Carceller-Carceller, JM.; Mifsud, M.; Climent Olmedo, MJ.; Iborra Chornet, S.; Corma Canós, A. (2020). Production of chiral alcohols from racemic mixtures by integrated heterogeneous chemoenzymatic catalysis in fixed bed continuous operation. Green Chemistry. 22(9):2767-2777. https://doi.org/10.1039/c9gc04127c

ARTICLE

Production of chiral alcohols from racemic mixtures by integrated heterogeneous chemoenzymatic catalysis in fixed bed continuous operation

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Valuable chiral alcohols have been obtained from racemic mixtures with an integrated heterogeneous chemoenzymatic catalyst in a two consecutive fixed catalytic bed continuous reactor system. In the first bed the racemic mixture of alcohols is oxidized to the prochiral ketone with a Zr-Beta zeolite and using acetone as hydrogen acceptor. In the second catalytic bed the prochiral ketone is stereoselectively reduced with an alcohol dehydrogenase (ADH) immobilized on a two dimensional (2D) zeolite. In this process, the alcohol (isopropanol) formed by reduction of acetone in the first step, reduces the cofactor in the second step, and the full reaction cycle is in this way internally closed with 100 % atom economy. Conversion about 95 % with ~100 % selectivity to either the (R) or the (S) alcohol have been obtained for a variety of racemic mixtures of alcohols.

Introduction

One of the main demands of modern chemistry is the easy syntheses of optically pure compounds,[1] and resolution of racemic mixtures is the most common method to prepare enantiomerically pure compounds at an industrial scale.[2,3] Chiral secondary alcohols are valuable intermediates in the fine chemical industry. For example, the pure chiral (R or S)-2-octanol and (S)-2-pentanol are important precursors for the preparation of several drugs for the treatment of Alzheimer.[4–8] The easy accessibility and commercial availability of an abundant variety of racemic alcohols have promoted the development of biocatalytic routes to produce enantiopure alcohols from racemic mixtures.[9] Most protocols being used to produce chiral alcohols are based on kinetic resolutions (KR) of racemic secondary alcohols with enzymes such as lipases and esterases.[10,11]

Enzymatic kinetic resolution is often very efficient in terms of selectivity but suffers of important drawbacks, such as the maximum theoretical yield is limited to 50 % due to the consumption of only one enantiomer, while it requires a laborious separation of the product from the remaining substrate. An attractive method to overcome those drawbacks is the dynamic kinetic resolution (DKR) in which racemisation of unwanted enantiomer is coupled in one-pot with KR, and then a theoretical maximum yield of up to 100 % can be achieved.[12–14]

Another strategy for deracemization of secondary alcohols that has attracted much attention is based on simple oxidation-reduction sequences that are usually accomplished by multienzymatic combinations.[15–18] However, it is also possible to achieve deracemization by combining a chemocatalyzed non-selective oxidation step of the racemic alcohols with stereoselective (enzymatic) reduction of the prochiral ketone. This protocol has been comparatively less explored, and it is usually accomplished using homogeneous chemoenzymatic catalytic systems based in the combination of and oxidant such as TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl) or AZADO (2-azaadamantane N-oxyl) with redox biocatalysts such alcohol deshydrogenases (ADH).[19–22]

Here, we present an efficient process to obtain valuable chiral alcohols from racemic mixtures with an integrated heterogeneous chemo/enzymatic two steps relay catalyst.[23] With this strategy we have coupled two steps in a continuous process: the first step is the oxidation of a racemic mixture of secondary alcohols into a prochiral ketone through the Oppenauer Oxidation using a Lewis acid zeolite (Zr-Beta) followed by the stereoselective reduction of the prochiral obtained ketone to alcohol with the enzyme alcohol dehydrogenase (ADH) immobilized on a two-dimensional (2D) zeolite (ITQ-2) (Scheme 1). Finally, the alcohol formed as byproduct in the Oppenauer reaction reduces the cofactor closing the system with 100 % atom economy. This strategy allows obtaining optically pure alcohols without having to isolate and purify intermediates, reducing the number of separation steps with the corresponding benefit from the environmental and economic point of view.

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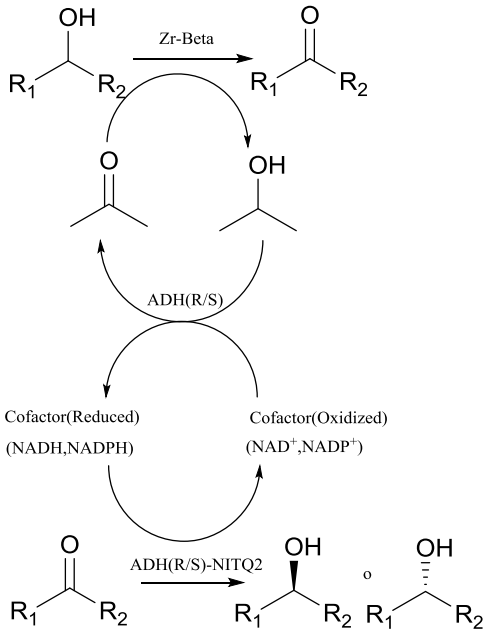
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x



Scheme 1. Coupling Oppenauer oxidation of racemic mixtures of alcohol with stereoselective reduction of the prochiral ketone with immobilized ADH

The Meerwein-Ponndorf-Verley (MPV) reduction of aldehydes and ketones and the reverse Oppenauer oxidation of alcohols (MPVO) are valuable tools in organic synthesis since they are chemoselective reactions in which other reducible or oxidizable groups are not reacted, moreover they can be performed under mild conditions. Basically, in the MPVO reaction, a carbonyl compound is reduced to an alcohol molecule while an alcohol is oxidized to the corresponding carbonyl compound through a hydride transfer process. The conventional catalysts used in MPVO reactions are aluminum alkoxides, however the stoichiometric amount of alkoxide required, its water sensitivity, along with the required neutralization at the end of the reaction and wastes generated are important disadvantages. Therefore, in the last years a variety of heterogeneous catalysts have been developed and successfully applied in this process. Among them, Al- and Ti-Beta zeolites have been reported as excellent catalysts for MPVO reactions showing that Lewis acid sites are the active sites of the catalyst.[24–27] Compared with Al- and Ti-Beta zeolites, better performances for the MPVO reactions were found using Sn- and Zr-Beta zeolites which was attributed to the more adequate Lewis acidity of the Sn and Zr sites in the zeolite framework.[28–31] A computational study indicates that the mechanism of Sn-Beta and Zr-Beta catalysts is similar and consists of three steps: adsorption of both ketone and the alcohol on the Lewis acid site, deprotonation of the alcohol, carbon-to-carbon hydride transfer, and proton transfer from the catalyst and product exchange.[30] The exceptional characteristics of Sn-Beta and Zr-Beta zeolites as highly active and stable catalysts for the MPVO reaction, prompted us to select the Zr-Beta zeolite as catalyst to produce the oxidation of racemic alcohols into prochiral ketones as the first step of the process proposed here. On the other hand, alcohol dehydrogenases (ADH) are enzymes that reversibly catalyse the reduction of aldehydes or ketones to primary or secondary alcohols respectively. They play an increasingly important role for the production of chiral alcohols, hydroxyl-acids or aminoacids.[32,33] However, comparatively with hydrolytic enzymes, such as lipases, reductases have been considerably less used in the production of optically active compounds, due to an important limitation for industrial applications of ADH is the need of stoichiometric amounts of expensive co-factors (NADH or NADPH). Reductions using ADH are only economically feasible if an efficient regeneration of the reduced cofactor is possible. Efficient methods for the regeneration of the cofactor allowing their use in catalytic amounts are the use ADH in combination with other substrate/enzyme systems such as formate/formate dehydrogenase or glucose/glucose dehydrogenase. However, one of the cheapest method for cofactor regeneration is by using isopropanol as a substrate of ADH.[34–36] The use of isopropanol is very convenient since it can also be used as a co-solvent for homogeneous solutions of organic substrates.

In the case of enzymes, their immobilization on solids supports may be adapted for increasing the stability and reaction kinetics of the enzyme[37,38] and, more importantly from the industrial point of view, the immobilization allows the easy separation from the reaction mixture, enzyme recyclability and the possibility to be used in continuous operation process. In this context the co-immobilization of enzymes and cofactor on solid materials is an interesting approach for large scale biotransformations.[39–42] For instance, Lopez-Gallego et al.[40] have integrated enzymes and phosphorylated cofactors on agarose microbeads activated with polyethyleneimine. They reported the first example of asymmetric reduction of ketones using co-immobilized ADH and NAD^+ in continuous flow reactor observing an insignificant NAD^+ lixiviation, which is one of the main limitations of the immobilized nicotinamide cofactor.

