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Reusable ω-transaminase sol–gel catalyst for the preparation of amine enantiomers

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ABSTRACT

Heterogeneous ω-transaminase sol–gel catalysts were prepared and characterized in terms of immobilization degree, loading capacity and catalytic behavior in the kinetic resolution of racemic 1-phenylethylamine (a model compound) with sodium pyruvate in phosphate buffer (pH 7.5). The catalyst obtained when ω-transaminase from *Arthrobacter* sp. was encapsulated from the aqueous solution of the enzyme, isopropyl alcohol and polyvinyl alcohol in the sol–gel matrices, consisting of the 1:5 mixture of tetramethoxysilane and methyltrialkoxysilane, proved to be optimal including the reuse and storage stabilities of the catalyst. The optimized immobilizate was shown to perform well in the kinetic resolution of four structurally different aromatic primary amines in aqueous DMSO (10, v/v-%). The enzyme preparation showed synthetic potential by enabling the catalyst reuse in five consecutive preparative scale kinetic resolutions using 100 mM 1-phenylethylamine in aqueous DMSO (10, v/v-%). It was typical to fresh catalyst preparations that the kinetic resolution tended to exceed 50% before the reaction stopped leaving the (*S*)-amine unreacted while thereafter in reuse the reactions stopped at 50% conversion as expectable to highly enantioselective reactions.

1. Introduction

Importance of enantiopure amines in pharmaceutical and fine chemical industry has led to efforts to establish viable synthesis methods for their production. The methods include highly effective reactions with enzymes as chiral and green chemistry catalysts [1–7]. Among such approaches, the utilization of ω-transaminase (EC 2.6.1.x) enzymes has recently attracted considerable attention [3–7], the highlight being the application of the engineered (*R*)-enantioselective enzyme derived from *Arthrobacter* sp. KNK168 in sitagliptin (Diabetes II drug) manufacture [8–10]. This and other (*R*)-selective enzymes are of high importance as ω-transaminases used to be predominantly (*S*)-selective [11–13]. ω-Transaminases are unique pyridoxal-5′-phosphate (PLP)-dependent enzymes which catalyze the transfer of an amino group from an amine donor to a ketone acceptor without the requirement of an adjacent carboxylic acid moiety, which is obligatory with other transaminases. The enzymes show relatively broad substrate specificity, enabling the kinetic resolution of racemic amines [theoretical yield 50%, an (*R*)-selective enzyme producing (*S*)-amines as unreactive enantiomers] as well as asymmetric synthesis [theoretical yield 100%, the same enzyme now yielding (*R*)-amines] [3]. In addition, a two enzyme process has been described for deracemization of racemic amines with 100% theoretical yields [14].

Thanks to computational methods combined with rational design and mutagenesis procedures both (*R*)- and (*S*)-selective ω-transaminases are available today. Moreover, possibilities to transform enzyme powders into heterogeneous immobilized catalysts have been investigated to enable the catalyst reuse in successive reactions and to improve both storage and operational stability of the catalysts when used under harsh reaction conditions of organic synthesis. In order to choose an immobilization method, the particularities of an enzyme and its eventual synthetic application need consideration [15,16]. Mostly cells containing the desired ω-transaminase have been immobilized, the methods used including encapsulation into calcium alginate beads [17,18] and a polyvinylalcohol hydrogel (LentiKats®) [19] as well as incorporation into a chitosan precipitate [20]. ω-Transaminases have also been covalently attached on chitosan beads [21,22] and entrapped into sol–gel matrices [23,24]. The recent advances in using ω-transaminase catalysis in organic solvents rather than in aqueous solutions have provided new opportunities to utilize lyophilized ω-transaminases as such and when adsorbed on hydrophobic supports [25–27].

The studies herein aimed for the preparation of a reusable, enantioselective and robust ω-transaminase catalyst for the production of primary amine enantiomers. The sol–gel entrapment of the

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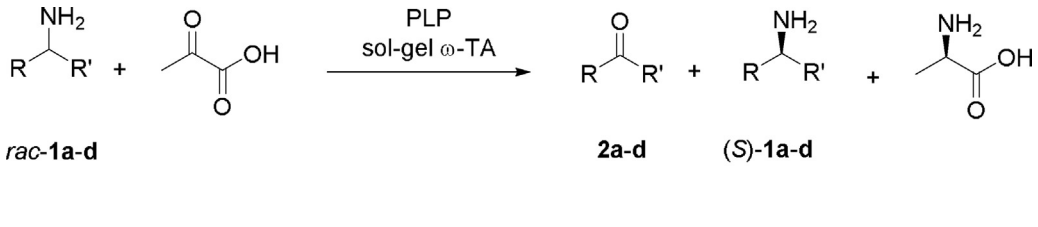


Scheme 1. Kinetic resolution of *rac*-**1a–d** using sol–gel entrapped ω-transaminase.

