most employed cross-linking agents is glutaraldehyde, which is commercially available at a low cost as an aqueous solution [48–51]. This dialdehyde is able to create a three-dimensional network through the formation of Schiff bases between the carbonyl groups and the free NH_2 groups of the enzyme.

The efficiency of the cross-linking process was evaluated through the comparison of the results obtained in the reduction of **1c** catalyzed by EMIN028@SiO_2 and the immobilized sample after cross-linking the named EMIN028@SiO_2-glut (Figure 3).

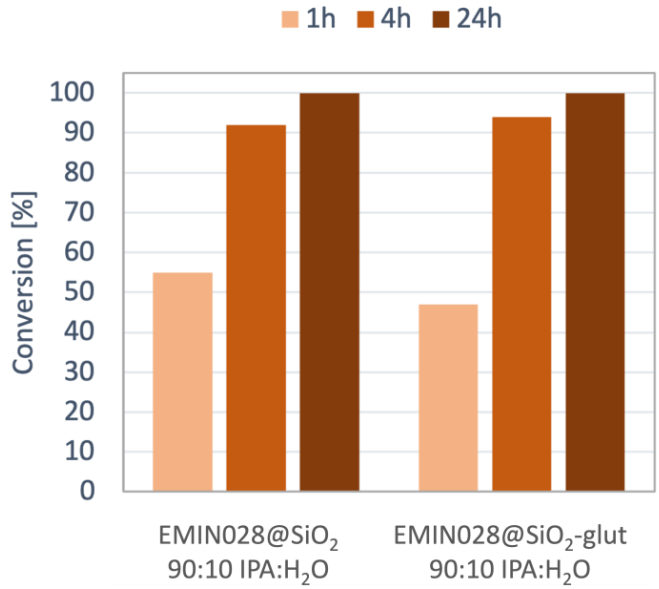


Figure 3. Enantioselective reduction of **1c** to (*S*)-**2c** catalyzed by EMIN028@SiO_2 and EMIN028@SiO_2-glut at 30 °C.

As shown in Figure 3, complete conversion of the substrate **1c** was achieved in 24 h with both immobilized samples, leading to the synthesis of the enantiopure product (*S*)-**2c** (ee > 99%). These results confirmed that the catalytic performance of the studied enzyme was not compromised by the cross-linking step, even conducting the reaction in a solution of 90% organic solvent and without a buffer.

3. Materials and Methods

3.1. Materials

EMIN001 and EMIN028 enzymes were kindly donated by Enzymaster Deutschland GmbH (Dusseldorf, Germany). ADH105 enzyme was purchased from Johnson Matthey (Cambridge, United Kingdom). All three ADH samples were provided as lyophilized powders. The silica gel (particle size of 200–500 μm, pore diameter of 300 Å, and surface area of 300 m^2/g) and the aminopropyl-derivatized silica gel (particle size of 70–200 μm, pore diameter of 110 Å, surface area of 500 m^2/g) were purchased from SiliCycle (Quebec City, Canada) and Merck (Darmstadt, Germany), respectively. Acetophenone > 99% was purchased from Carlo Erba (Milano, Italy), while 3′,5′-bis(trifluoromethyl)-acetophenone > 98% and *p*-methoxy-acetophenone > 99% were purchased from Zentek (Milan, Italy). NADH as well as NAD^+ cofactors and potassium phosphate salts were purchased from Merck (Darmstadt, Germany). If not specified, the solvents used in this work were HPLC-grade, purchased from Merck (Darmstadt, Germany) and Alfa Aesar (Karlsruhe, Germany) and used without any further purifications.