ADH have been immobilized by entrapment, covalent, or electrostatic interactions over different carriers. For instance, ADH has been immobilized on glyoxyl-agarose,[43] silica nanotubes,[44] magnetic nanoparticles,[45,46] polyaniline coated silver nanoparticles,[47] magnetic graphene oxide nanocomposites[48] and more recently over mesoporous silica[49] and titania nanoparticles.[50] However, when working with mesoporous materials, and depending on the size of the enzyme, hindered diffusion of the coenzyme and/or substrate through the porous system of the support can occurs limiting mass transport, affecting negatively the catalytic activity. Therefore, in this work we have selected as support for immobilization ADH a pure silica 2D zeolite (ITQ-2 zeolite). ITQ-2 zeolite can be prepared by delamination of a layered MWW zeolite precursor or by a dual templating one step

synthesis.[51–54] The resulting ITQ-2 zeolite is a crystalline material highly stable in aqueous media formed by thin zeolite sheets (2.5 nm thick) containing regularly distributed silanol groups and high external surface area (~ 600 m^2g^{-1}). On these structured inorganic sheets, the enzyme can be easily grafted and diffusional problems of the coenzyme and substrate are not expected. The large external surface area of ITQ-2 zeolite along with their high stability in aqueous media converts this material in an excellent and stable enzyme carrier for industrial biotransformations.[55,56]

Taking into account the above considerations, we have envisaged here a cascade process to produce chiral secondary alcohols starting from racemic mixtures in a continuous process by combining two fixed bed continuous reactor. In the first bed the alcohol racemic mixture is oxidized to a prochiral ketone through a hydrogen transfer reaction, the Oppenauer oxidation, using acetone as hydrogen acceptor and Zr-Beta zeolite as Lewis acids catalyst. In this reaction, isopropanol is produced as by-product. Then, the mixture of the prochiral ketone and isopropanol goes through the second catalytic bed containing the immobilized ADH on ITQ-2 in where after adjusting the amount of isopropanol and cofactor (NADH or NADPH) the stereoselective reduction of the ketone is produced, while the acetone used in the first step is regenerated.

Results and discussion

Characterization of the support and immobilization of ADH

For enzyme immobilization through electrostatic interactions, the surface of the pure silica ITQ-2 zeolite was functionalized with amino groups by treating the zeolite with (3-aminopropyl)triethoxysilane (material labelled as NITQ-2). Elemental analysis of the sample confirmed the presence of 2,5 wt % of nitrogen on the material which corresponds to a functionalization density of amino groups of 1.8 mmol/g (see Table S1). The FTIR analysis of the ITQ-2 and NITQ-2 are presented in Figure S1. Stretching vibration bands corresponding to Si–OH groups are observed in the ITQ-2 pure silica sample at 3740 cm^{-1} (Figure S1(a)). After chemical modification with (3-aminopropyl)triethoxysilane, the stretching vibration bands associated to amino groups at 3426 cm^{-1} and 1658 cm^{-1} along with, the stretching vibration of –CH2 groups (2928 cm^{-1}, 2866 cm^{-1}) associated to the alkyl chain of the propyl group are observed (Figure S1(b)), confirming the presence of the aminopropyl groups. Moreover the intensity of the 3740 cm^{-1} corresponding to Si-OH groups has very strongly decreased due to the anchoring the (3-aminopropyl)triethoxysilane. The Brunauer-Emmett-Teller surface area of the unmodified ITQ-2 was calculated to be 756 m^2/g, and after modification with (3-aminopropyl)triethoxysilane the BET surface area was decreased up to 387 m^2/g, however it still represents a high surface area support where the enzyme can be anchored (see Table S2).

The isoelectronic point (ip) of the ADH was determined to be at pH= 4.5 (see experimental), while the ip of the aminopropilated siliceous support should be in basic range,[49] therefore a pH= 5.5 was selected for immobilization of the enzyme through electrostatic interactions. Thus, the immobilization of the enzyme (ADH(S) and ADH(R) was performed by incubating the enzyme (10 mg) with the NITQ-2 (200 mg) in a phosphate buffer solution at pH 5.5 during 24 h. The amount of protein immobilized was determined by analyzing the amount of free enzyme in the supernatant by bicinchoninic acid protein test. After 24 h of incubation it was determined that the amount of immobilized ADH was 86 % while the activity recovery[57] was 80 %, which agrees with the calculated amount of enzyme immobilized.

Properties of Immobilized ADH

To determine the different properties of immobilized ADH on NITQ-2 we selected as reaction model the reduction of 2-octanone into (S)-2-octanol. Reactions were performed at 25 ^oC using a molar ratio substrate/NAD^+ of 10/1 which is the recommended ratio by the supplier.[46,58,59]

First, we studied the effect of enzyme loading on the catalytic activity by varying the amount of enzyme on the support as showed in Table 1. As can be observed, during the preparation of the supported enzyme, when the ratio enzyme/support (wt/wt) is duplicated (entries 1 and 2), the amount of immobilized enzyme decreases from 86 % (entry 1) to 75 % (entry 2). When the ratio of enzyme/support (entry 3) was reduced the amount of immobilized enzyme resulted 100 %. When the catalytic activity for the reduction of 2-octanone was tested for these different samples (Figure S2), it is possible to see that samples of entry 1 and 2 perform similar, achieving total conversion of the ketone after 30 min reaction time (see Figure S2). These results indicate that there is a range of enzyme loadings (between 0.05 and 0.1 g of enzyme per gram of support) for which the enzymatic derivative maintains very good performance. When the ratio enzyme/support was further decreased (entry 3), as expected, lower initial reaction rate was found, but practically total conversion could still be achieved after 90 min. Notice that the initial reaction rate per mg of enzyme supported remains practically the same independently of the enzyme loading, indicating good accessibility of the substrate to active sites in all cases (Table 1). The sample of entry 1, i.e. those prepared from 10 mg of ADH on 200 mg of NITQ-2 was selected for performing all the subsequent studies.

Table 1. Effect of enzyme loading on the catalytic activity for the reduction of 2-octanone

Entry	Enzyme (mg)	NITQ-2(mg)	Immobilized Enzyme (mg)	Initial reaction rate (μmol/min)	Initial reaction rate/mg enzyme
1	10	200	8.6	33.5	3.89
2	10	100	7.5	29.1	3.88
3	2.5	100	2.5	11.4	4.56

Immobilization conditions: 10 mg or 2.5 mg of ADH(S) in 5 mL of phosphate buffer, pH= 5.5; 200 mg or 100 mg of NITQ-2, under agitation for 24 h. Reaction conditions: 2-octanone, 0.3 mmol (30 mM), NAD^+, 0.03 mmol, in 10 mL of solvent (Isopropanol/phosphate buffer solution pH 7 (50/50 v/v), at 25 °C.

Catalytic activity as a function of pH and thermal stability

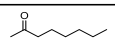
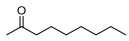
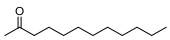
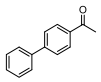
To study the effect of pH on the conversion of 2-octanone into (S)-2-octanol the activity of ADH(S)@NITQ-2 was studied at pH 5.5, 7 and 10 at 25 ^oC and compared with the free enzyme (see Figure S3). As can be observed free and immobilized ADH showed maximum activity at pH=7, while acidic pH (5.5) or basic pH (10) was detrimental for the enzyme activity. However, the immobilized enzyme was more resistant to the pH changes, showing higher activity than the native enzyme in a wider range of pH.

The thermal stability of ADH(S)@NITQ-2 was determined by summiting the supported enzyme at different temperatures: 25, 35, and 55 ^oC during 1 h at pH 7. After that, the substrate 2-octanone was added and the residual catalytic activity was determined under the conditions described in the experimental section. In Figure S4 the residual activity of the free and immobilized enzyme after the thermal treatment is presented. As can be seen there, at 25 ^oC, the free and immobilized enzyme perform almost the same, giving ~80-90 % conversion after 1 h reaction time. After treatment at increasing temperatures both the immobilized and native enzyme undergo a strong loss of activity. In fact, 48.3 % conversion with free ADH was achieved at 35 ^oC while the ADH(S)@NITQ-2 gave slightly higher conversion (55.3 %). However, the free enzyme lost completely its activity at 55 ^oC, while the immobilized enzyme retains a residual activity. Immobilization of ADH can restrict unfolding of the enzyme, and therefore the thermal stability could be somewhat improved with the increase of temperature.[48] However, these results evidence that for practical uses of the ADH@NITQ-2 the working temperature of the process should not surpass the 25 ^oC.

Scope of the reduction with ADH@NITQ-2

To study the scope of the reaction we performed the reduction of different ketones using the enzymatic derivative ADH(S)@NITQ-2 under the optimal reaction conditions. Several linear, cyclic and aromatic ketones were selected and as can be observed in Table 2 excellent yields to the (S) enantiopure alcohol with an enatiomeric excess > 99 % were obtained, showing the wide scope of the ADH (S)@NITQ-2 biocatalyst. When the reduction of 2-octanone was performed with the immobilized enantiocomplementary enzyme (ADH(R)@NITQ-2), NADPH dependent, (entry 1), yield and enantioselectivity to (R)-2-octanol was also very high although longer reaction time was required.

Table 2. Reduction of different ketones using the ADH@NITQ-2 as biocatalyst.

Entry	Substrate	ADH	time (h)	Conversion (Selectivity) (%)	ee (%)
1		S	1	99 (100)	>99
		R[a]	6.5	98 (100)	>99
2		S	1.5	89 (100)	>99
			2	99 (100)	
3		S	1	99 (100)	>99
4		S	1.5	99 (100)	>99
5		S	1.5	99 (100)	>99
6		S	2	56 (100)	>99
			4	98 (100)	
7		S	2	89 (100)	>99
			4	95 (100)	

Reaction Conditions: ADH(S)@NITQ-2 (208.6 mg), ketone 0.3 mmol (30 mM), NAD^+, 0.03 mmol (molar ratio substrate/cofactor = 10), in 10 mL of solvent (Isopropanol/phosphate buffer solution, pH 7, (50/50 v/v), at 25 °C. [a]ADH(R)@NITQ-2 (208.6 mg), ketone 0.3 mmol (30 mM), $NADP^+$, 0.03 mmol, in 10 mL of solvent (Isopropanol/phosphate buffer solution pH 7, 1 mM $MgCl_2$ (50/50 v/v), at 25 °C.