(*R*)-selective ω-transaminase from *Arthrobacter* sp. KNK168 (commercially available as ATA-117) was the method of choice due to our previous good experiences with lipase immobilizations [28,29]. The focus herein was on improving operational and storage stability of the enzyme. The ω-transaminase sol–gel catalysts prepared were characterized in terms of immobilization degree, loading capacity and ability to catalyze the kinetic resolution of racemic 1-phenylethylamine (*rac*-**1a**, a model compound) in successive reactions. We chose to study kinetic resolution to keep the reaction system as simple as possible, as means to shift the enzymatic reaction equilibrium toward the side of the products is required in asymmetric synthesis. Substrate scope of the optimized catalyst was studied using *rac*-**1a**–**d** as substrates (Scheme 1). Finally, the synthetic potential of the optimized catalyst was investigated performing five successive kinetic resolutions with the enzyme preparation and separating the unreacted (*S*)-**1a** from the combined resolution mixtures.

2. Materials and methods

2.1. Materials

ω-Transaminase powder (ATA-117) was a product of Codexis, and it was used as received in immobilizations. Solvents, amines, sodium pyruvate, isopropyl alcohol (IPA), poly(vinyl alcohol) (PVA, M_w 9000–10,000) and Celite (Celite analytical filtering aid, CAFA) were purchased from Sigma–Aldrich and were used as received.

2.2. Analytical methods

The protein contents of the ω-transaminase powder and the aqueous washing solutions of sol–gels were determined using the bicinchonic acid assay with bovine serum albumin as the standard protein [30,31]. The immobilization degrees of the sol–gel ω-transaminases were calculated as the percentual difference between the protein content of the lyophilized enzyme powder used in the immobilization and the protein content of the aqueous washing solution. The loading capacities of the ω-transaminase sol–gel catalysts were calculated as the percentual difference between the protein content of the entrapped enzyme powder and protein content of the aqueous washing solution divided by the mass of the obtained sol–gel catalyst. The progression of the reactions was monitored by an achiral reverse phase HPLC and the enantiomeric excess values of the amines **1a**–**d** by a chiral GC. The columns, separation conditions and retention times are presented in Table 1. Optical rotations were determined with a Perkin-Elmer 341 polarimeter and the $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. All enzymatic reactions were performed at 30 °C.

2.3. Preparation of ω-transaminase sol–gel catalysts

Phosphate buffer (0.1 M, pH 7.5, 390 μL or 490 μL producing a sol–gel/water ratio of 1:10) was added to a vial containing ω-transaminase powder (50 or 100 mg) and possible solid [Celite 50 mg or Celite 50 mg together with sucrose (5 mg)] or liquid additives [IPA (50 μL) and aqueous PVA (4% (w/v), 100 μL)] before the addition of the aqueous NaF (1 M, 50 μL) under effective agitation. TMOS and RTMS (total amount of 3 mmol) were added and the mixture was stirred effectively until heat-formation started (observed as warming of the vessel at hands). The vial was cooled on an ice bath and the mixture was gently shaken until increase in viscosity was observed. Closed vials were stored at room temperature overnight for maturation. ω-Transaminase sol–gel preparations were gently crushed with spatula and sequentially washed (IPA 10 mL, water 10 mL, *n*-hexane 10 mL; procedures are given in Table 2), dried at room temperature for 72 h and stored at 4 °C.

2.4. Kinetic resolution of rac-*1a–d*

All experiments were carried out keeping the protein content constant (8 mg mL^{-1}) if not otherwise stated. One of the substrates *rac*-**1a–d** (50 mM), sodium pyruvate (50 mM) and pyridoxal-5′-phosphate monohydrate (0.2 mg mL^{-1}) in phosphate buffer (1 mL, 0.1 M, pH 7.5) containing IPA or DMSO as a possible cosolvent (10, v/v-%) was added to a 2 mL Eppendorf tube, containing the ω-transaminase sol–gel catalyst (25 or 50 mg). The reaction was shaken (170 rpm) at 30 °C. After 24 h the reaction was stopped by centrifuging the mixture and removing the solution by pipette. The solid catalyst was reused while conversion was monitored by taking a sample (5 μL) from the solution and diluting it with the HPLC eluent (500 μL). The sample was filtered and analyzed for conversion by HPLC. A sample (400 μL) for $ee^{(S)-1}$ analysis was taken, and aqueous NaOH (2 M, 50 μL) was added followed by the extraction of the amine into ethyl acetate (400 μL). The organic phase (300 μL) was dried with Na_2SO_4, and after filtration the amine in the sample (200 μL) was derivatized with acetic anhydride (10 μL) to determine the enantiomeric excess of (*S*)-**1** by GC.

