Title: Immobilization of ω-transaminase for industrial application: screening and characterization of commercial ready to use enzyme carriers

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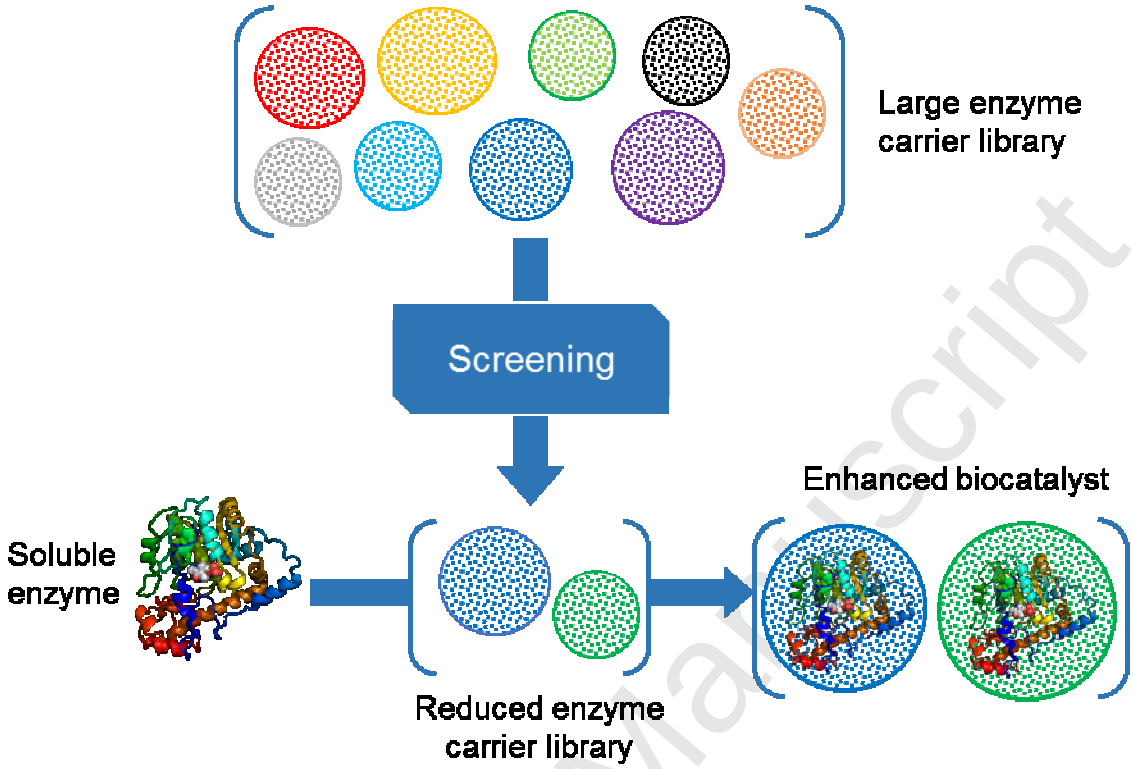
Graphical Abstract



Highlights

- Screening and characterisation of enzyme carriers is presented.
- A library containing 6 enzyme carriers (hydrophobic and ionic exchangers) have been used.
- The selected carriers improved the enzymes stability towards increased temperature.
- The selected carriers allowed increased storage stability at room temperature.
- It was possible re-use the immobilized enzyme for 8 cycles of 24 hours each.

Immobilization of ω-transaminase for industrial application: screening and characterization of commercial ready to use enzyme carriers.

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CFE_{Sol}	Soluble cell-free extract
CFE_{Imm}	Immobilized cell-free extract
WC	Whole cells
ω-TAm	ω-Transaminase
MBA	Methylbenzylamine
PYR	Pyruvate
PLP	Pyridoxal-5'-phosphate
PPB	Potassium phosphate buffer
IPA	Isopropylamine
IPAc	Isopropyl acetate
iPrOH	Isopropyl alcohol
APH	Acetophenone
BA	Benzylacetone
APB	3-Amino-1-phenylbutane
OD	Optical density

ABSTRACT:

Despite of the advantages that enzyme immobilisation can bring to industrial biocatalysis, its utilisation is still limited to a small number of enzymes and processes. Transaminase catalysed processes are a good example where immobilisation can be of major importance and even decisive for economic feasibility. This work presents results obtained for screening of enzyme carriers for immobilisation of ω-Transaminase for industrial application. A total of 6 commercial enzyme carriers (polymeric resins) were screened and two suitable enzyme carriers were selected for immobilization of both (*S*)- and (*R*)-selective ω-transaminases. These carriers allowed the re-use of the immobilized enzyme for 8 cycles of 24 hours each, under relevant process conditions, corresponding to approximately 250 hours of operation, with more than 50% of the initial activity retained. Likewise the stability towards higher temperatures and possibility to store the biocatalyst for more than 70 days (at room temperature) were obtained as result of the immobilization on the selected supports.