Enzymatic reduction of prochiral ketone in a fixed bed reactor

Owing to the high activity showed by the ADH@NITQ-2 in batch mode, and following our initial objective, we performed the reduction of the prochiral ketone in a flow reactor. For doing that the ADH(S)@NITQ-2 (208.6 mg) was diluted with silica 1.8 g SiO_2 and packed in a stainless steel reactor coupled with a peristaltic pump for feeding 2-octanone, the cofactor (in a substrate/ cofactor molar ratio of 10) and as solvent a mixture of isopropanol/phosphate buffer pH 7, 100 mM (50/50 v/v). Temperature was fixed at room temperature, and the contact time was evaluated keeping constant the flow and increasing the amount of 2-octanone. As we can observe (Figure S5 and Table S3) the conversion was maintained ~90 % at contact time above 1.3 h. For a contact time of 2 h a conversion of 96 % is already achieved, being the selectivity to (S)-2-octanol maintained at ~100 %.

Then, we selected a contact time of 1.3 h to check the stability of the enzymatic derivative. As we can be seen in Figure 1, the

conversion of 2-octanone (90 %) could be maintained during 24 h of operation, showing that deactivation does not occur in the studied period. In Figure 2 is showed that using a contact time of 4 h (that correspond to WHSV of 0.25 h^{-1}) the conversion of 2-octanone was maintained to 97 % during 100 h without deactivation. In fact, the reactor was kept under operation during 16 days, and no deactivation of the catalytic system was observed. Similar data were obtained using the ADH(R)@NITQ-2, obtaining in this case (R)-2-octanol with ~100 % selectivity.

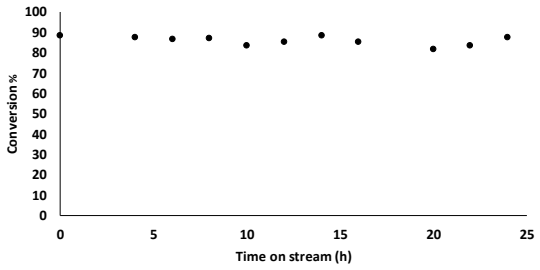


Figure 1. Evaluation of the stability of the reactor at contact time of 1.3 h for the reduction of 2-octanone to (S)-2-octanol. Reaction conditions: ADH(S)@NITQ-2 (208.6 mg), 2-octanone (94 mmol/L), NAD^+ (9,4 mmol/L), solvent (Isopropanol/phosphate buffer solution pH 7 (50/50 v/v), flow 0.55 mL/h 25 °C.

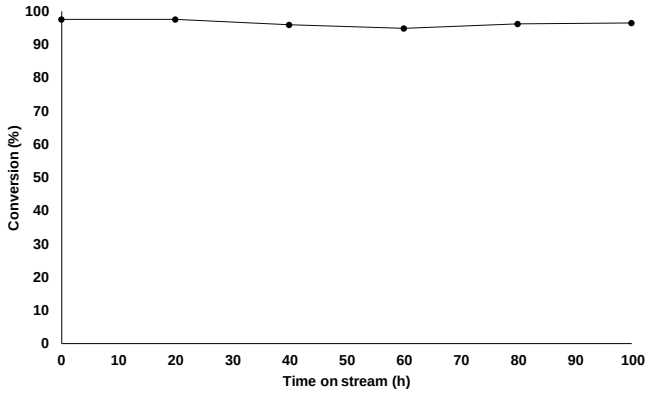


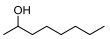
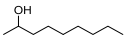
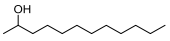
Figure 2. Results of the reduction of 2-octanone into (S)-2-octanol in continuous reactor using ADH(S)@NITQ-2 as biocatalyst. Reaction conditions: ADH(S)@NITQ-2 (208.6 mg), 2-octanone (30 mmol/L), NAD^+ (3 mmol/L), solvent (Isopropanol/phosphate buffer solution pH 7 (50/50 v/v), flow 0.55 mL/h, 25 °C.

Oxidation of racemic alcohols into prochiral ketones by Oppenauer reaction in batch mode

Up to now, we have showed that ADH immobilized on NITQ-2 is an active and stable biocatalyst to perform the steroselective reduction of prochiral ketones into the corresponding enantiopure alcohols. Then, the next step was to check the possibility to perform the oxidation of racemic mixtures of alcohols into prochiral ketones using acetone as hydrogen acceptor that will give isopropanol that will be later used for the enzymatic step, through the Oppenauer oxidation using Zr-Beta zeolite as Lewis acid catalyst. We selected first, the oxidation of 2-octanol using an excess of acetone (acetone/2-octanol molar ratio of 51) as the reaction model. The reaction was performed in batch mode at 50 °C in the presence of Zr-Beta as catalyst. As can be observed in Figure S6, the catalyst performs the oxidation selectively, achieving practically total conversion of the rac-2-octanol with 100 % selectivity to 2-octanone.

Then, the scope of the reaction was tested with a variety of secondary alcohols of different structure. As can be seen in Table 3, the Zr-Beta catalyst performs the reaction with excellent results in all cases. However, depending on the structure of the rac-alcohol, the reaction temperature had to be adjusted in order to achieve high conversion although the selectivity was 100 % in all cases.

Table 3. Reduction of different ketones using the ADH@NITQ-2 as biocatalyst.

Entry	Substrate	(%) Conversion (Selectivity)
1		99 (100)[a]
2		98 (100)[b]
3		94 (100)[b]
4		99 (100)[a]
5		99 (100)[a]
6		96 (100)[b]
7		99 (100)[b]

Reaction conditions: Zr-Beta (32 mg), alcohol (0.4 mmol), acetone (1.5 mL, 20.4 mmol), 20 h, [a] at 50 °C. [b] at 80 °C.

Oxidation of racemic alcohol in a fixed bed reactor

For the experiments in flow reactor**,** 304 mg of Zr-Beta zeolite was diluted with silica (1.7 g) and packed in a stainless steel reactor coupled with an electric heater controller and a peristaltic pump for feeding the reactor with a solution of rac-2-octanol in acetone and maintaining the temperature at 50 °C. The contact time was evaluated by varying the concentration of 2-octanol in the feed while the flow was maintained at 0.5 mL/h. As we can be seen in Figure S7 the conversion could be maintained very high (95-98 %) with 100 % selectivity.

The catalyst stability was evaluated using the contact time of 25 h (that correspond to WHSV of 0.04 h^{-1}), and as can be seen in Figure 3, the catalytic system was stable for 100 h of operation, being the average conversion of 98 % with 100 % selectivity to the prochiral ketone.

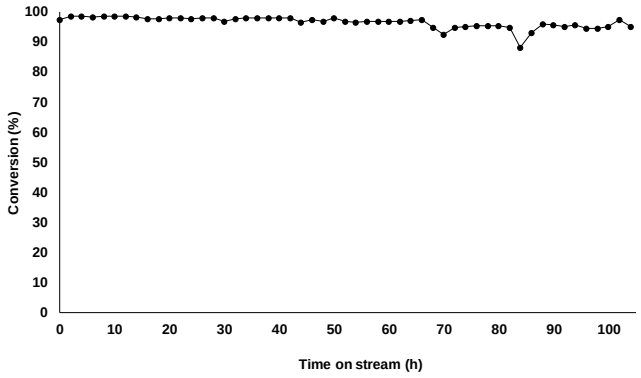


Figure 3. Evaluation of oxidation of rac-2-octanol in continuos reactor. Reaction conditions: Zr-Beta (304 mg), acetone (10 mL, 136 mmol), 2-octanol 191 mmol/L, flow 0.5 mL/h at 50 °C.

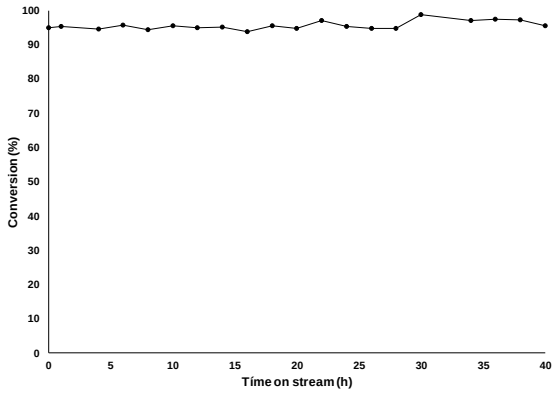


Figure 4. Total yield of (S)-2-octanol obtained from deracemization of rac-2-octanol by the two coupled flow reactors.

Coupling Oxidation-Reduction flow reactors

To explore the efficacy of our proposal for deracemization of secondary alcohols, both steps were coupled in a continuous flow reactor with two catalytic beds. Thus, in a first bed, Zr-Beta zeolite was placed and the reactor was fed with a solution of rac-2-octanol in acetone (191 mmol/L) using a contact time of 25 h (WHSV 0.04 h^{-1}) flow 0.5 mL/h, while the temperature was maintained at 50 °C. With an intermediate addition of the cofactor and solvent isopropanol/phosphate buffer pH 7, 100 mM (50/50 v/v), the flow coming out from the first catalytic bed went through the second catalytic bed where the ADH(S)@NITQ-2 was placed. The second catalytic bed was maintained at 25 °C, and the contact time was 4 h (WHSV 0.25 h^{-1}). However, under these conditions (entry 1, Table 4) the reduction of 2-octanone was decreased to 70 %. This was attributed to the presence of acetone in the feed, coming to the enzymatic bed and which reduction competes with the reduction of 2-octanone. To check that, we performed additional experiments where acetone was partially or totally eliminated from the feed going into the second reactor. As can be seen in Table 4, when the amount of acetone was reduced (entry 2) or removed (entry 3) from the feed to the second catalytic bed, 96 % conversion with 99 % ee to (S)-2-octanol was achieved. In good agreement with the results presented above in Figure 2, the catalytic activity was maintained for at least 40 h without observing any deactivation (Figure 4).