3.2. HPLC Analysis

The substrate-to-product conversion was analyzed by a Jasco (Jasco Europe, Cremella, Italy) HPLC system equipped with a PU-2089 Plus pump, CO-2060 Plus column oven, AS-2057 Plus autosampler, MD-910 Diode Array Detector, and Phenomenex (Castel Maggiore, Italy) reverse-phase column (Kinetex C18, 150 × 4.6 mm, 5 μm). For **1a**/**2a** and **1b**/**2b**, the elution was carried out with H_2O:MeCN:TFA 70:30:0.1 at a flow rate of 1 mL/min, 25 °C, and detection at 211 nm. For **1c**/**2c**, the elution was carried out with H_2O:MeCN:TFA 50:50:0.1 at a flow rate of 1 mL/min, 25 °C, and detection at 211 nm.

The retention times for **2a** and **1a** were 3.7 min and 6.2 min, respectively.
The retention times for **2b** and **1b** were 5.1 min and 6.9 min, respectively.
The retention times for **2c** and **1c** were 6.2 min and 9.3 min, respectively.

The enantiomeric excess was determined by a Jasco HPLC system equipped with a PU-980 Plus pump, CO-2060 Plus column oven, MD-910 Multiwavelength Detector, and Phenomenex chiral column (Lux Cellulose-1, 250 × 4.60 mm, 3 μm). Elution was carried out with Hex:IPA 95:5 at a flow rate of 0.5 mL/min, 25 °C, and detection at 220 nm.

The retention times for (*R*)-**2a** and (*S*)-**2a** were 12 min and 13 min, respectively.
The retention times for (*S*)-**2b** and (*R*)-**2b** were 10 min and 12 min, respectively.
The retention times for (*S*)-**2c** and (*R*)-**2c** were 13 min and 14 min, respectively.

The absolute configurations were assigned via a comparison of the elution orders with those of authentic samples of known configurations.

3.3. Biocatalyst Characterization

3.3.1. Protein Concentration

The protein concentration of the commercial sample of ADHs was determined according to a Bradford protein assay [52] by using bovine serum albumin as the standard in a concentration range of 0.095–2 mg/mL and Coomassie Brilliant Blue G-250 dye. In each assay, 20 μL of the sample was mixed with 1 mL of Bradford's dye. Absorbance data were monitored at 595 nm using a Shimadzu UV-2600i spectrophotometer after 10 min of contact. The samples (including blank experiments) were assayed in duplicate.

The protein purities of the commercial samples of EMIN001, EMIN028, and ADH105 were 0.6, 0.8, and 0.92 mg/mg of lyophilized powder, respectively.

3.3.2. Activity Assay

The activity tests were carried out in triplicate on the reference substrate acetophenone. For the enzyme in the soluble form, the reaction mixture contained the following: 840 µL of phosphate buffer (pH = 7, 0.1 M), 100 µL of NADH solution (10 mM in H_2O), 50 µL of enzyme solution (3 mg/mL in potassium phosphate buffer, pH = 7, 0.1 M), and 10 µL of acetophenone solution (100 mM in IPA). For the immobilized enzyme, the reaction mixture contained the following: 890 µL of potassium phosphate buffer (pH = 7, 0.1 M), 100 µL of NADH solution (10 mM in H_2O, 10 mg of immobilized sample, and 10 µL of substrate solution (100 mM in IPA). The reactions were performed at 30 °C for 10 min and the concentration of the product was determined via HPLC.

The specific activities of the commercial samples of EMIN001, EMIN028, and ADH105 were 0.086 ± 0.012, 0.241 ± 0.008, and 0.302 ± 0.005 U/mg protein, respectively.

3.4. General ADH Immobilization Procedure

3.4.1. Immobilization onto Silica Gels

The enzyme solution (12.5 mg/mL) was prepared by dissolving the lyophilized ADH enzyme into a potassium phosphate-buffered solution (100 mM, pH = 7.0) to obtain a gel/buffer ratio of 1:4 (*w*/*v*). The silica was first transferred to the immobilization vessel (100 mL flask) and the immobilization solution containing the enzyme was then added. The slurry was gently mixed for 24 h at 25 °C. The gel was washed 3 times 1:4 (*w*/*v*) with a potassium phosphate-buffered solution (100 mM, pH = 7.0) and filtered. The immobilized biocatalyst was then dried under a vacuum until a constant weight was reached.