1 INTRODUCTION

Considering the advantages that the use of immobilized biocatalysts offer compared to soluble formulations (easy recovery and reuse, improved stability and possibility for continuous operation in packed bed reactors, to name a few), one might expected many processes running using immobilized biocatalysts as well as a large number of commercially available immobilized enzymes. However, this is not the reality. Lipase B from *Candida antarctica* (Cal B) is probably the most successful case of a commercially available immobilized enzyme which is supplied by several companies such as Novozymes A/S, Denmark (Novozym®435),

Roche Molecular Biochemicals, Germany (Chirazyme L-2), Purolite Ltd, United Kingdom (CalB ImmoPlus™) as well as similar preparations from SPRIN Technologies S.p.A (Italy), c-LEcta GmbH (Germany) and CLEA Technologies B.V (The Netherlands) [1].

The usual loss of activity commonly observed in the immobilisation process could be one reason for this observation. On the other hand, another explanation could be the lack of a generally applicable, and simple to use, method for immobilization. Today, most usually a trial and error approach is required to immobilize enzymes. Moreover, the loss in activity due to introduction of mass transfer limitations and loss of active enzyme by leaching or irreversible denaturation, which are commonly observed in immobilization of enzymes, can also be a limiting factor. Another contribution could be related to the increase in biocatalyst cost contribution to the production as a result of the introduction of an immobilization step in the biocatalyst formulation. However, the cost contribution of the immobilized enzyme for an implemented full scale process should in principal be lower than for a soluble enzyme, since the immobilized enzyme may be re-used for many reaction cycles [2].

For challenging processes where several processing strategies need to be applied in order to achieve industrially relevant yields, and where the cost associated with the biocatalyst production or purchase are high (which often is associated with the small scale of production of the biocatalyst), immobilizing the enzyme can be instrumental in determining process feasibility.

The asymmetric synthesis of chiral amines using ω-transaminases (ω-TAms) (EC 2.6.1.1) is a good example of such a process [2]. ω-TAm can be used as suitable catalysts for the production of optically pure chiral amines, which are important building blocks in the (agro)-chemical and pharmaceutical industries [2-10]. The enzyme transfers an amine group from a

donor molecule to an acceptor (pro-chiral ketone), yielding a chiral amine and a carbonyl co-product (Figure 1). The reaction can either be run as a kinetic resolution [3, 5, 10, 11] (limited to a maximum return yield of 50%) or in an asymmetric synthesis [4, 6, 7, 12], which is preferable since in principle it can give 100% yield. However, despite these promising features, ω-TAms are known to be often severely inhibited both by the ketone substrate and the chiral amine product and frequently affected by an unfavourable equilibrium (Keq<<1) in the synthetic direction [2]. Thus, several process strategies often need to be implemented to help shift the equilibrium and alleviate substrate and product inhibition in order to allow acceptable yield and product concentration [2]. Some of these strategies make use of external agents such as organic solvents or polymeric resins which, in principle, can decrease the biocatalyst stability and activity or interfere with its availability in the reactor (by binding onto a resin column for example). In such cases, immobilizing the biocatalyst can be advantageous, especially when operating with cell-free extract (CFE). Additionally, ω-TAm catalysed reactions are relatively new processes and therefore production is frequently at small scale. This means that the cost for production and/or purchase of the enzyme is potentially high. In principle, immobilization of the enzyme can reduce these costs since it allows biocatalyst to be re-used for several cycles (increasing the biocatalyst yield – $g_{product}/g_{biocatalyst}$) and also decreasing the cost associated with the downstream processing, since immobilization simplifies the separation of the biocatalyst from the products.