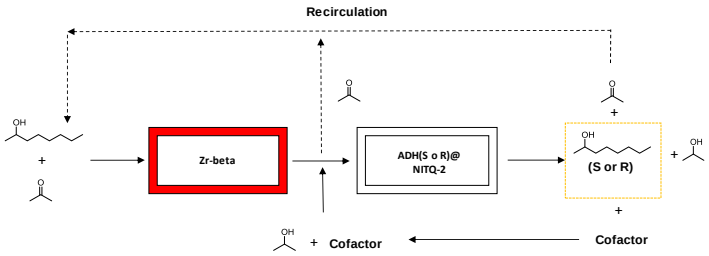
Table 4. Effect of the concentration of acetone on the reduction process

Entry	Acetone (%)(v/v)	Conversion of 2-octanone(%)
1	16	70
2	8	79
3	-	96

Reaction conditions: (First step) Oxidation reaction of racemic 2-octanol in a continuous-flow reactor using Zr-Beta (304 mg), 2-octanol 191 mmol/L, flow 0.5 mL/h at 50 °C. (Second step) Reduction reaction of prochiral 2-octanone in a continuous-flow reactor using ADH(2)@NITQ-2 (209 mg), 2-octanone 30 mmol/L, NAD^+ 3 mmol/L flow 0.55 mL/h.

Finally, the oxidation-reduction process have been extended to alcohols of different structures, i.e the 2-dodecanol, 1-cyclohexylethanol and 4-biphenylmethylcarbinol, also with excellent results (see Figures S8, S9 and S10 respectively).

The results presented above show that it is feasible to couple both steps (oxidation of rac-alcohols with stereoselective reduction of the prochiral ketone intermediate) in a continuous process. Since removal the excess of acetone after the first step is very convenient, we believe that this could be achieved by flash evaporation after the first oxidation reactor. This can be possible without losing the isopropanol formed during the oxidation because the acetone has a boiling point of 56 °C and the isopropanol 82.5 °C. After that, the acetone could be reused again in the first step of the process (see Scheme 2).



Scheme 2. Possible combination of oxidation-reduction system for an industrial process

Experimental Section

Materials

Crude cell extract of prelog Alcohol Dehydrogenases (ADH030) (S) (NADH dependent) and antiPrelog (ADH270) (R) (NADPH dependent) were purchased from Evocatal. 2-Octanol, 2-octanone, 1-cyclohexylethanol, 1-cyclohexylethanone, 2-pentanol, 2-pentanone, 2-hexanol, 2-hexanone, 2-nonanol, 2-nonanone, 2-dodecanol, 2-dodecanone, 4-biphenylmethylcarbinol, 4-acetylbiphenyl, the pure chiral compounds (R and S) 2-octanol, (R and S) 2-pentanol, (R and S)

2-hexanol, (R and S) 2-nonanol and the cofactors $NADP^+$, NAD^+ were purchased from Sigma Aldrich. The (R and S) 2-dodecanol was purchased from Boc Sciences and the (R and S) 4-biphenylmethylcarbinol and (R and S) 1-cyclohexylethanol were purchased from Enamine Store.

Catalyst preparation and characterization

Synthesis of the pure silica MWW (MCM-22) zeolite

The synthesis of pure silica MCM-22 zeolite was performed following the literature.[51] An example of the procedure for the synthesis of MCM-22 is as follows: 0.95 g of NaCl are dissolved in 50.70 g of a solution 0.42 M of N,N,N-trimethyl-1-adamantanamonium hydroxide, previously diluted in 21.33 g of water. Then, 2.62 g of hexamethyleneimine are added to this solution, followed by 4.88 g of silica (Aerosil 200, Degussa) under continuous stirring. The reaction mixture is heated in a Teflon lined stainless steel autoclave at 150 ^oC rotated at 60 rpm for 5 days. After filtering, the white solid obtained is washed until pH was less than 9, and finally dried at 100 ^oC.

Preparation of pure silica ITQ-2 zeolite

The synthesis of the pure silica ITQ-2 zeolite was carried following literature.[51] Typically, 5 g of the pure silica MCM-22 zeolite were dispersed in 20 g of water. Then, 100 g of an aqueous solution of hexadecyltrimethylammonium hydroxide (25% by weight, 50 % exchange Br/OH), and 30 g of an aqueous solution of tetrapropylammonium (40 % by weight, 30 % exchange Br/OH) were added. The resulting mixture (pH 12.5) was heated to 55 ^oC and stirred vigorously for 16 h to facilitate swelling between zeolitic sheets. At this point, the suspension was treated in an ultrasonic bath (50 W, 50 Hz) for 1 h to disperse the zeolitic sheets. By adding HCl (6 M), the pH was decreased to about 3, to facilitate flocculation of the delaminated solid, which is recovered by centrifugation. Then the solid was washed with distilled water, dried at 60 ^oC for 12 h, and calcined at 540 ^oC, first for 3 h in N_2 atmosphere, and then for 6 h in air.

Functionalization of the support with amino groups

The support (zeolite ITQ-2 pure silica) (500 mg) was activated at 200 ^oC under vacuum for 2 hours. After cooling at room temperature, 50 mL of anhydrous toluene and 240 µL of (3-aminopropyl) triethoxysilane were added to the solid and the mixture was refluxed for 24 h at 120 ^oC. After this time, the solid was filtered under vacuum and washed with toluene and n-hexane, obtaining the material functionalized with amino groups that were labelled as NITQ-2.[56,60]

Preparation of Zr-Beta zeolite

Nanocrystalline zeolite Beta seeds were synthesized following the procedure described in the bibliography.[61,62] A synthesis gel of molar composition 1.0 SiO2:0.56 TEAOH:0.02 Al2O3: 6.5 H2O crystallized in a Teflon-lined stainless steel autoclave at 140 ^oC for 72 h. The product was separated by centrifugation, washed with deionized water, and dried in air at 100 ^oC. One gram of the as-made sample was treated with 50 mL of 6 M HNO3 at 80 ^oC for 24 h to remove the aluminium. The solid was recovered by filtration, washed with deionized water, and dried at 100 ^oC.

The synthesis of zeolite Zr-Beta was done according with the bibliography.[30,63] Typically, Al-free Zr-Beta zeolite was synthesized in a fluoride medium. Tetraethylorthosilicate (TEOS) was hydrolyzed in an aqueous solution of 35 % tetraethylammonium hydroxide (TEAOH) under stirring. A solution of $ZrOCl2{\cdot}8H_2O$ in water was added and the mixture was stirred until the ethanol formed by hydrolysis of TEOS was evaporated. HF was added to the clear solution and a thick paste was formed. Finally, an aqueous suspension of the dealuminated zeolite Beta seeds was added. The final gel composition was 1.0 SiO_2: 0.008 ZrO_2: 0.54 TEAOH: 7.5 H_2O: 0.54 HF. Crystallization was carried out in a Teflon-lined stainless steel autoclave at 140 ^oC for 14 days. The solid product obtained was filtered, washed with deionized water, dried at 100 ^oC and calcined at 580 ^oC for 3 h. The amount of Zr (1% wt) on the catalysts was measured by ICP AES analysis, the Brunauer-Emmett-Teller surface area was determined showing that the zeolite has 475 m^2/g and the micropore volume 0.22 m^3/g.

Immobilization of Alcohol dehydrogenase

The immobilization of the enzyme ADH(S) and ADH(R) was performed as follows: 10 mg of ADH (R or S) was dissolved in 5 mL of phosphate buffer solution 100 mM (PBS) pH=5.5, subsequently, 200 mg of NITQ-2 was added and left in closed flask under gentle agitation for 24 hours. After this time, to determine the amount of protein immobilized, the protein in the supernatant was analyzed by bicinchoninic acid protein test. To do that, 2 mL of the bicinchoninic acid test solution was added to 0.1 mL sample and incubated 30 minutes at 37 ^oC and then the absorbance at $\lambda_{562\ nm}$ was measured.[64] Finally, the biocatalyst was recovered by centrifugation, washed thoroughly and stored at 4 ^oC.

Catalytic Experiments

Biocatalyst reactions in batch mode

Alcohol dehydrogenase reactions were done by incubating the immobilized enzyme ADH(S)@NITQ-2 with the ketone (2-octanone) (0.3 mmol) (30 mM), the cofactor (NAD^+) (molar ratio substrate/cofactor = 10), in 10 mL of solvent (Isopropanol/phosphate buffer solution, pH 7, 100 mM) (50/50 v/v), at 25 ^oC while stirring at 250 rpm. For ADH(R)@NITQ-2 reactions, the cofactor used was ($NADP^+$) in a molar ratio substrate/cofactor = 10, in 10 mL of solvent (Isopropanol/phosphate buffer solution, pH 7, 100 mM and MgCl2 1 mM (50/50 v/v). After reaction, the enzymatic derivative was separated from the mixture by centrifugation at 6000 rpm during 5 minutes, and washed with phosphate buffer (pH 7) thoroughly and stored at 4 ^oC until use. It is interesting to point out that in all cases, and for economic reasons, we used the cofactor in the oxidized form (NAD+ and

NADP+) which in the presence of isopropanol and ADH is rapidly reduced into the required cofactor (NADH or NADPH).
The reaction mixture was extracted using n-hexane and dried with anhydrous MgSO4. Then organic phase was analysed on a Varian 3900 gas chromatograph equipped with a capillary column HP-5 (30 m × 0.25 mm × 0.25 µm) and flame ionization detector (FID). Dodecane was used as external standard. The molar balance was in all cases > 97 %.
The identification of the products was carried out by GC-MS on an Agilent 5973 Network Mass selective Detector equipped with a capillary column HP5-MS Ultrainert (30 m × 0.25 mm × 0.25 µm) and FID detector. The retention time and GC-MS spectra of the alcohols produced were compared with that of the pure commercial alcohols.
Enantiomeric excess of the secondary alcohol was determined on a Varian 3900 gas chromatograph equipped with a (Supelco β-Dex225 30 m x 0.25 mm x 0.25 µm) and FID detector, previous derivatization of the alcohols with trifluoroacetic anhydride following the method described in the literature.[65]
Typically, in a solution of alcohol (5 mmol), triethylamine (6 mmol), and 4-(dimethylamino)pyridine (0.5 mmol) in CH_2Cl_2 (5 mL) was added trifluoroacetic anhydride (6.2 mmol) at 0 ^{o}C. After the mixture was stirred at 0 ^{o}C for 2 h and then at room temperature for 2 h, H_2O was added to the mixture and organic phase was separated. The aqueous phase was extracted with CH_2Cl_2 (four times) and the combined organic phase was dried over MgSO4 and analysed by GC with Supelco β-Dex column. The identity of the chiral products was verified by comparing the retention times with the pure chiral commercial alcohols, under the same derivatization conditions.