3.4.2. Immobilization onto Amino-Functionalized Silica Gels

The silica was pre-activated with a 2% (*v*/*v*) glutaraldehyde solution in a potassium phosphate-buffered solution (100 mM, pH = 7.0) to obtain a gel/buffer ratio of 1:4 (*w*/*v*) and gently mixed at 25 °C for 1 h. The glutaraldehyde solution was removed via filtration and the gel was washed 3 times 1:4 (*w*/*v*) with a potassium phosphate-buffered solution (100 mM, pH = 7.0) and filtered. The enzyme solution (12.5 mg/mL) was prepared by dissolving the lyophilized ADH enzyme into a potassium phosphate-buffered solution (100 mM, pH = 7.0) to obtain a gel/buffer ratio of 1:4 (*w*/*v*). The amino-derivatized silica was first transferred to the immobilization vessel (100 mL flask) and the immobilization solution containing the enzyme was then added. The slurry was gently mixed for 24 h at 25 °C. The gel was washed 3 times 1:4 (*w*/*v*) with a potassium phosphate-buffered solution (10 mM, pH = 7.0) and filtered. The immobilized biocatalyst was then dried under a vacuum until a constant weight was reached.

3.5. Asymmetric Ketone Reduction with Soluble ADHs

The reduction of ketones to chiral alcohols was conducted in a 5 mL Eppendorf tube inserted into an Eppendorf Thermomixer C, which combines mixing and temperature control.

Then, 25 mg of a liquid substrate (**1a**, **1b**, **1c**) was solubilized directly in the reaction vessel in 125 µL of DMSO and 375 µL of IPA, to ensure the complete homogenization of the starting material. Then, 3 mL of a potassium phosphate-buffered solution (100 mM, pH = 7.0) and 0.5 mL of a NAD^+ solution (100 mM in water) were added. The solution was pre-incubated at the reaction temperature for 5 min. After this, 1 mL of an ADH enzyme suspension 25 mg/mL in a potassium phosphate-buffered solution (100 mM, pH = 7.0) was added, and the reaction mixture was stirred at 30 °C and 800 rpm.

Reactions were sampled by withdrawals of 50 µL at different time intervals, quenched with 200 µL of MeCN, diluted into 950 µL of 50:50 H_2O:MeCN, centrifuged, filtered, and analyzed via HPLC. After 24 hours, the reaction was quenched with 1 volume of ethyl acetate and the enzyme was separated from the supernatant via centrifugation and filtration. The filtrate was concentrated under a reduced pressure to remove the IPA from the solution and the aqueous phases was extracted with 2 volumes of ethyl acetate. The organic phases were combined, dried over Na_2SO_4, filtered, and concentrated under a reduced pressure.

In entries where complete conversion was achieved, products were recovered as white crystalline solid or colorless liquids. The conversion of the substrates and the enantiomeric excess of the products were determined via HPLC using a reversed-phase column and a chiral column, respectively.

3.6. Enantioselective Ketone Reduction with Immobilized ADHs

3.6.1. Bioreduction under Batch Conditions

The reduction of ketones to chiral alcohols was conducted in a 5 mL Eppendorf tube inserted into an Eppendorf Thermomixer C, which combines mixing and temperature control. To being, 25 mg of liquid substrate was solubilized directly in the reaction vessel in 125 µL of DMSO and 375 µL of IPA, to ensure complete homogenization in the starting material. Then, 4 mL of a potassium phosphate-buffered solution (100 mM, pH = 7.0) and 0.5 mL of a NAD^+ solution (100 mM in water) were added. The solution was pre-incubated at the reaction temperature for 5 min. Then, 200 mg of immobilized ADH enzyme sample was added and the reaction mixture was stirred at 30 °C and 800 rpm.