2.5. Preparation of (S)-*1a*: reuse of ω-transaminase sol–gel catalyst

Phosphate buffer (4 mL, 0.1 M, pH 7.5, DMSO, 10 (v/v-%)) containing *rac*-**1a** (100 mM), sodium pyruvate (100 mM) and pyridoxal-5′-phosphate monohydrate (0.2 mg mL^{-1}) was added to a reaction vessel containing the optimized ω-transaminase sol–gel catalyst (200 mg). The reaction was shaken (170 rpm) at 30 °C. After 24 h the enzyme preparation was recovered by centrifuging the reaction mixture and the solution was replaced by a fresh solution of substrates. After five consecutive reaction cycles aqueous HCl (6 M) was added to the collected reaction solutions before extraction with dichloromethane (10 mL × 4) to remove acetophenone. Addition of aqueous NaOH (6 M) to the solution was followed by extraction with dichloromethane (15 mL × 4). The organic phase was dried with Na_2SO_4, evaporation yielding (*S*)-1-phenylethylamine in 41% yield (*ee* = 98%, $[\alpha]_D^{25}$ = 29.5 (c 1, $CHCl_3$) {lit. [28] $[\alpha]_D^{25}$ = 30.6 (c 1, $CHCl_3$ *ee* > 99%}.

3. Results and discussion

3.1. Catalyst optimization

Based on the sol–gel immobilization method of Reetz and his coworkers, the (*R*)-selective ω-transaminase (ATA-117) was entrapped into SiO_2 matrices [32,33]. The method exploits fluoride ion catalysis in the hydrolysis and subsequent cross-linking condensation of tetramethoxysilane [$Si(OMe)_4$, TMOS] and an alkyltrialkoxysilane [$RSi(OMe)_3$, R being a non-hydrolyzable alkyl moiety] mixtures in the presence of the enzyme and possible additives. RTMS provides hydrophobicity to the microenvironment of the entrapped enzyme and to the external surface of the catalyst facing to the reaction medium, allowing an easy fine-tuning of physical and chemical properties of the matrix for a given reaction

Table 1
Retention times and analysis conditions for *rac*-**1a**–**d** and **2a**–**d**.

Entry	Compound	t_R (min)	Conditions for analysis
1	*rac*-**1a**	1.8	Zorbax Eclipse XDB-C18 (4.6 mm × 159 mm, 5 μm) achiral HPLC column, a mixture of phosphate buffer (25 mM, pH 7) and acetonitrile (40:60, v/v)
	2a	2.5	
2	*rac*-**1a**	12.4/13.6[a]	Chrompack CP-Chirasil-DEX CB (25 m × 0.25 mm) chiral GC column at 140 °C
3	*rac*-**1b**	1.7	Zorbax Eclipse XDB-C18 (4.6 mm × 159 mm, 5 μm) achiral HPLC column, a mixture of phosphate buffer (25 mM, pH 7) and acetonitrile (40:60, v/v)
	2b	2.9	
4	*rac*-**1b**	6.7/7.1[a]	Chrompack CP-Chirasil-DEX CB (25 m × 0.25 mm) chiral GC column at 140 °C
5	*rac*-**1c**	1.7	Zorbax Eclipse XDB-C18 (4.6 mm × 159 mm, 5 μm) achiral HPLC column, a mixture of phosphate buffer (25 mM, pH 7) and acetonitrile (40:60, v/v)
	2c	2.9	
6	*rac*-**1c**	19.8/20.8[a]	Chrompack CP-Chirasil-DEX CB (25 m × 0.25 mm) chiral GC column at 150 °C
7	*rac*-**1d**	1.8	Zorbax Eclipse XDB-C18 (4.6 × 159 mm, 5 μm) achiral HPLC column, a mixture of phosphate buffer (25 mM, pH 7) and acetonitrile (40:60, v/v)
	2d	2.3	
8	*rac*-**1d**	35.2/39.3[a]	Chrompack CP-Chirasil-DEX CB (25 m × 0.25 mm) chiral GC column at 140 °C

[a] Retention times for enantiomers (*S*)/(*R*).

system [15,16,32–34]. The sol–gel immobilizate obtained is often gently crusted and utilized in an easy to handle powdered form.