There are already a few reports in the scientific literature regarding the use of immobilized ω-TAm both formulated as WC and CFE. Interestingly most of the reports using immobilized WCs show reduced performance, mostly due to diffusional limitations [5, 13]. This has been overcome in another unrelated work where WC and permeabilized cells have been

immobilized by entrapment in PVA-gel (Lentikats®) resulting in low diffusional limitations [14]. In another study, immobilization of *E. coli* by crosslinking with glutaraldehyde and by entrapment with carrageenan and polyacrylamide were reported unsuccessful, with more than 50% of activity being lost in the case of entrapment in polyacrylamide, while the entrapment in carrageenan resulted in a mechanically unstable preparation [15]. In the same work, the authors reported immobilization of WC using hydrous titanium oxide (surface adsorption), calcium alginate (entrapment) and chitosan (by cell flocculation). The former was reported to show very poor loading capacity (less than 0.1 $g_{WC}/g_{carrier}$, on a dry weight basis) and consequently decreased immobilization yield, while the preparation using calcium alginate showed decreased activity due to mass transfer limitations already at low loadings of 0.2 $g_{WC}/ g_{carrier}$. On the other hand, the preparation using chitosan allowed loadings of up to 3.2 $g_{WC}/ g_{carrier}$ (dry weight) and more than 60% residual activity.

Immobilization of CFE of ω-TAm has been achieved both by covalent linkage to different solid support materials and by entrapment in sol–gel matrices [16, 17] with reported immobilization yields of 20–50% (immobilized protein/total protein) and with less than 20% of activity remaining [18], respectively. Other studies have reported low immobilization yields as well as poor residual activity (<50%) after immobilization by covalent attachment to carriers [19-21]. On the other hand, the same studies reported increased storage stability, which could be associated with conformational stabilisation of the enzyme as result of the immobilization.

Enzyme recycle studies using immobilized ω-TAm enzymes have also been sought and reported by serval or the above mentioned authors [14, 17, 18].

In a recent study, Truppo and co-workers [22] reported the development of an immobilized ω-TAm capable of operating in organic solvents (namely isopropyl acetate, isopropanol and toluene). However, it is not clear how much of this is a direct result of the immobilization itself or a result of prior improvements made to the enzyme through protein engineering techniques [23]. Nevertheless, the immobilized preparation allowed 10 times recycling in optimized reaction conditions, corresponding to approximately 200 hours of operation.

In contrast to the mentioned study by Truppo, most of the reports mentioned earlier have in common the fact that non-commercial supports, such as calcium alginate, hydrogels or hydrous titanium oxide are used. These supports require labour intensive preparation steps prior the immobilization itself. In order to be attractive and suitable for industrial application, the procedure for immobilization should be quick, robust, scalable and reproducible, while preserving the enzyme activity [24].

The use of commercial ready-to-use supports, such as polymeric resins can in principle avoid these concerns. Furthermore, these supports are known to be mechanically stable, which is a feature that needs to be considered when scaling up reactions using an immobilized biocatalyst [25, 26].

These supports are mainly made of polystyrene, polyacrylate, polyvinyls, polyamide and polypropylene. They are commercialized as purely adsorptive supports, ion exchangers or with epoxy functional groups (allowing covalent immobilisation) by several companies, under different trade names such as Amberlite, Duolite and Dowex (Dow Chemicals. USA), Lewatit (Lanxess, Germany), Diaion, Sepabeads and Relizymes (Resindion, Italy), Purolite ECR® (Purolite, USA), among others [27].

The present study focuses on the application of organic supports of synthetic origin for immobilisation and present a step-by-step screening process for selection and characterization of a suitable support for immobilization of ω-TAm. A library of supports was selected for this study containing 6 organic supports of synthetic origin (hereafter simply referred to as "resins" or "enzyme carriers") establishing hydrophobic and ion exchange interactions with the enzyme through direct interaction with the octadecyl and ethylamino functional groups, respectively, present on the surface of the supports.

2 ENZYME CARRIERS USED IN THIS STUDY

The library of 6 resins were purchased from Resindion Srl. (Milan, Italy) (Table 1) and were composed of two different types of mechanism of interaction with the enzyme (hydrophobicity and ion exchange) and two distinct mean pore diameters (10-20 nm and 40-60 nm). Finally, different lengths of functional groups were also represented in the library as shown in Table 1.

3 SCREENING METHODOLOGY FOLLOWED

Suitable supports for immobilization can be selected based on different factors, depending on the features desired. Often, the immobilization of enzymes is carried out in order to increase the enzyme stability, especially in non-conventional media (e.g. organic solvent, high pH or temperature), while in other cases the possibility of re-using the biocatalyst is sought, as discussed earlier. In these study both these features were explored and used to screen and select a suitable support to immobilize ω-TAm. The screening methodology followed was divided in three steps as described below and depicted in Figure 2.

- **Step I – Immobilization efficiency:** In this step, the resins were tested for their capacity to immobilize the enzyme. This was evaluated through the ***immobilization yield***, ***loading capacity*** and ***residual relative activity*** of the immobilized enzyme. A threshold of 90% for the residual relative activity of the immobilized enzyme was defined in this study in order to select only the most suitable resins. Immobilized preparations showing residual activity lower than 90% were discarded.