Reactions were sampled by withdrawals of 50 µL at different time intervals, quenched with 200 µL of MeCN, diluted into 950 µL of 50:50 H_2O:MeCN, centrifuged, filtered, and analyzed via HPLC. After 24 h, the reaction was quenched with 1 volume of ethyl acetate and the immobilized enzyme was separated from the supernatant via filtration. The filtrate was concentrated under reduced pressure to remove the IPA from the solution and the aqueous phases were extracted with 2 volumes of ethyl acetate. The organic phases were combined, dried over Na_2SO_4, filtered, and concentrated under a reduced pressure. In entries where complete conversion was achieved, products were recovered as white crystalline solids or colorless liquids. The conversions of the substrates and the enantiomeric excess of the products were determined via HPLC using a reversed-phase column and a chiral column, respectively.

3.6.2. Bioreduction under Flow Conditions

Of the immobilized EMIN028@SiO_2 sample, 600 mg was carefully packed into a PEEK column with dimensions of 4 cm length and 4 mm inner diameter, connected to a pump and maintained at a constant temperature of 30 °C. The reaction mixture was prepared in a 5 mL glass container with a tightly sealed cap. The components included 150 µL of a 100 mM NAD^+ solution in H_2O, another 150 µL of pure H_2O, 2.7 mL of IPA, and 150 mg (200 mM) of substrate **1c**. The starting mixture volume was 3 mL (90:10 IPA:H_2O). The final amounts employed were as follows: 600 mg of the immobilized enzyme sample, cofactor 5 mM, and substrate 200 mM. The resulting solution was then heated to 30 °C and flow reactions were carried out by systematically pumping the mixture through the system.

At distinct intervals, samples of the supernatant (50 µL) were extracted, swiftly quenched with 200 µL of MeCN, and subsequently diluted into a 1:1 mixture of MeCN and H_2O (250 µL). The mixture was centrifuged and analyzed via HPLC. After 24 h, the reaction mixture was sampled and extracted from the flow system, and the column underwent a thorough wash with the reaction solvent mixture to ensure the removal of both the initial materials and desired products. The reaction mixture, along with the washing fractions, were concentrated under a reduced pressure. When complete conversion was achieved, (*S*)-**2c** was recovered as a white crystalline solid. The immobilized ADH sample EMIN028@SiO_2 was reused in five consecutive reactions, conducted under identical experimental conditions.

3.7. Cross-Linking with Glutaraldehyde

The immobilized EMIN028@SiO_2 sample was briefly washed with a potassium phosp hate-buffered solution (100 mM, pH = 7.0) and then incubated in the same buffer containing 0.1% (*v*/*v*) glutaraldehyde for 30 min. After washing with a phosphate buffer (100 mM, pH = 7.0), the capping of unreacted aldehyde groups was performed with a Tris–HCl buffer

(100 mM Tris, pH = 8.0). The final sample was dried under a vacuum until a constant weight was reached and then properly stored at 4 °C.

4. Conclusions

In this study, three commercially available ADHs were immobilized on different silica-based supports via adsorption and covalent bond formation. The prepared supported biocatalysts were fully characterized and employed in the enantioselective reduction of three aromatic substrates. In particular, prochiral ketones with an acetophenone-type structure have been reduced by using ADH samples in both soluble and immobilized forms.

Two of the three immobilized enzymes allowed the obtaining of the corresponding products with good to high conversions (50–100%) and excellent enantioselectivities (ee > 99%). These chiral alcohols represent industrially relevant intermediates for the synthesis of active pharmaceutical ingredients (APIs). The best results were obtained with the enzymes adsorbed on a non-functionalized silica gel support.