Enzyme entrapment using the [TMOS]/[MeTMS] ratio of 1:5 was previously optimal for lipase immobilization [28,29,32,33]. The ratio of 1:5 with the 3 mmol total amount of the silanes and a 50 mg enzyme loading were used as the starting point in the present study. ω-Transaminase sol–gel catalysts without additives were prepared, and the catalysts (50 mg mL^{-1}, protein content of 8 mg mL^{-1}) were subjected to the kinetic resolution of *rac*-**1a** (50 mM) with sodium pyruvate (50 mM) in the presence of pyridoxal-5′-phosphate (0.2 mg mL^{-1}) in phosphate buffer (0.1 M, pH 7.5). The results are shown in Table 2. Washing of the maturated sol–gel with suitable solvents is a pivotal part of catalyst preparation to dispose of methanol produced by silane hydrolysis, the enzyme not entrapped and other soluble material. The protein contents of the ATA-117 powder (86, w/w-%) and the combined washing solutions were determined to obtain immobilization degrees and loading capacities of each catalyst. While the immobilization degrees (90–93%) and loading capacities (12–17%) were relatively little affected by a washing protocol and the nature of a solvent [water, isopropyl alcohol (IPA) and *n*-hexane in different combinations], the effect on the conversion of (*R*)-**1a** into the ketone **2a** in 24 h (used as a rough measure of reactivity) was significant (Table 2). IPA rather than water or *n*-hexane as a final washing solvent was beneficial to conversion and enantioselectivity (entries 3 and 5 vs. 2 and 4). When water was the last solvent used, the catalyst was left visibly moist affecting the loading capacity (12%, entry 4). A considerable decrease in reactivity was evident with *n*-hexane as the last solvent (entry 2). In both cases, the enzymatic enantioselectivity [*E* (enantiomeric ratio) values 19 and 27] was considerably reduced. It is worth to emphasize that the *E* values herein are more or less guiding as something else, in addition to transamination, seems to distort conversion values as is discussed in Section 3.3. For this reason, *E* values are omitted except in Table 2. To conclude, the washing procedure utilizing water and IPA in succession (entry 5) was adopted for further catalyst optimization.

ω-Transaminase sol–gel catalysts with different [TMOS]/[RTMS] ratios (total silane content 3 mmol and enzyme loading 50 mg) were prepared without additives as above. As a purpose to examine catalyst behavior and reuse capacity, each catalyst (protein content of 8 mg mL^{-1}) was applied to the kinetic resolution of *rac*-**1a** in three successive reactions (Table 3). Immobilization degrees decreased from 97% to 91% while the values of loading capacities inconsistently varied between 17% and 14% with [TMOS]/[MeTMS] ratios between 1:1 and 1:7 (entries 1–3 and 6). In spite of this, the loading capacity with the 1:5 [TMOS]/[MeTMS] ratio was reproducible from patch to patch when the catalyst was prepared under the same conditions. The most promising sol–gel catalyst had the ratio of 1:5 giving sufficiently high conversions in 24 h to leave the unreacted (*S*)-**1a** enantiopure in reuse experiments (entry 3). Considerably reduced conversions without any proper reuse possibilities were detected when lower and higher proportions of MeTMS were applied (entries 1, 2 and 6). The ratio of 1:7 was the limit beyond which the MeTMS content cannot be increased in terms of feasible sol–gel formation. The ω-transaminase sol–gel catalysts {50 mg mL^{-1}, ATA-117 (50 mg of the enzyme powder), silanes (3 mmol), [TMOS]/[MeTMS] = 1:5} and {25 mg mL^{-1}, ATA-117 (100 mg of the enzyme powder), silanes (3 mmol), [TMOS]/[MeTMS] = 1:5} gave similar successful kinetic resolutions of *rac*-**1a** in reuse (entries 3 and 5). However, when the first-mentioned catalyst was applied in 25 mg mL^{-1} (protein content of 4 mg mL^{-1}), the catalyst performance was insufficient (entry 4 vs. 3). When the more hydrophobic PrTMS and iBuTMS ([TMOS]/[RTMS] = 1:5) were used as silane precursors in the place

Table 2
Characterization of ω-transaminase sol–gel catalysts[a] (50 mg mL^{-1}) and the kinetic resolution of **1a** (50 mM) with sodium pyruvate (50 mM) in the presence of pyridoxal-5′-phosphate (0.2 mg mL^{-1}) in phosphate buffer (0.1 M, pH 7.5); reaction time 24 h.

Entry	Sol–gel ω-transaminase			Kinetic resolution	
	Washing procedure	Immobilization degree[b] (%)	Loading capacity[c] (%, w/w)	Conversion (%)	*ee* (%) [*E*]
1	–[d]	–	–	52	>99 [>100]
2	IPA, water, IPA, hex	–[e]	–[e]	49	76 [19]
3	IPA, water, IPA	93	16	58	>99 [>100]
4	IPA, water	94	12	38	54 [27]
5	water, IPA	93	17	59	>99 [>100]

[a] The catalyst prepared using ATA-117 (50 mg) and silanes (3 mmol, [TMOS]/[MeTMS] = 1:5).
[b] 100 m(protein content in the sol–gel preparation)/m(protein content in the ATA-117 powder) %.
[c] 100 m(protein content in the sol–gel preparation)/m(ω-transaminase sol–gel catalyst) %.
[d] ATA-117 powder (2 mg mL^{-1}) instead of sol–gel catalyst; reaction time ½ h.
[e] Reliable values were not obtained.