Activity recovery determination

Activity recovery was determined according to literature,[57] 2-octanone (30 mM), the cofactor (NAD^+) 3 mM, in 10 mL of solvent (Isopropanol/phosphate buffer solution, pH 7, 100 mM) (50/50 v/v) was submitted to reduction using 10 mg of free ADH(S). The activity was measured and expressed as the rate of S-2-octanol produced (µmol·min^{-1}). Then, 10 mg of ADH(S) were put in contact with 200 mg of NITQ-2 during 24 hours as was described above. After that the solid was recovered by centrifugation and the activity of the total amount of immobilized enzyme was determined under the same conditions as the free enzyme. Activity recovery is expressed as a percentage: (activity of immobilized enzyme/activity of free enzyme) x 100.

Determination of catalytic activity in function of pH and thermal stability

The effect of the pH on the catalytic activity of the ADH-S free and immobilized form were determined by incubating the enzyme during 1 hour in the presence of the substrate (2-octanone) in the same conditions that we describe above, in a pH range from 5.5 to 10. The thermal stability of the enzyme was determined by heating during 1 hour the free and the immobilized ADH-S at temperatures between 25 and 55 ^{o}C. Subsequently the cofactor and the substrate (2-octanone) were added and the reaction was carried out under the reaction conditions describe above.

Oxidation of racemic alcohols using Zr-Beta in batch mode

Typically, in a glass reactor were placed 0.4 mmol of (rac)-2-octanol in 1.5 mL of acetone (hlpc grade) and 32 mg of Zr-Beta zeolite (1 wt %). The solution was heated at 50 °C during 24 h while stirring at 750 rpm. Finally, the catalyst was removed by centrifugation 6000 rpm during 5 min. The reaction was followed taking samples at regular periods that were analysed by Varian 3900 gas chromatograph equipped with a capillary column HP-5 (30 m × 0.25 mm× 0.25 µm) and FID detector. Dodecane was used as external standard. The molar balance in all cases was > 96 %. The identification of the products was carried out by GC-MS on an Agilent 5973 Network Mass selective Detector equipped with a capillary column HP5-MS Ultrainert (30 m × 0.25 mm × 0.25 µm) and FID detector. The retention time and GC-MS spectra of the ketones produced were compared with that of the pure commercial ketones.

Enzymatic reduction of prochiral ketone in a fixed bed reactor

The experiments in flow reactor were carried out as follows: a dry sample of ADH@NITQ2 (209 mg) was diluted with silica (SiO_2 ≥ 0.25 µm, 1.8 g) and packed in a stainless steel reactor (diameter 1 mm, 35 mm height). The catalyst dilution with inert solid particle, having the same size of the catalytic particles, reduces the local hot-spots and improves the temperature distribution along the catalytic bed. The dilution of the catalyst was taken following the literature,[66] where is recommended that the volume of the bed (catalyst plus inert) has to fit in a range depending on the diameter and length of the reactor used. Temperature was fixed at room temperature for all experiments, and the reactor was coupled with a peristaltic pump for feeding. To evaluate the contact time increasing concentrations of 2-octanone in isopropanol/phosphate buffer (pH 7, 100 mM) (50/50 v/v) were used as feed, while keeping constant the temperature, the flow rate (0.55 mL/h), and the molar ratio (substrate/NAD^+ = 10). The contact time has been calculated as the inverse of Space velocity WSHV (h^{-1}) which is defined as: WSHV (h^{-1})=limiting reactant (g·h^{-1})/gram of catalyst.

Oxidation of racemic alcohol in a fixed bed reactor

The continuous experiments were carry out as follows: 304 mg of dry catalyst Zr-Beta (0.2-0.4 µm) was diluted with silica (SiO_2 ≥ 0.25 µm, 1.7 g) and packed in a stainless steel reactor (diameter 1 mm, 35 mm height) coupled with an electric heater controller and a peristaltic pump for feeding the reactor. The temperature was fixed at 50 ^{o}C and stability and contact time conditions were evaluated. For this propose, increasing concentrations of 2-octanol in acetone were used as feed, while keeping constant the flow rate (0.5 mL/h).

Coupling Oxidation-Reduction in a flow reactor

The coupling of both reactors were carried out as follows: the oxidation of 2-octanol was done using 304 mg of dry catalyst Zr-Beta (0.2-0.4 μm) diluted with silica (SiO_2 ≥ 0.25 μm; 1.7 g) and packed in a stainless steel reactor (diameter 1 mm, 35 mm height) coupled with an electric heater controller and a peristaltic pump for feeding the reactor, the temperature was fixed at 50 °C. The reactor was feed with a solution of 2-octanol in acetone (191 mmol/L) with a flow of 0.5 mL/h and contact time 25 h. After that, the solution of 2-octanone obtained after the first reactor (10 mL) was diluted with isopropanol/phosphate buffer (pH 7, 100 mM) (50/50 v/v) until a volume of 62 mL (which contains 16 v/v% acetone) (Entry 1, Table 4). To this solution of 2-octanone (30 mM) the cofactor (NAD^+) (3 mM) was added and used for feeding the second reactor (where was the ADH(S)@NITQ-2) at a flow of 0.55 mL/h.

In a second experiment, the amount of acetone of the solution obtained after the first reactor was reduced by evaporation up to 8 v/v %, and then proceeded as above (entry 2 Table 4). Finally, in the last experiment the acetone was completely evaporated from the solution obtained from the first reactor and proceeded as above (entry 3, Table 4).

Analytical methods

Infrared analysis of the support was performed with a IR Vertex Burker DTGS (Detector), and a conventional quartz infrared cell Quartz KR55 windows connected to a vacuum dosing system. The samples were pressed into self-supporting pellets and treated under vacuum (10^{-4} to 10^{-5} Pa) at 400 °C for 24 hours.

The specific surface areas of the supports were calculated by the Brunauer-Emmett-Teller (BET) method by means of nitrogen adsorption at -196 °C, using an ASAP 2420 (V2.09 J). Nitrogen adsorption isotherms of ITQ-2, NITQ-2 and ADH8.6mg@NITQ-2 are presented in Figures S11-13 respectively, and the pore size distribution of ITQ-2 and ADH8.6mg@NITQ-2 are presented in Figure S14.

Elemental analysis (Table S1) were performed in a Euro EA3000 Elemental Analyzer (EuroVector), using sulfanilamide as reference.

Isoelectronic point of ADH(S) was determined according with the literature.[67] Typically, 10 mg ADH(S) were dissolved in 5 mL of phosphate buffer pH from 3 to 7 (100 mM), then the solutions were stirred at 450 rpm for 1 h at 50 °C, the precipitated protein was recovered by centrifugation 10 min at 6000 rpm. Finally, the samples were dried at 60 °C for 24 h and the amount of precipitated protein was determinate by analytical balance.

The metal contents in the samples and metal leaching were analysed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Varian 715-ES.

Conclusions

A new process for producing chiral alcohols starting from racemic mixtures has been developed. The process involves two catalytic steps. In the first one, the alcohol is oxidized to a prochiral ketone using acetone as hydrogen acceptor with a very efficient solid Zr-Beta catalyst. In the second step the ketone is stereoselectively reduced with an alcohol dehydrogenase immobilized in a 2D zeolite. The process can be carried out within two consecutive CSTR reactor system or with a two fixed beds continuous reactor. In both cases conversions above 95 % with ~100 % selectivity to the desired R or S alcohol is obtained. This combined heterogeneous chemical/enzymatic catalytic process can be applied with excellent results to a large variety of racemic alcohols. The catalyst is very stable and the two beds continuous system has been operated for more than 16 days without deactivation.

Conflicts of interest

There are no conflicts to declare

Acknowledgements

The research leading to these results has received funding from the Spanish Ministry of Science, Innovation and Universities through “Severo Ochoa” Excellence Programme (SEV-2016-0683) and the PGC2018-097277-B-100(MCIU/AEI/FEDER, UE) project. J.M.C thanks to Universitat Politécnica de Valencia for a predoctoral fellowship.

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Supporting Information

Production of chiral alcohols from racemic mixtures by integrated heterogeneous chemoenzymatic catalysis in fixed bed continuous operation

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Table S1. Elemental analysis of the support

Material	N%	H%	C%
ITQ-2	-	0.92	0.31
N-ITQ2	2.5	2.21	9.96

Table S2. Surface area of ITQ-2 zeolite, surface modified NITQ-2 and ADH@NITQ-2 catalyst.