The best performing biocatalyst was used for the optimization of the enantioselective reduction reaction of 3′,5′-bis(trifluoromethyl)acetophenone (**1c**) to (*S*)-1-[3,5-bis-(trifluoromethyl)phenyl]ethanol (**2c**), which is an important intermediate. The immobilized biocatalyst used in a mainly organic medium (90% IPA) allowed for obtaining the desired product with complete conversion and high ee (>99%) after 24 hours. The reusability of the biocatalysts was also investigated in a flow system under optimized reaction conditions. A further development of the process optimization was obtained through the cross-linking of the immobilized enzyme with glutaraldehyde.

In conclusion, this research demonstrated that silica-based supports could represent an alternative to organic polymeric carriers for the preparation of immobilized ADHs, which can be used for the synthesis of chiral compounds. In conclusion, this research demonstrated that silica-based supports could represent an alternative to organic polymeric carriers for the preparation of immobilized ADHs, which can be used for the synthesis of chiral compounds.

Author Contributions: Conceptualization, D.A., O.P., and A.P.; methodology, D.A.; validation, D.A.; investigation, D.A.; data curation, D.A. and A.P.; writing—original draft preparation, D.A.; writing—review and editing, O.P. and A.P.; visualization, D.A.; supervision, O.P. and A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Pisa, Italy (Fondi di Ateneo), and by the Studio di Consulenza Scientifica—SCSOP, Sirtori (LC), Italy.

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors thank Enzymaster Deutschland GmbH for kindly donating the alcohol dehydrogenase enzymes employed to obtain the experimental results shown in this work.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

@	Immobilized on
ADH	Alcohol dehydrogenase
DMSO	Dimethyl sulfoxide
ee	Enantiomeric excess
Hex	Hexane
IPA	2-propanol
MeCN	Acetonitrile
PEEK	Polyether ether ketone
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane

References

1. Bell, E.L.; Finnigan, W.; France, S.P.; Green, A.P.; Hayes, M.A.; Hepworth, L.J.; Lovelock, S.L.; Niikura, H.; Osuna, S.; Romero, E.; et al. Biocatalysis. *Nat. Rev. Methods Primers* **2021**, *1*, 46. [CrossRef]
2. Chapman, J.; Ismail, A.E.; Dinu, C.Z. Industrial applications of enzymes: Recent advances, techniques, and outlooks. *Catalysts* **2018**, *8*, 238. [CrossRef]
3. Carrea, G.; Riva, S. Properties and synthetic applications of enzymes in organic solvents. *Angew. Chem. Int. Ed.* **2000**, *39*, 2226–2254. [CrossRef]
4. Doukyu, N.; Ogino, H. Organic solvent-tolerant enzymes. *Biochem. Eng. J.* **2010**, *48*, 270–282. [CrossRef]
5. Cao, L. *Carrier-Bound Immobilized Enzymes: Principles, Application, and Design*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2005.
6. Sheldon, R.A.; Brady, D. Broadening the scope of biocatalysis in sustainable organic synthesis. *ChemSusChem* **2019**, *12*, 2859–2881.
7. Sheldon, R.A. The E factor 25 years on: The rise of green chemistry and sustainability. *Green Chem.* **2017**, *19*, 18–43. [CrossRef]
8. Abdussalam-Mohammed, W.; Ali, A.Q.; Errayes, A.O. Green chemistry: Principles, applications, and disadvantages. *Chem. Methodol.* **2020**, *4*, 408–423.
9. Faber, K. *Biotransformations in Organic Chemistry*, 7th ed.; Springer: Berlin/Heidelberg, Germany, 2018.
10. Moore, J.C.; Pollard, D.J.; Kosjek, B.; Devine, P.N. Advances in the enzymatic reduction of ketones. *Acc. Chem. Res.* **2007**, *40*, 1412–1419. [CrossRef]
11. de Miranda, A.S.; Milagre, C.D.; Hollmann, F. Alcohol dehydrogenases as catalysts in organic synthesis. *Front. Catal.* **2022**, *2*, 900554. [CrossRef]
12. Zheng, Y.G.; Yin, H.H.; Yu, D.F.; Chen, X.; Tang, X.L.; Zhang, X.J.; Xue, Y.P.; Wang, Y.J.; Liu, Z.Q. Recent advances in biotechnological applications of alcohol dehydrogenases. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 987–1001. [CrossRef]
13. Younus, H. Oxidoreductases: Overview and Practical Applications. In *Biocatalysis: Enzymatic Basics and Applications*; Husain, Q., Ullah, M., Eds.; Springer: Berlin/Heidelberg, Germany, 2019; pp. 39–55.
14. Koesoema, A.A.; Standley, D.M.; Senda, T.; Matsuda, T. Impact and relevance of alcohol dehydrogenase enantioselectivities on biotechnological applications. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 2897–2909. [CrossRef] [PubMed]
15. Raynbird, M.Y.; Sampson, J.B.; Smith, D.A.; Forsyth, S.M.; Moseley, J.D.; Wells, A.S. Ketone reductase biocatalysis in the synthesis of chiral intermediates toward generic active pharmaceutical ingredients. *Org. Process. Res. Dev.* **2020**, *24*, 1131–1140. [CrossRef]
16. Sardauna, A.E.; Abdulrasheed, M.; Nzila, A.; Musa, M.M. Biocatalytic asymmetric reduction of prochiral bulky-bulky ketones. *Mol. Catal.* **2023**, *541*, 113099. [CrossRef]