Table 3
ω-Transaminase sol–gel catalysts[a] (50 mg mL^{-1}) in successive kinetic resolutions of **1a** (50 mM) with sodium pyruvate (50 mM) in the presence of pyridoxal-5′-phosphate (0.2 mg mL^{-1}) in phosphate buffer (0.1 M, pH 7.5); reaction time 24 h.

Entry	Sol–gel ω-transaminase				Conversion (%)/*ee* (%)		
	R	[TMOS]/[RTMS]	Immobilization degree (%)	Loading capacity (%, w/w)	Cycle 1	Cycle 2	Cycle 3
1	Me	1:1	97	17	45/45	34/40	28/38
2	Me	1:3	97	14	47/62	40/65	36/63
3	Me	1:5	93	17	59/>99	50/>99	50/>99
4[b]	Me	1:5	93	17	34/44	24/34	22/31
5[b,c]	Me	1:5	93	27	55/>99	50/>99	48/95
6	Me	1:7	91	15	42/57	29/52	26/45
7	Pr	1:5	93	16	39/39	33/41	30/41
8	*i*-Bu	1:5	92	31	60/>99	50/>99	50/>99
9[b]	*i*-Bu	1:5	92	31	51/83	47/83	43/79
10[b,c]	*i*-Bu	1:5	92	42	54/>99	49/>99	50/>99

[a] The catalyst prepared using ATA-117 (50 mg) and silanes (3 mmol, [TMOS]/[MeTMS] = 1:5).
[b] ω-Transaminase sol–gel content 25 mg mL^{-1}.
[c] The catalyst prepared using ATA-117 (100 mg) and silanes (3 mmol, [TMOS]/[RTMS] = 1:5).

of MeTMS, immobilization degrees remained high (entries 7–10). The presence of PrTMS in the catalyst {50 mg mL^{-1}, ATA-117 (50 mg of the enzyme powder), silanes (3 mmol)} resulted in similar loading capacity. On the other hand, reactivity considerable decreased compared to the conversion reached with the catalyst containing MeTMS (entry 7 vs. 3). With iBuTMS, the value of loading capacity increased (entries 8–10) as the condensation reaction was slow and a significant part of the sol–gel mixture was washed off as liquid precursors, leading to low yields of the sol–gel matrix. Keeping the protein content at 8 mg mL^{-1} (corresponding with catalyst content of 25 mg mL^{-1}), slight reactivity decrease and lowered reuse possibilities were observed in the kinetic resolution of *rac*-**1a** (entry 9). When the catalyst content was doubled to 50 mg mL^{-1} (entry 8) or the catalyst was prepared with 100 mg enzyme loading and applied using 25 mg mL^{-1} of the preparation (entry 10), both reactivity and reusability turned excellent. In practice, it is reasonable to use high enzyme loading in immobilization, as then the amount of the sol–gel catalyst preparation can be kept low and the enzyme content is high in the reaction mixture.

Considerable case-specific activity and enantioselectivity enhancements upon introduction of additives in enzyme sol–gel catalysts have been reported [15,16,32–34]. However, any universal tendencies for their effects on the outcome of enzyme sol–gel catalysts have not been possible to conclude. The optimization was continued with efforts to improve the TMOS/MeTMS and TMOS/iBuTMS catalysts {ATA-117 (50 and 100 mg), silanes (3 mmol), [TMOS]/[RTMS] = 1:5} with additives. In these studies we decided to reduce the protein content to 4 mg mL^{-1} in the kinetic resolution of *rac*-**1a** as then possible behavioral changes caused by the additives were easier to monitor. According to the lines found good with our previous lipase sol–gel catalysts [27,28] and to a reported successful ω-transaminase sol–gel Celite preparations [24], Celite with and without sucrose was first added before initiation of gelation. Dramatically reduced conversions and reuse capacities were observed in both cases (Table 4, entries 2 and 3 vs. 1). On the other hand, IPA and PVA, both known to stabilize enzymes in the sol–gel process [33], induced improved conversion values of close to 50% in 24 h in the first cycle and improvements in reuse capacities in comparison with the case without additives (entries 4 and 5 vs. 1). When IPA and PVA were used together the catalytic outcome was somewhat further improved (entry 6 vs. entries 4 and 5). As expected (see data in Table 3), excellent results in terms of conversion and reuse capacity were obtained when 100 mg of the ω-transaminase powder (instead of 50 mg) was immobilized and the protein content used in the kinetic resolution was 8 mg mL^{-1} (Table 4, entry 7). The simultaneous use of IPA and PVA in the corresponding [TMOS]/[iBuTMS] (1:5) sol–gel catalyst