Material	BET (m^2/g)	External surface area (m^2/g)
ITQ-2	756	592
NITQ-2	387	347
ADH@NITQ-2	219	96

Table S3. Evaluation of the contact time in flow reactor for the reduction of 2-octanone to (S)-2-octanol.

mmol/L	CT (h)	(%) Conversion (Selectivity)
30	4.0	97(100)
60	2.0	96(100)
94	1.3	90(100)
114	1.1	85(100)
160	0.8	54(100)

Conditions: ADH(S)@NITQ-2 (208.6 mg), NAD^+, (molar ratio substrate/cofactor = 10), in 10 mL of solvent (Isopropanol/phosphate buffer solution pH 7 100 mM (50/50 v/v), at 25 °C, flow 0.55 mL/h.

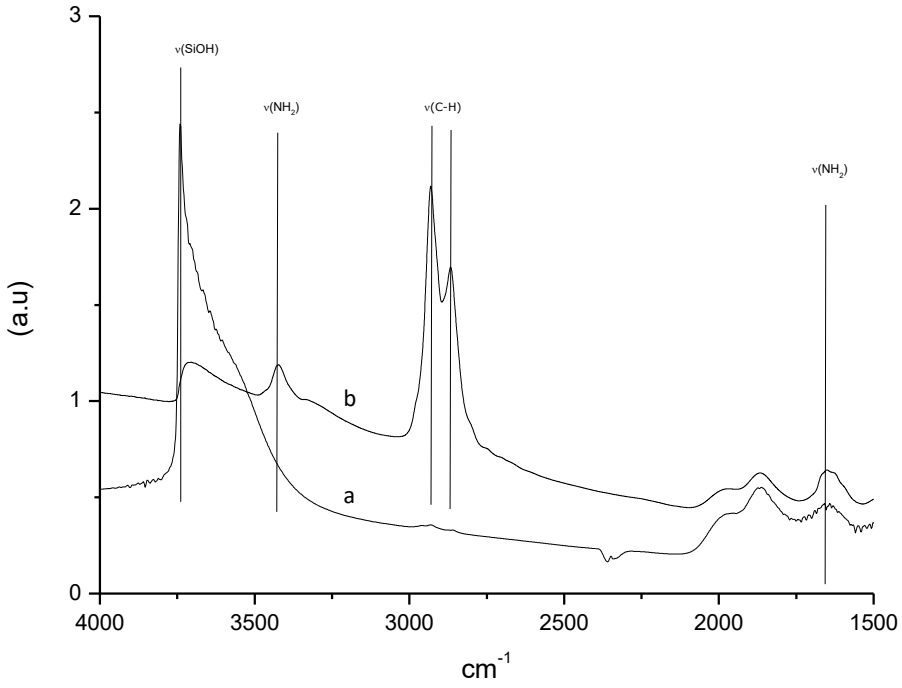

Figure S1. FTIR spectra of ITQ-2 pure silica (a); NITQ-2 (b)

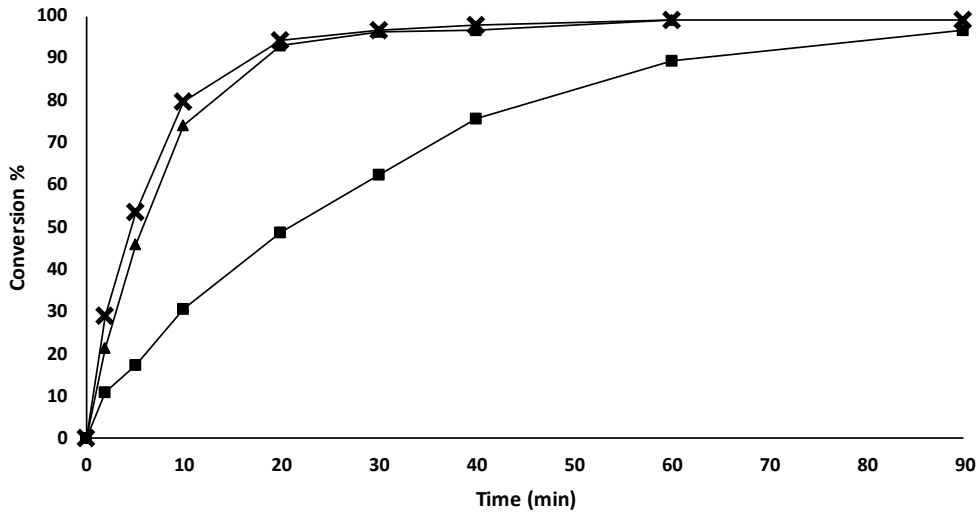

Figure S2. Catalytic activity of ADH(S)@NITQ-2 with different loadings in the conversion of 2-octanone into (S)-2-octanol. (✖) ADH8.6mg@NITQ-2, (▲) ADH7.5mg@NITQ-2, (■) ADH2.5mg@NITQ-2. Reaction conditions: 2-octanone, 0.3 mmol (30 mM), NAD^+, 0.03 mmol (molar ratio substrate/cofactor = 10), in 10 mL of solvent (Isopropanol/phosphate buffer solution pH 7 100 mM (50/50 v/v).

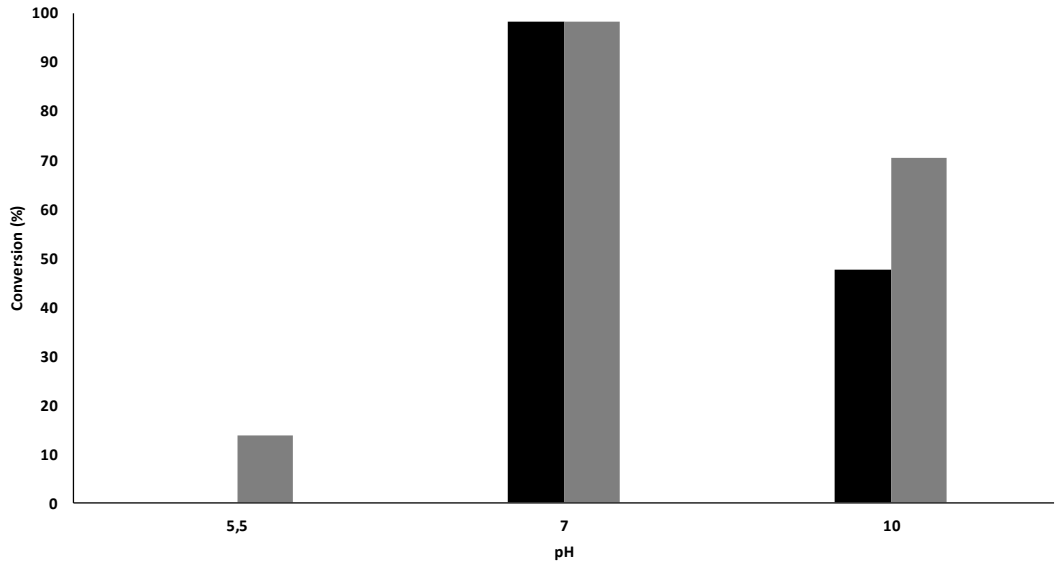

Figure S3. Effect of pH on the activity of ADH in the reduction of 2-octanone into (S)-2-octanol. (■) ADH(S) free, (■) ADH(S)@NITQ-2. Reaction conditions: ADH(S) (8.6 mg) or ADH(S)@NITQ-2 (208.6 mg), 2-octanone, 0.3 mmol (30 mM), NAD^+, 0.03 mmol (molar ratio substrate/cofactor = 10), in 10 mL of solvent (Isopropanol/phosphate buffer solution of pH 5.5, 7 and 10 (50/50 v/v), 1 h, at 25 °C.

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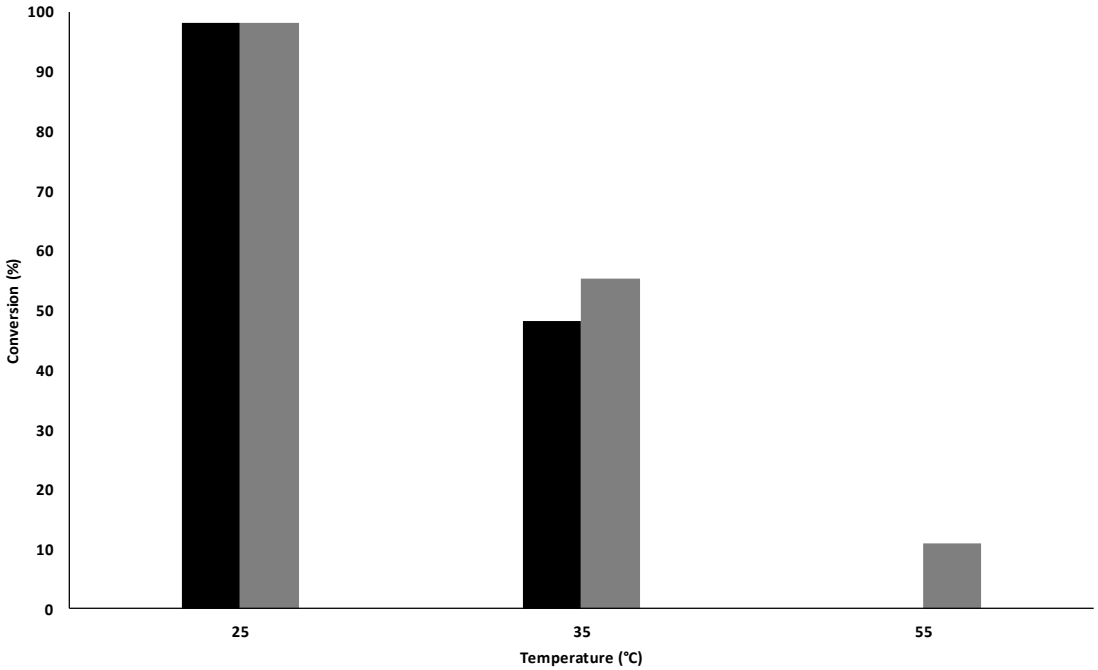

Figure S4. Thermal stability of the immobilized or free enzyme ADH(S). (■) ADH(S) free y (■) ADH(S)@NITQ-2. Reaction conditions: ADH(S) (8.6 mg) or ADH(S)@NITQ-2 (208.6 mg), 2-octanone, 0.3 mmol (30 mM), NAD^+, 0.03 mmol (molar ratio substrate/cofactor = 10), in 10 mL of solvent (Isopropanol/phosphate buffer solution pH 7 (50/50 v/v), 1 h, at 25 °C.