- **Step II – Operational stability:** The remaining immobilized preparations were tested for their activity for several cycles under reaction conditions. A total of 8 cycles of 24 hours or more were made and the immobilized preparations showing relative residual activities lower than 50% were discarded.

- **Step III: Activity under alternative conditions:** The selected immobilized preparations were tested for activity at high temperatures (50 °C) and in organic solvent, as well as for activity after a long storage period (up to 70 days) at room temperature.

4 EXPERIMENTAL SECTION

All the resins were purchased from Resindion S.R.L (Milan, Italy). The chemicals were all obtained from Sigma-Aldrich (Buchs, Switzerland). The (*S*)-ω-TAm (ATA-47), formulated as lyophilized CFE, was obtained from c-Lecta GmbH (Leipzig, Germany) and the (*R*)- ω-TAm (Ate-TA) formulated as WC was obtained by DSM Innovative Synthesis (Geleen, The Netherlands). With exception for assays regarding temperature stability, all the experiments were run in duplicate.

Pre-treatment of (S)-ω-TAm enzyme:

The enzyme was obtained already formulated as a lyophilized CFE powder. An enzyme solution of 5 g_{CFE}/L (dry weight) was gently prepared in 100 mM potassium phosphate buffer (PPB) pH 7 containing 8 mM of pyridoxal-5'-phosphate (PLP). 1 mL of this solution was added to each of the resins.

Pre-treatment of (R)-ω-TAm enzymes:

The enzyme was obtained as frozen centrifuged fermentation broth. The cells were re-suspended in 100 mM PPB pH 7 containing 8 mM of PLP solution (with a buffer/WC mass ratio of 2:1). The OD at 620 nm was measured to be approximately 0.3 (after a 30-fold dilution). The suspension was sonicated using a Sonics vibra-cell TM, sonicator Model"CV.18 9836A (Newtown, CT, USA), set for 10 minutes with 75% of amplitude and a pulse of 10. The OD was once again measured to be approximately 0.03 (after a 30-fold dilution). The sonicated broth was then centrifuged (Eppendorf Model 5415 R, Hamburg, Germany) for 10 minutes at 1300 rpm and the resulting pellet discarded. The supernatant was diluted with 100 mM PPB pH 7 containing 8 mM of PLP with a ratio 1:9. The final enzyme solution had approximately 7.9 g_{CFE}/L (dry weight) when lyophilized.

Lyophilisation: Samples were frozen at -80˚C and left overnight prior to lyophilisation at -54˚C for 6 hours under vacuum ($10E^{-2}$ mBar) using a Heto LyoLab 3000 from Thermo Scientific (Waltham, MA, USA).

Immobilization procedure:

Resins (50 mg for (*S*)-ω-TAm and 100 mg for (*R*)-ω-TAm) were washed with 1 mL 100 mM PPB pH 7 at room temperature. After 1 minute the washing buffer was discarded by means of

a syringe with a needle. Subsequently, 1 mL of the enzyme solution was added to each of the resins. Preparations were placed in an orbital shaking incubator (IKA ® KS, Model 130 Basic - Staufen, Germany) at room temperature (25-27 ˚C) for 48 h at 400 rpm.

Activity assay:

The activity assay was carried out in 4 mL vials at 30 ˚C at approximately 400 rpm (IKA ® KS, Model 130 Basic – Staufen, Germany). All the immobilised enzyme prepared was used (approx. 50 mg for (*S*)- and 100 mg for (R) ω-TAm). 1 mL of reaction mixture composed of 1 M isopropylamine (IPA), 30 mM acetophenone (APH), 2 mM PLP and 100 mM PPB pH 7 was used for (*S*)-ω-TAm and 2 mL of reaction mixture composed by 50 mM DL-(-)-MBA, 10 mM benzylacetone (BA), 2 mM PLP and 100 mM PPB pH 7 was used for (*R*)-ω-TAm. Samples of 100 µL were taken at minutes 1, 3 and 5 for Step I, at 0, 1.5, 3, 6 and 24 hours for Step II and at 0 and 1.5 hours for Step III. Samples were diluted into 400 µL of 1 M HCl and centrifuged for 10 minutes (Eppendorf Model 5415 R – Hamburg, Germany) at 13000 rpm, prior to analysis.