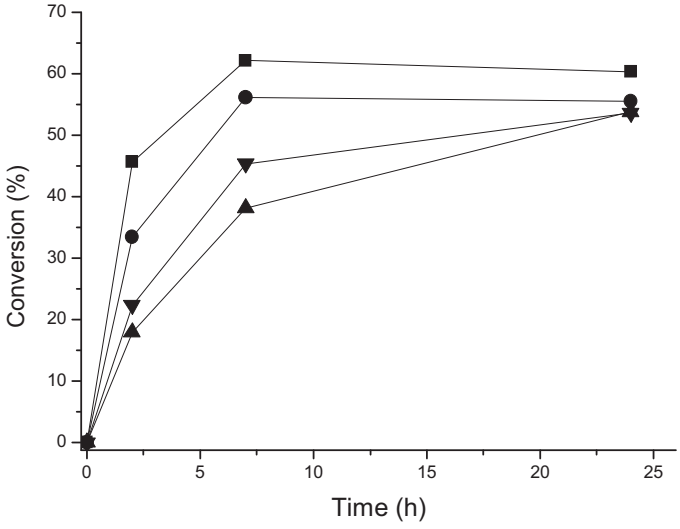


Fig. 1. Progression curves for the ω-transaminase IPA + PVA sol–gel catalyst in the kinetic resolution of **1a** (50 mM) with sodium pyruvate (50 mM) and in the presence of pyridoxal-5′-phosphate (0.2 mg mL^{-1}) in phosphate buffer (0.1 M, pH 7.5): 50 mg mL^{-1} of the catalyst (■), 25 mg mL^{-1} of the catalyst (●), 10% IPA and 25 mg mL^{-1} of the catalyst (▲) and 10% DMSO and 25 mg mL^{-1} catalyst loading (▼).

led to reduced conversions in comparison with the equivalent additive-free catalyst (entries 10 and 11).

Water soluble cosolvents are traditionally used in aqueous reaction media of enzymatic reactions to enhance the solubility of organic molecules. When enzyme sol–gel catalysts are used in aqueous solutions, cosolvents may play an especially important role, alleviating the diffusion of substrates and products in and out of the sol–gel network where the enzyme molecules are entrapped. IPA and dimethyl sulfoxide (DMSO) were studied as cosolvents for the kinetic resolution of *rac*-**1a** (Fig. 1 and Table 4). The presence of the solvents (10, v/v-%) retarded reactivity when compared to that in aqueous buffer although the reactions reached full conversions in 24 h providing the enantiopure (*S*)-**1a** (Fig. 1). The reuse capacity in aqueous DMSO was excellent (Table 4, entry 9). Moreover, the catalyst stored for 4 months at 4 °C and thereafter applied in the kinetic resolution of *rac*-**1a** in aqueous DMSO (10, v/v-%) gave the same result as a freshly prepared catalyst (entry 9), indicating storage stability in addition to reuse stability of the catalyst.

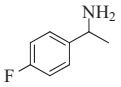
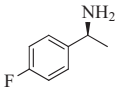
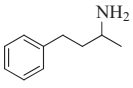
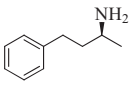
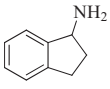
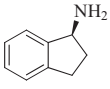
On the basis of the results in Table 4 (entries 7 and 9), the ω-transaminase IPA + PVA sol–gel catalyst {ATA-117 (100 mg), silanes (3 mmol), [TMOS]/[RTMS] = 1:5} was chosen as the optimal catalyst to continue the studies with. This catalyst (25 mg mL^{-1}) was used

Table 4
ω-Transaminase sol–gel catalysts[a] (25 mg mL^{-1}) in successive kinetic resolutions of **1a** (50 mM) with sodium pyruvate (50 mM) in the presence of pyridoxal-5′-phosphate (0.2 mg mL^{-1}) in phosphate buffer (0.1 M, pH 7.5); reaction time 24 h.