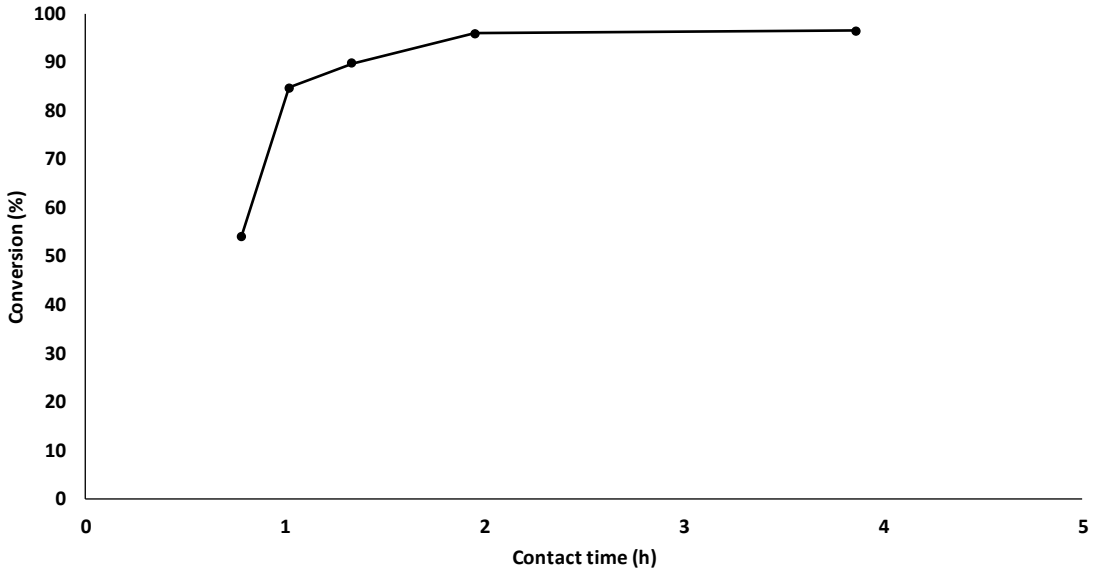

Figure S5. Evaluation of the contact time in flow reactor for the reduction of 2-octanone to (S)-2-octanol. Reaction conditions: ADH(S)@NITQ-2 (208.6 mg), (molar ratio substrate/cofactor = 10), 10 mL of solvent (Isopropanol/phosphate buffer solution pH 7 (50/50 v/v), flow 0.55 mL/h 25 °C.

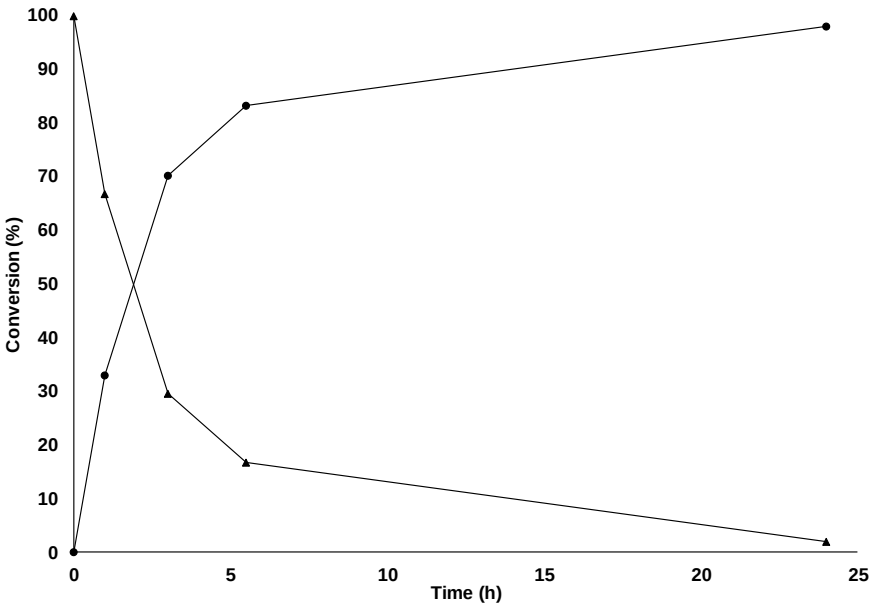

Figure S6. Oppenauer oxidation of rac-2-octanol using Zr-Beta zeolite.(▲) 2-octanol, (●) 2-octanone. Conditions: Zr-Beta (32 mg), 2-octanol (0.4 mmol), acetone (1.5 mL, 20.4 mmol), at 50 ºC.

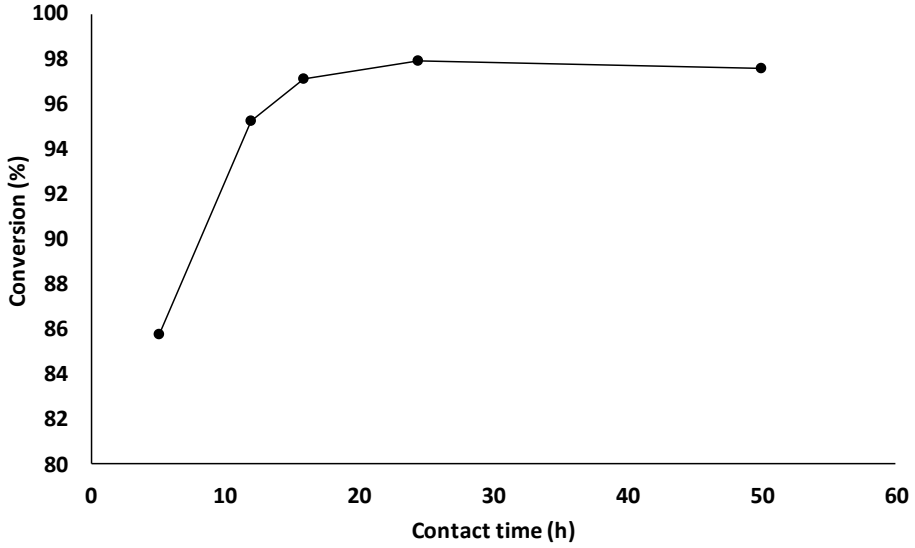

Figure S7. Evaluation of contact time in a continuos oxidation reactor. Reaction conditions: Zr-Beta (304 mg), acetone (10 mL, 136 mmol), flow 0.5 mL/h at 50 ^{o}C.

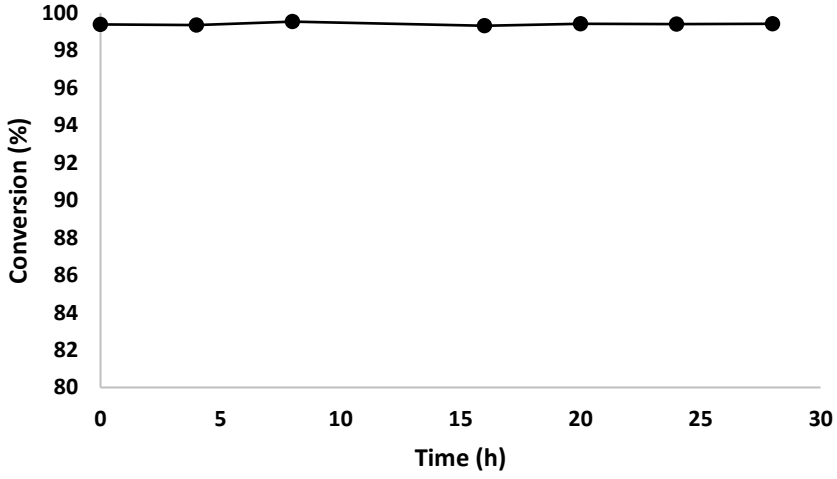

Figure S8. Evaluation of oxidation-reduction of rac-2-dodecanol in continuos reactor. Reaction conditions: First step: Zr-Beta (301 mg), acetone (10 mL, 136 mmol), 2-dodecanol 126 mmol/L, flow 0.5 mL/h at 50 ^{o}C. Second step: ADH(S)@NITQ-2 (208.6 mg), 2-dodecanone (30 mmol/L), NAD^{+} (3 mmol/L), solvent (Isopropanol/phosphate buffer solution pH 7 (50/50 v/v), flow 0.55 mL/h, 25 °C.