Analytical:

All samples were analysed by measuring the concentration of (S)-MBA (for reactions catalysed by (*S*)-ω-TAm) and (*R*)-3-Amino-1-phenylbutane ((*R*)-APB) (for reactions catalysed by (*R*)-ω-TAm) using HPLC (Agilent 1100 Series). Compounds were separated on a Grace ™ Prevail ™ C18 (250x4.6 mm), 5 µm column (Grace, Columbia, MD, USA) at a flow rate of 2 mL/min using a multi-step gradient flow of aqueous 100 mM perchloric acid and pure acetonitrile, with the following percentage of acetonitrile: 0 min (0%), 1 min (10%), 2.5 min (10%), 5.9 min (60%), 6 min (0%), 7 min (0%). Compounds were detected at 210 nm. The quantitative analysis was performed based on peak areas, calibrated against external standards.

Step I of the screening methodology – Immobilization efficiency

Following immobilization, samples were taken from the supernatant and assayed for residual activity in order to estimate the amount of residual un-adsorbed enzyme. The remaining supernatant was discarded and the resins were washed twice (for 2 minutes and 1h30 respectively) with 100 mM PPB pH 7.0 under mild agitation (400 RPM) at room temperature (25-27 ˚C). Samples of 100 µL were taken from the supernatant after the second washing and analysed for activity in order to quantify the leakage from the carriers (unconjugated protein). The remaining washing buffer was discarded using a syringe and the reaction mixture was added to the immobilised enzyme. Activity assay was carried out to estimate the residual activity in the immobilized enzyme. Soluble enzymes were treated under the same conditions as a reference.

Step II of the screening methodology – Operational stability

Both (*S*)- and (*R*)-selective ω-Tam, immobilized as previously described, were re-used for 8 cycles of 24 hours each (with exception for cycle number 5 which lasted 72 hours). Samples were taken at 1.5, 3, 6, and 24 or 72 h. After each cycle, the remaining reaction mixture was discarded and the resins were washed once with 1 mL of 100 mM PPB pH 7. The washing buffer was removed (by means of a syringe with a needle) and fresh reaction mixture was added to start the new cycle.

Step III of the screening methodology – Activity under alternative process conditions

Activity at 50 °C

Soluble enzymes were incubated for 12 hours at 50 °C in 100 mM PPB pH 7 solution containing 2 mM PLP. After the incubation, the reaction mixture was added to the enzymes and the activity assay was initiated at 50 °C. The immobilized enzymes were incubated at 50°C in the reaction mixture used for the activity assay. After the incubation, the liquid was discarded and the immobilized enzymes were rinsed with 100 mM PPB pH7 solution before fresh reaction mixture was added to start the activity assay at the same temperature. These were compared to free and immobilized enzyme assayed at 30 °C.

Activity in organic solvents

Lyophilized enzymes (5 mg for (*S*)-ω-TAm and 7.9 mg for (*R*)-ω-TAm – dry weight) and immobilized enzymes (prepared as previously described and followed by a drying step with Nitrogen sweeping) were tested for activity in water saturated toluene, isopropyl acetate, cyclohexane and 50 % isopropanol. Solvents were saturated with an equal volume of aqueous solution (100 mM PPB pH 7, containing 2 mM of PLP) for 72 hours under vigorous mixing to ensure proper saturation of both phases. The aqueous phase was discarded and IPA and APH (final concentrations of 1 M and 30 mM, respectively) were added to the saturated organic phase for the reaction using (*S*)-ω-TAm, while DL-MBA and BA (final concentration of 50 mM and 10 mM respectively) were added to reactions using (*R*)-ω-TAm. The activity assay was carried out in 4 mL vials at 30 ˚C at approximately 400 rpm (IKA ® KS, Model 130 Basic –

Staufen, Germany). Samples of 100 µL were taken at 1.5, 3, 6 and 24 h, diluted in 400 µL acetonitrile, centrifuged and analysed by HPLC.

Activity after storage

Free and immobilized enzymes were left in the fume cabinet for 20 days at room temperature (which was measured to oscillate between 25-27 °C). The soluble enzymes were prepared in 100 mM PPB pH 7.0, 8 mM PLP solution, while the immobilized enzymes were left in a semi wet state (the excess of immobilization buffer was removed by means of a syringe).

In addition, the immobilized enzymes previously used in Step II were washed with 100 mM PPB pH 7.0, 8 mM PLP solution after the 8th cycle, the excess washing buffer was then removed by means of a syringe and the immobilized preparations were left in the fume hood (25-27 °C) for 60 days. After the 'storage' period, the biocatalysts were added to the reaction mixtures and an activity assay was initiated.