Entry	Sol–gel ω-transaminase				Conversion (%)/*ee* (%)		
	R	Additive(s)	Immobilization degree (%)	Loading capacity (%, w/w)	Cycle 1	Cycle 2	Cycle 3
1	Me	–	93	17	34/44	24/34	22/31
2	Me	Celite	93	13	21/18	13/20	14/18
3	Me	Celite + sucrose	93	13	15/11	16/19	20/25
4	Me	IPA	93	15	53/94	40/82	37/68
5	Me	PVA	92	15	46/65	31/49	26/40
6	Me	IPA + PVA	93	16	55/>99	47/91	40/74
7[b]	Me	IPA + PVA	94	26	56/>99	52/>99	51/>99
8[b,c]	Me	IPA + PVA	94	26	54/>99	49/99	49/96
9[b,d]	Me	IPA + PVA	94	26	54/>99	52/>99	50/>99
10	*i*-Bu	–	92	31	51/83	47/83	43/79
11	*i*-Bu	IPA + PVA	90	23	23/23	18/23	17/21

[a] The catalyst prepared using ATA-117 (50 mg) and silanes (3 mmol, [TMOS]/[MeTMS] = 1:5).
[b] The catalyst prepared using ATA-117 (100 mg) and silanes (3 mmol, [TMOS]/[MeTMS] = 1:5).
[c] IPA as a cosolvent (10, v/v-%).
[d] DMSO as a cosolvent (10, v/v-%).

Table 5
The optimized ω-transaminase IPA + PVA sol–gel catalyst[a] (25 mg mL^{-1}) in the kinetic resolution of *rac*-**1a–d** (50 mM) with sodium pyruvate (50 mM) in the presence of pyridoxal-5′-phosphate (0.2 mg mL^{-1}) in phosphate buffer (0.1 M, pH 7.5, 10 (v/v-%) DMSO); reaction time 24 h.

Entry	Substrate	Product	Conversion (%)	*ee* (%)
1			54	>99
2			56	>99
3			54	>99
4			44/64[b]	80/>99[b]

[a] The catalyst prepared using ATA-117 (100 mg) and silanes (3 mmol, [TMOS]/[MeTMS] = 1:5).
[b] ω-Transaminase sol–gel content 50 mg mL^{-1}.

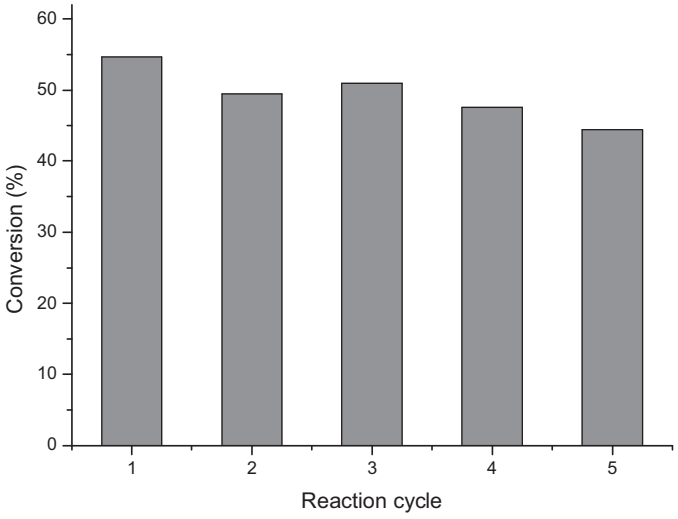


Fig. 2. ω-Transaminase IPA + PVA sol–gel catalyst (50 mg mL^{-1}) in five successive kinetic resolutions of **1a** (100 mM) with sodium pyruvate (100 mM) in the presence of pyridoxal-5′-phosphate (0.2 mg mL^{-1}) in phosphate buffer [4.0 mL, 0.1 M, pH 7.5, DMSO (10, v/v-%)]; reaction time 24 h.

for substrate scope studies by subjecting *rac*-**1a–d** under kinetic resolution conditions in phosphate buffer (0.1 M, pH 7.5) containing DMSO (10, v/v-%). DMSO was added to ensure substrate and product solubilities. The results in Table 5 indicate that the catalyst is appropriate for all substrate types studied. Enantiopure (*S*)-**1a–c** were left unreacted at 54–56% conversions after 24 h (entries 1–3). Although the reaction of *rac*-**1d** reached only 44% conversion in 24 h enzymatic enantioselectivity was high as indicated by the *ee* value of 80% at the given conversion (entry 4). When higher catalyst content (50 mg mL^{-1}) was applied, the reaction produced (*S*)-**1d** with *ee* > 99% in 24 h.