17. Kulig, J.; Simon, R.C.; Rose, C.A.; Husain, S.M.; Häckh, M.; Lüdeke, S.; Zeitler, K.; Kroutil, W.; Pohl, M.; Rother, D. Stereoselective synthesis of bulky 1, 2-diols with alcohol dehydrogenases. *Catal. Sci. Technol.* **2012**, *2*, 1580–1589. [CrossRef]
18. Sheldon, R.A.; Basso, A.; Brady, D. New frontiers in enzyme immobilisation: Robust biocatalysts for a circular bio-based economy. *Chem. Soc. Rev.* **2021**, *50*, 5850–5862. [CrossRef]
19. Basso, A.; Serban, S. Industrial applications of immobilized enzymes—A review. *Mol. Cat.* **2019**, *479*, 110607. [CrossRef]
20. Bolivar, J.M.; Wilson, L.; Ferrarotti, S.A.; Guisan, J.M.; Fernandez-Lafuente, R.; Mateo, C. Improvement of the stability of alcohol dehydrogenase by covalent immobilization on glyoxyl-agarose. *J. Biotechnol.* **2006**, *125*, 85–94. [CrossRef] [PubMed]
21. Li, G.Y.; Li, Y.J. Immobilization of Saccharomyces cerevisiae alcohol dehydrogenase on hybrid alginate–chitosan beads. *Int. J. Biol. Macromol.* **2010**, *47*, 21–26.
22. Bolivar, J.M.; Rocha-Martín, J.; Mateo, C.; Guisan, J.M. Stabilization of a highly active but unstable alcohol dehydrogenase from yeast using immobilization and post-immobilization techniques. *Process. Biochem.* **2012**, *47*, 679–686. [CrossRef]
23. Alsafadi, D.; Parisi, F. Covalent immobilization of alcohol dehydrogenase (ADH2) from *Haloferax volcanii*: How to maximize activity and optimize performance of halophilic enzymes. *Mol. Biotechnol.* **2014**, *55*, 240–247. [CrossRef]
24. Shinde, P.; Musameh, M.; Gao, Y.; Robinson, A.J.; Kyratzis, I.L. Immobilization and stabilization of alcohol dehydrogenase on polyvinyl alcohol fibre. *Biotechnol. Rep.* **2018**, *19*, e00260. [CrossRef] [PubMed]
25. Solé, J.; Brummund, J.; Caminal, G.; Schürman, M.; Álvaro, G.; Guillén, M. Ketoisophorone synthesis with an immobilized alcohol dehydrogenase. *ChemCatChem* **2019**, *11*, 4862–4870. [CrossRef]
26. Musa, M.M.; Phillips, R.S. Recent advances in alcohol dehydrogenase-catalyzed asymmetric production of hydrophobic alcohols. *Catal. Sci. Technol.* **2011**, *1*, 1311–1323. [CrossRef]
27. Adebar, N.; Gröger, H. Flow process for ketone reduction using a superabsorber-immobilized alcohol dehydrogenase from Lactobacillus brevis in a packed-bed reactor. *Bioengineering* **2019**, *6*, 99. [CrossRef]
28. Hartmann, M.; Kostrov, X. Immobilization of enzymes on porous silicas—Benefits and challenges. *Chem. Soc. Rev.* **2013**, *42*, 6277–6289. [CrossRef] [PubMed]
29. Magner, E. Immobilisation of enzymes on mesoporous silicate materials. *Chem. Soc. Rev.* **2013**, *42*, 6213–6222. [CrossRef] [PubMed]
30. Hartmann, M.; Jung, D. Biocatalysis with enzymes immobilized on mesoporous hosts: The status quo and future trends. *J. Mater. Chem.* **2010**, *20*, 844–857. [CrossRef]
31. Pietricola, G.; Dosa, M.; Ottone, C.; Fino, D.; Piumetti, M.; Tommasi, T. Covalent Immobilization of Aldehyde and Alcohol Dehydrogenases on Ordered Mesoporous Silicas. *Waste Biomass Valorization* **2022**, *13*, 4043–4055. [CrossRef]