5 RESULTS AND DISCUSSION

Step I – Immobilization efficiency

The relative residual activity of the immobilized preparation, together with the loading capacity are of high importance, since low residual activity and/or low loading capacity are often translated into an economically infeasible immobilization process. Often, a reference loading capacity of 0.1 $gCFE_{Imm}/g_{Resin}$ is used in scientific literature as ideal [1, 27]. In this step, these parameters as well as the leakage quantification were used to evaluate the immobilization efficiency for the different preparations. The effect of the pore diameter and length of the functional groups were also evaluated.

With exception for Sepabeads EC-OD, all the resins allowed immobilisation yield above 60% (Figure 3).

Leakage quantification:

For all the preparations, the results suggest no significant enzyme leakage after 1.5 h stirring in 100 mM PPB pH 7. No activity was detected in the washing buffer (data not shown).

Effect of functional group and its length on the immobilization:

The results obtained suggest an overall better immobilisation yield for resins establishing ionic interactions with the enzyme (Sepabeads EC-EA, Relizyme EA403, Sepabeads EC-HA and Relizyme HA403), in comparison with those establishing hydrophobic interactions (Sepabeads EC-OD and Relizyme OD403) (Figure 3). Interestingly, within this group, the resins having long length functional groups appeared to have slightly better immobilisation yield. This was observed for Sepabeads EC-HA and Relizyme HA403 when compared with Sepabeads EC-EA, Relizyme EA403 . This is normally associated with obstruction of the enzyme's catalytic centre upon immobilization in the case of short length functional groups due to the proximity of the enzyme to the carrier surface, causing enzyme inactivation. This is often overcome by the use of spacers [25, 27, 28].

Effect of pore size on the immobilization:

For all cases, resins with larger pore sizes (Relizyme: 40-60 nm) showed higher immobilization yields than those having smaller pore sizes (Sepabeads: 10-20 nm). For instance, immobilizations with Relizyme HA403 showed higher immobilization yield than immobilization with Sepabeads EC-HA. The same can be observed for Relizyme EA403 in comparison with Sepabeads EC-EA and Relizyme OD403 *vs* Sepabeads EC-OD (Figure 3).

Following these results, all resins but Sepabeads EC-OD, which had very low immobilisation yield, were selected for further steps of characterization. All these five selected resins had a loading capacity comparable to the reference of 0.1 $gCFE_{Imm}/g_{Resin}$ used in the scientific literature [1, 27] (data not shown).

Step II – Operational stability

The ability to re-use the biocatalyst for several cycles is in most cases the key advantage achieved by immobilization. In this step of the screening, the five selected resins were used to prepare new immobilised enzymes using this time no only the (*S*)-ω-TAm, but a (*R*)-selective ω-TAm as well. The immobilization yields for this step was above 80% for immobilisation with (*S*)-ω-TAm and about 50% for immobilisation with (*R*)-ω-TAm. The difference in the immobilization yields between the two enzymes is mainly due to the different initial formulations in which these enzymes were presented resulting in different amount of protein being immobilised. Additionally, considering that both enzymes come from different suppliers, different level of purity and expression are to be expected.

These two set of immobilised enzymes were re-used for 8 cycles of 24 hours each under the process conditions (with exception for cycle number 5 which lasted 72 hours). In total, the preparations were subjected to approximately 250 hours of operation (pH 7 and temperature of 30 ˚C with orbital agitation).

The performance for recycling of both enzymes immobilized on the selected resins were evaluated by following the conversion over time throughout the 8 cycles (Figure 4Error! Reference source not found.) and also by following the initial rate measurements for each cycle (Figure 5).

Based on the maximum conversion achieved at the end of each cycle, the immobilizations using the resins Relizyme OD403, Sepabeads EC-HA and Relizyme HA403 showed the best performance among the five for (*S*)-ω-TAm (Figure 4A). For (*R*)-ω-TAm, all the five immobilized preparations showed maximum conversion possible after 24 hours for all cycles (Figure 4B), suggesting an excellent activity retention despite the lower immobilization yield.

In order to have a clearer overview of the performance of the immobilized enzymes in each cycle, it is important to look at the initial rates as well, since this dictates how much of the activity is lost in each cycle, due to enzyme deactivation or leaching.

Due to the high immobilization yield, all the preparations for (*S*)-ω-TAm presented in the first cycle an activity comparable to the free enzyme (illustrated by the dashed line in Figure 5A). However, in the second cycle the activity decreases almost 50% for all of the preparations and from this point the loss of activity for the following 6 cycles is not so accentuated. This can be observed especially for the immobilizations using the resins Relizyme OD403, Sepabeads EC-HA and Relizyme HA403 which kept approximately 50-60% of the initial activity throughout the 8 cycles.