3.2. Preparation of (*S*)-**1a** in successive catalyst reuse

The synthetic potential of the optimized ω-transaminase IPA + PVA sol–gel catalyst {50 mg mL^{-1}, ATA-117 (100 mg), silanes (3 mmol), [TMOS]/[RTMS] = 1:5} was studied in preparative scale reusing the catalyst in five successive reactions of **1a** (100 mM) in aqueous DMSO (10, v/v-%). The successive reactions were performed by allowing each reaction cycle to proceed for 24 h, after which the reaction solution was replaced with a fresh solution of the reagents in aqueous DMSO (10, v/v-%). The use of DMSO allowed the substrate concentration to be increased from 50 mM, used in optimization, to 100 mM. Every reaction cycle reached close to 50% conversion (Fig. 2), although a slight tendency of decreasing reactivity might be interpreted. One possible reason for the decrease is catalyst recovery loss rather than actual loss in reactivity (centrifugation and removal the reaction solution before the addition of new reagents). Furthermore, the catalyst loss is likely to accumulate in each cycle. When the five resolved mixtures were combined, (*S*)-**1a** was purified with 41% isolated yield and 98% *ee*, indicating good reuse stability of the catalyst. In addition, the data in Table 6 (reaction volume 1 mL) together with the results in Fig. 2 (reaction

Table 6
Sol–gel ω-transaminase (50 mg) catalyzed successive kinetic resolution of **1a** (100 mM) with sodium pyruvate (100 mM) in the presence of pyridoxal-5′-phosphate (0.2 mg mL^{-1}) in phosphate buffer (1 mL, 0.1 M, pH 7.5, 10 (v/v-%) DMSO) at 30 °C after 24 h.

Entry	Conversion (%)/*ee* (%)				
	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
1	59/>99	51/>99	51/>99	51/>99	51/>99
2	59/–[a]	53/–[a]	51/–[a]	51/–[a]	51/–[a]

[a] *ee* values not determined.

volume 4 mL) indicate that the kinetic resolution of *rac*-**1a** with the ω-transaminase IPA + PVA sol–gel catalyst is highly reproducible.

3.3. Insight into the kinetic resolution results

Discussion about enzymatic enantioselectivity in terms of *E* values was practically neglected above as the equation used to calculate *E* is valid only when conversion exclusively represents the transformation of a substrate into the product [35]. Our precaution in this respect came from the fact that the first reaction cycle of the kinetic resolution of *rac*-**1a** with the best catalyst preparations tended to exceed 50% conversion (Table 3, entries 3, 8 and 10; Table 4, entry 7; Table 6; Figs. 1 and 2), although the reactions should have stopped at 50% conversion leaving the enantiopure (*S*)-**1** unreacted. The precaution is reasonable as can clearly be seen in the progression curves for the kinetic resolution of *rac*-**1a** with the optimal sol–gel catalyst in Fig. 1. The reactions with catalyst contents of 25 (●) and 50 mg mL^{-1} (■) had already halted after 7 h at 56% and 62% conversions, respectively, without any changes in conversion even at extended reaction times. Thus, higher conversions were reached with the higher catalyst content. We concluded that the amine substrate might accommodate in the hydrophobic sol–gel matrix. As a support, 20% of *rac*-**1a** disappeared in aqueous buffer in 2 h in the presence of the sol–gel matrix (50 mg mL^{-1}) which did not contain the enzyme, the amount thereafter staying unchanged. Evidently the equilibrium of the amine between the aqueous solution and the matrix more or less already exists when the second cycle starts. For the same reason the initial rate constants and activities of the prepared ω-transaminase sol–gel catalysts in the kinetic resolution of *rac*-**1a** cannot be discussed herein.

4. Conclusions

Several (*R*)-enantioselective ω-transaminase sol–gel catalysts were prepared and characterized in terms of immobilization degree, loading capacity and catalytic behavior in the successive kinetic resolution of *rac*-**1a** with sodium pyruvate in phosphate buffer. While immobilization yields were high (>90%), loading capacities of the catalysts varied. Important factors in the catalyst preparation were shown to be (1) the use of the silane ratio of [TMOS]/[MeTMS] = 1:5, (2) the washing procedure of the maturated sol–gel preparation ending with IPA rather than with water or *n*-hexane as the last washing solution and (3) sufficiently high protein content in the catalyst leading to conversions where (*S*)-**1** was obtained enantiopure in successive kinetic resolutions. The catalysts containing the more hydrophobic silane precursors, PrTMS and iBuTMS in the place of MeTMS were characterized as less favorable.

The catalyst optimized with respect to reactivity, reusability and storage stability was obtained by the entrapment of the enzyme powder (100 mg) in [TMOS]/[MeTMS] = 1:5 matrix (total silane content of 3 mmol) in the presence of IPA and PVA as additives. Finally, the catalyst was shown to perform well in the kinetic resolution of four aromatic amines *rac*-**1a–d** in aqueous DMSO (10, v/v-%). The herein presented sol–gel immobilization allowed the successful reuse of the ω-transaminase catalyst in five consecutive preparative scale reactions using substrate concentration of 100 mM.

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