32. Engelmann, C.; Ekambaram, N.; Johannsen, J.; Fellechner, O.; Waluga, T.; Fieg, G.; Liese, A.; Bubenheim, P. Enzyme immobilization on synthesized nanoporous silica particles and their application in a bi-enzymatic reaction. *ChemCatChem* **2020**, *12*, 2245–2252. [CrossRef]
33. Sun, J.; Zhang, D.; Zhao, W.; Ji, Q.; Ariga, K. Enhanced activity of alcohol dehydrogenase in porous silica nanosheets with wide size distributed mesopores. *Bull. Chem. Soc. Jpn.* **2019**, *92*, 275–282. [CrossRef]
34. Liu, X.; Du, X.; Feng, J.; Wu, M.; Lin, J.; Guan, J.; Wang, T.; Zhang, Z. Co-immobilization of short-chain dehydrogenase/Reductase and glucose dehydrogenase for the efficient production of (±)-ethyl Mandelate. *Catal. Lett.* **2019**, *149*, 1710–1720. [CrossRef]
35. Dreifke, M.; Brieler, F.J.; Fröba, M. Immobilization of alcohol dehydrogenase from *E. coli* onto mesoporous silica for application as a cofactor recycling system. *ChemCatChem* **2017**, *9*, 1197–1210. [CrossRef]
36. Petkova, G.A.; Záruba, K.; Král, V. Synthesis of silica particles and their application as supports for alcohol dehydrogenases and cofactor immobilizations: Conformational changes that lead to switch in enzyme stereoselectivity. *Biochim. Biophys. Acta BBA-Proteins Proteom.* **2012**, *1824*, 792–801. [CrossRef]
37. Trivedi, A.; Heinemann, M.; Spiess, A.C.; Daussmann, T.; Büchs, J. Optimization of adsorptive immobilization of alcohol dehydrogenases. *J. Biosci. Bioeng.* **2005**, *99*, 340–347. [CrossRef]
38. Yang, G.; Wu, J.; Xu, G.; Yang, L. Comparative study of properties of immobilized lipase onto glutaraldehyde-activated amino-silica gel via different methods. *Colloids Surf. B Biointerfaces* **2010**, *78*, 351–356. [CrossRef] [PubMed]
39. Politano, V.T.; Diener, R.M.; Christian, M.S.; Hawkins, D.R.; Ritacco, G.; Api, A.M. The pharmacokinetics of phenylethyl alcohol (PEA) safety evaluation comparisons in rats, rabbits, and humans. *Int. J. Toxicol.* **2013**, *32*, 39–47. [CrossRef] [PubMed]
40. Scognamiglio, J.; Jones, L.; Letizia, C.S.; Api, A.M. Fragrance material review on phenylethyl alcohol. *Food Chem. Toxicol.* **2012**, *50*, S224–S239. [CrossRef] [PubMed]
41. Belsito, D.; Bickers, D.; Bruze, M.; Calow, P.; Dagli, M.L.; Fryer, A.D.; Greim, H.; Miyachi, Y.; Saurat, J.H.; Sipes, I.G. A toxicological and dermatological assessment of aryl alkyl alcohols when used as fragrance ingredients. *Food Chem. Toxicol.* **2012**, *50*, 5. [CrossRef] [PubMed]
42. Dong, Y.; Dong, L.; Gu, X.; Wang, Y.; Liao, Y.; Luque, R.; Chen, Z. Sustainable production of active pharmaceutical ingredients from lignin-based benzoic acid derivatives via "demand orientation". *Green Chem.* **2023**, *25*, 3791–3815. [CrossRef]
43. Chen, Y.; Zhou, Q.; Hankey, W.; Fang, X.; Yuan, F. Second generation androgen receptor antagonists and challenges in prostate cancer treatment. *Cell Death Dis.* **2022**, *13*, 632. [CrossRef] [PubMed]
44. Dorokhov, V.S.; Nelyubina, Y.V.; Ioffe, S.L.; Sukhorukov, A.Y. Asymmetric Synthesis of Merck's Potent hNK1 Antagonist and Its Stereoisomers via Tandem Acylation/[3, 3]-Rearrangement of 1, 2-Oxazine N-Oxides. *J. Org. Chem.* **2020**, *85*, 11060–11071. [CrossRef]
45. Jin, Y.; Wu, X.; Guan, Y.; Gu, D.; Shen, Y.; Xu, Z.; Wei, X.; Chen, J. Efficacy and safety of aprepitant in the prevention of chemotherapy-induced nausea and vomiting: A pooled analysis. *Support Care Cancer* **2012**, *20*, 1815–1822. [CrossRef]
46. Armani, D.; Piccolo, O.; Petri, A. Biocatalytic Asymmetric Synthesis of (*S*)-1-[3, 5-bis (trifluoromethyl) phenyl] ethanol by an Immobilized KRED in Batch and Flow Conditions. *ChemCatChem* **2023**, *15*, e202300809. [CrossRef]
47. Govardhan, C.P. Crosslinking of enzymes for improved stability and performance. *Curr. Opin. Biotechnol.* **1999**, *10*, 331–335. [CrossRef]
48. Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization. *RSC Adv.* **2014**, *4*, 1583–1600. [CrossRef]
49. Kim, M.I.; Kim, J.; Lee, J.; Shin, S.; Na, H.B.; Hyeon, T.; Park, H.G.; Chang, H.N. One-dimensional crosslinked enzyme aggregates in SBA-15: Superior catalytic behavior to conventional enzyme immobilization. *Microporous Mesoporous Mater.* **2008**, *111*, 18–23. [CrossRef]
50. López-Gallego, F.; Betancor, L.; Mateo, C.; Hidalgo, A.; Alonso-Morales, N.; Dellamora-Ortiz, G.; Guisán, J.M.; Fernández-Lafuente, R. Enzyme stabilization by glutaraldehyde crosslinking of adsorbed proteins on aminated supports. *J. Biotechnol.* **2005**, *119*, 70–75. [CrossRef] [PubMed]
51. Migneault, I.; Dartiguenave, C.; Bertrand, M.J.; Waldron, K.C. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *BioTechniques* **2004**, *37*, 790–802. [CrossRef]
52. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef] [PubMed]