With respect to (*R*)-ω-TAm, the results obtained showed excellent activity retention throughout the 8 cycles for all the preparations, with special emphasis to preparations using Relizyme OD403, Sepabeads EC-EA and Sepabeads EC-HA, which allowed retention of more than 90% of the initial activity (Figure 5B). Based on these results, the resin library was reduced to 3: Relizyme OD403, which showed good activity retention for both enzymes; Relizyme HA403 and Sepabeads EC-EA, which showed good performance for (S)-ω-TAm and (*R*)-ω-TAm, respectively.

Step III – Activity under alternative conditions

Similarly with the reusability, stability is one of the improvements often used to justify the need for immobilization. Several authors have reported improved biocatalyst stability as a result of immobilization using many different enzymes [29, 30].

Temperature stability is one of the improvements which can be achieved through immobilization. A more thermo stable biocatalyst may open the possibility of carrying out reactions at higher temperatures, which should mean higher reaction rates. In the specific case of ω-transaminase, carrying out the reaction at higher temperature also opens the possibility of new process options such as the evaporation of volatile co-products such as acetone in order to shift reaction equilibrium [1].

Furthermore, the activity in non-conventional conditions (or alternative conditions), such as in organic solvents, is often discussed and reported in the scientific literature [22, 29, 31]. Having a biocatalyst which can operate in organic solvents can make possible reaction in two phase system or in neat organic phase which could bring advantages such as increased substrate and product solubility [2].

Finally, the possibility to store the immobilized biocatalyst for a long period of time can also be advantageous and has been widely discussed in the scientific literature [27, 29, 32-34]. It can save time and allow immobilization of a large amount of enzyme in advance for future utilization as well as opening the possibility for commercialization of the immobilized enzyme.

Activity at 50°C

The immobilized enzymes were incubated for 24 hours at 50 °C and compared to soluble enzymes incubated at the same temperature but for 12 hours only. Afterwards, these were compared to soluble enzymes incubated at 30 °C for 24 hours. The results illustrated in Figure 6 show that soluble enzymes incubated at 50 °C lose about 80% and 100% of initial activity (for (*S*)-ω-TAm and (*R*)-ω-TAm respectively). None of the immobilized (*S*)-ω-TAm preparations showed substantial improvements, when compared with the free enzyme treated under the same conditions (50 °C). The best result obtained was observed for the preparations using the resin Relizyme HA403 which showed slightly higher residual activity in comparison to the soluble enzyme (Figure 6A).

On the other hand, the results were more prominent for the (*R*)-ω-TAm, which was found to retain 80% of activity when immobilized with the Relizyme OD403 and about 30% with Sepabeads EC-EA while the free soluble enzyme at the same temperature showed no residual activity (Figure 6B). These results illustrate an enhanced stability at 50 °C, achieved as direct result of immobilization.

Activity in organic solvent

Soluble and immobilized enzymes were used to run reactions in both water-immiscible and water-miscible solvents. Similar experiments have been previously reported where the immobilized ω-TAm shown remarkable performance well [22]. However, the results obtained in current study suggest that the biocatalyst activity in organic phase was not enhanced as result of immobilization. None of the immobilized preparations performed better than lyophilised soluble enzyme in any of the solvents with exception for the assay performed under 50% isopropanol, where almost 5% of residual activity was found in the preparation of

(*S*)-ω-TAm immobilized on Relizyme HA403 (data not shown). These could also be related to a poor mass transfer of the substrate from the organic phase to the biocatalyst itself, or the absence of the cofactor (PLP) in the reaction mixture, since PLP is not soluble in organic solvent.

Storage stability

The results obtained for the storage stability show an improved stability at room temperature for all the immobilized preparations (Figure 7). In all cases, the immobilized preparations retained more activity than the soluble enzymes (both (*S*)-ω-TAm and (*R*)-ω-TAm) treated under the same conditions.

Both enzymes immobilized (using both hydrophobic and ionic exchange resins) retained more activity after 20 and 70 days in comparison with their free formulations at room temperature. In general, the ion exchange resins (Sepabeads EC-EA and Relizyme HA403) allowed greater activity retention than the hydrophobic resin (Relizyme OD403) after 60 days. More than 50 % and 40% of residual activity was observed for (*S*)-ω-TAm and (*R*)-ω-TAm immobilized on the ionic exchange support, after 60 days. These results are more encouraging than those previously reported where losses of 30 % and 92% of activity of ω-TAm immobilized on chitosan and Eupergit® C, respectively, after 24 days at 4 °C were obtained [17]. It is expected that better activity retention could have be obtained if the preparations were stored at 4-5 °C instead of room temperature.