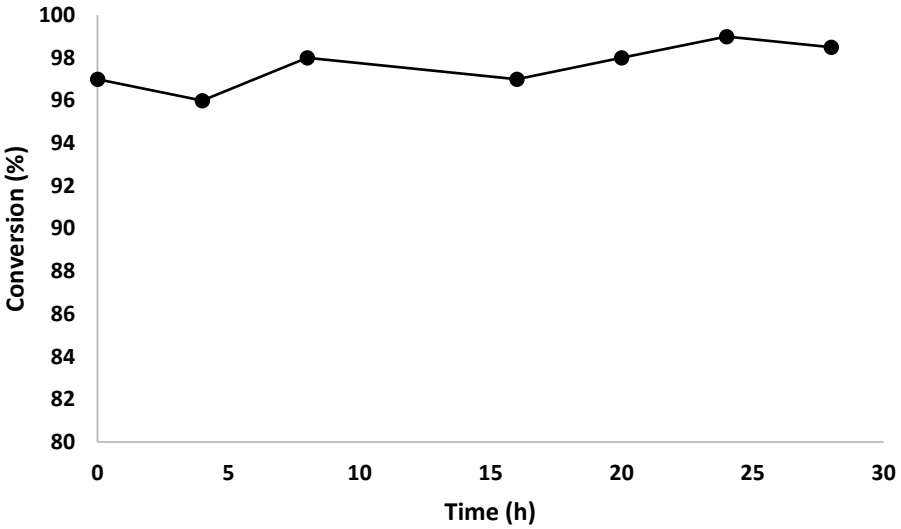

Figure S9. Evaluation of oxidation-reduction of rac-1-cyclohexylethanol in continuos reactor. Reaction conditions: First step: Zr-Beta (610 mg), acetone (10 mL, 136 mmol), rac-1-cyclohexylethanol 123 mmol/L, flow 0.2 mL/h at 50 ^{o}C. Second step: ADH(S)@NITQ-2 (208.6 mg), rac-1-cyclohexylethanone (30 mmol/L), NAD^{+} (3 mmol/L), solvent (Isopropanol/phosphate buffer solution pH 7 (50/50 v/v), flow 0.55 mL/h, 25 °C

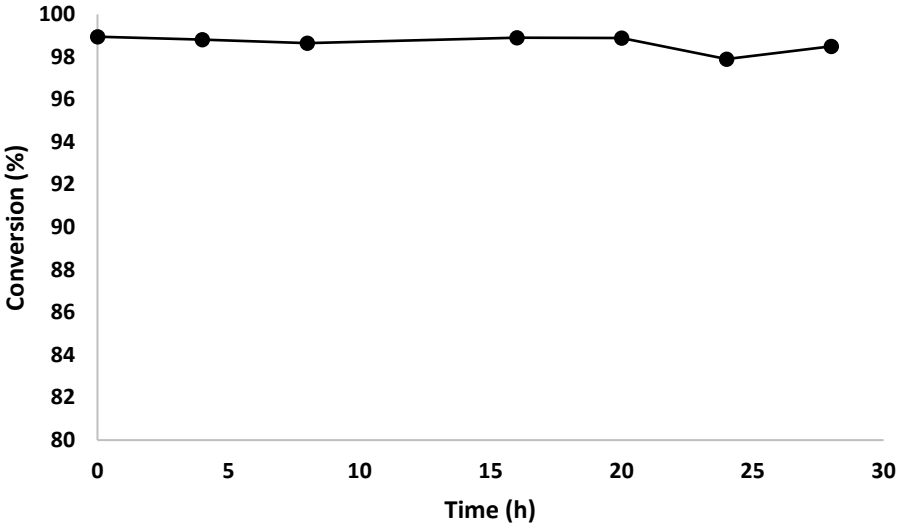

Figure S10. Evaluation of oxidation-reduction of rac-4-biphenylmethylcarbinol in continuos reactor. Reaction conditions: First step: Zr-Beta (610 mg), acetone (10 mL, 136 mmol), rac-4-biphenylmethylcarbinol 80 mmol/L, flow 0.2 mL/h at 50 ^{o}C. Second step: ADH(S)@NITQ-2 (208.6 mg), 4-acethylbiphenyl (30 mmol/L), NAD^{+} (3 mmol/L), solvent (Isopropanol/phosphate buffer solution pH 7 (50/50 v/v), flow 0.55 mL/h, 25 °C.

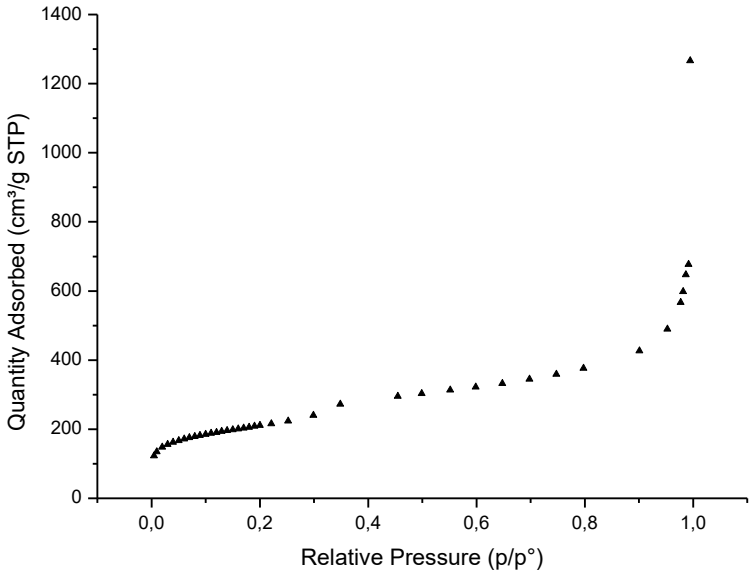

Figure S11. Nitrogen adsorption isotherm of ITQ-2 zeolite

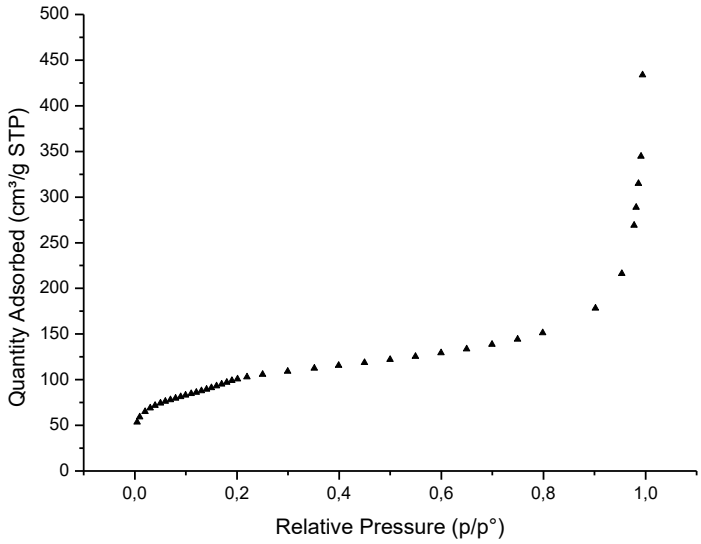

Figure S12. Nitrogen adsorption isotherm of NITQ-2 zeolite

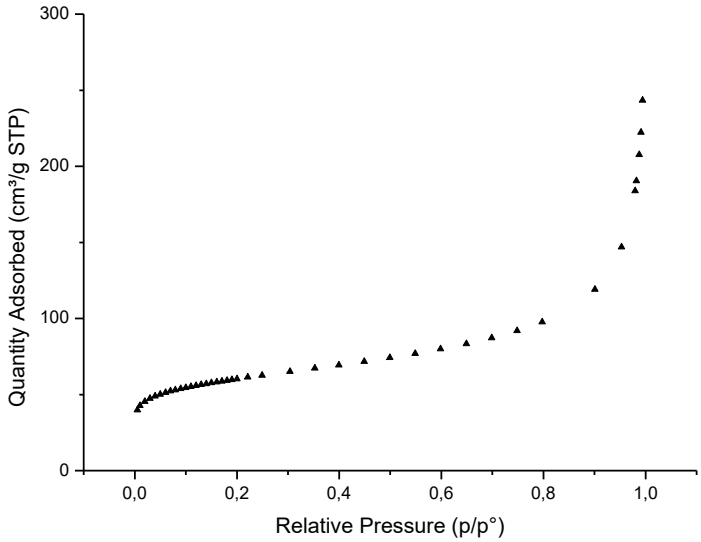

Figure S13. Nitrogen adsorption isotherm of ADH@NITQ-2 zeolite

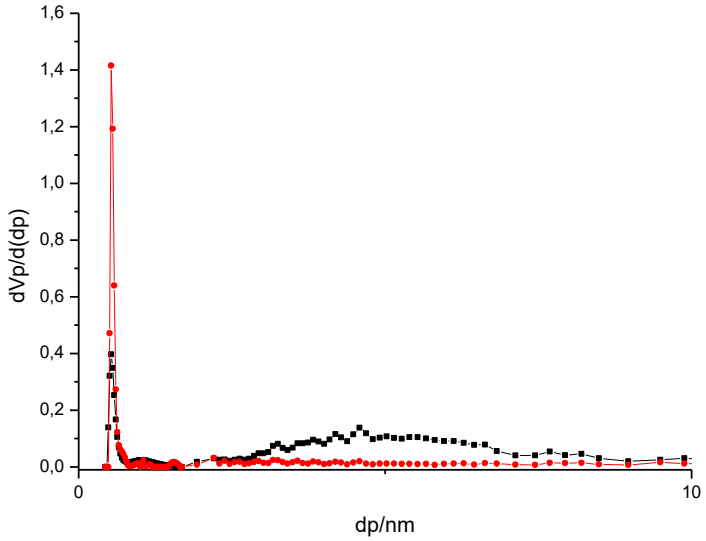

Figure S14. Pore volume distribution of ITQ2 zeolite (red) and ADH@NITQ2 (black)