6 CONCLUSIONS

Immobilisation of ω-TAms enzymes on commercially ready to use enzyme carrier allowed re-use of the immobilized preparation for 8 cycles (each of 24 hours), under the process

conditions, which corresponds to more than 250 hours of operation, retaining more than 50% initial activity. The resulting preparation showed increased enzyme stability at high temperatures in caparison to the soluble native enzymes and made it possible to store the enzyme for more than 60 days at room temperature.

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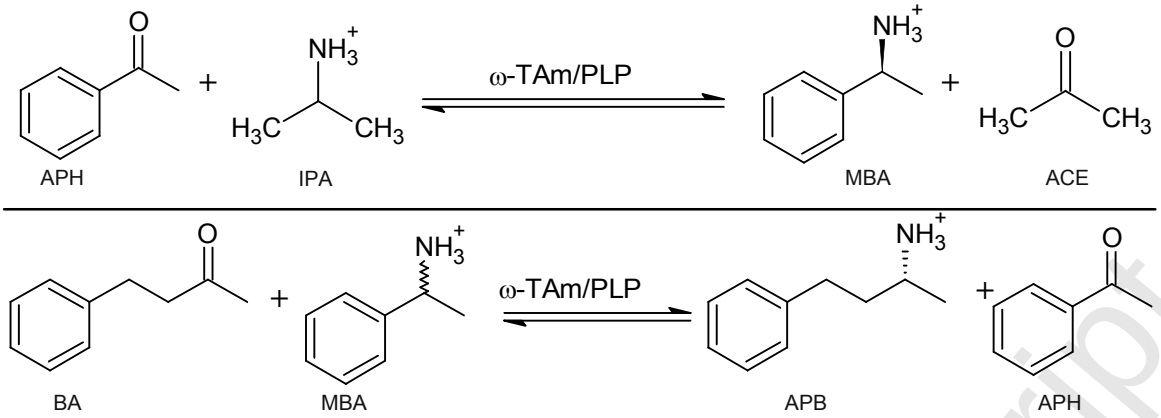


Figure 1: Asymmetric synthesis of chiral amines catalysed by ω-Transaminase

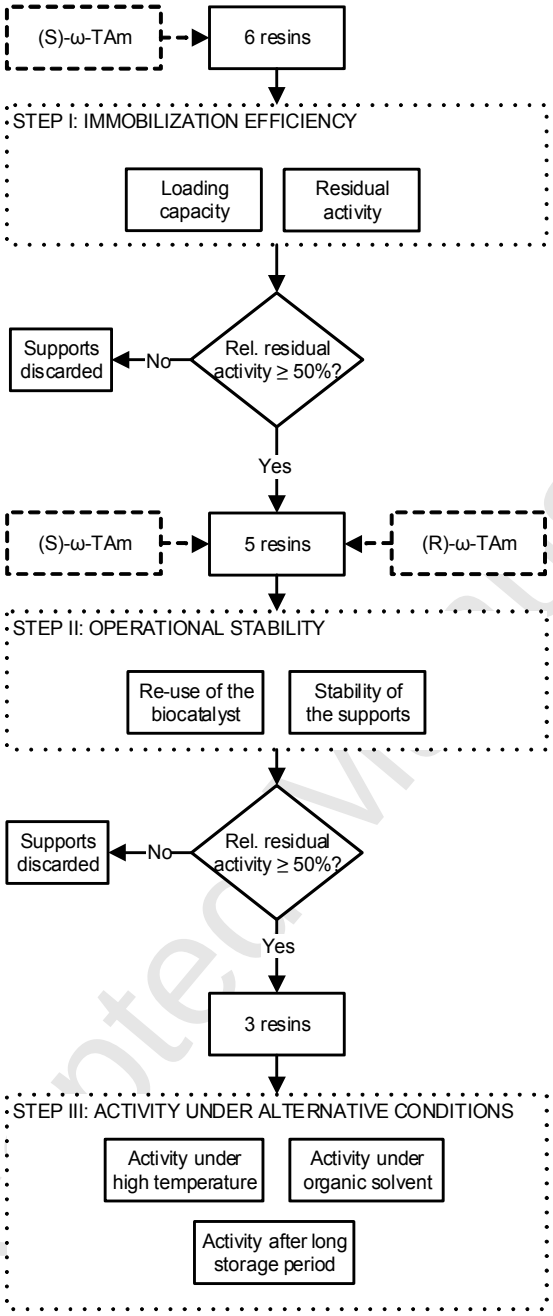


Figure 2: Summary of screening steps.

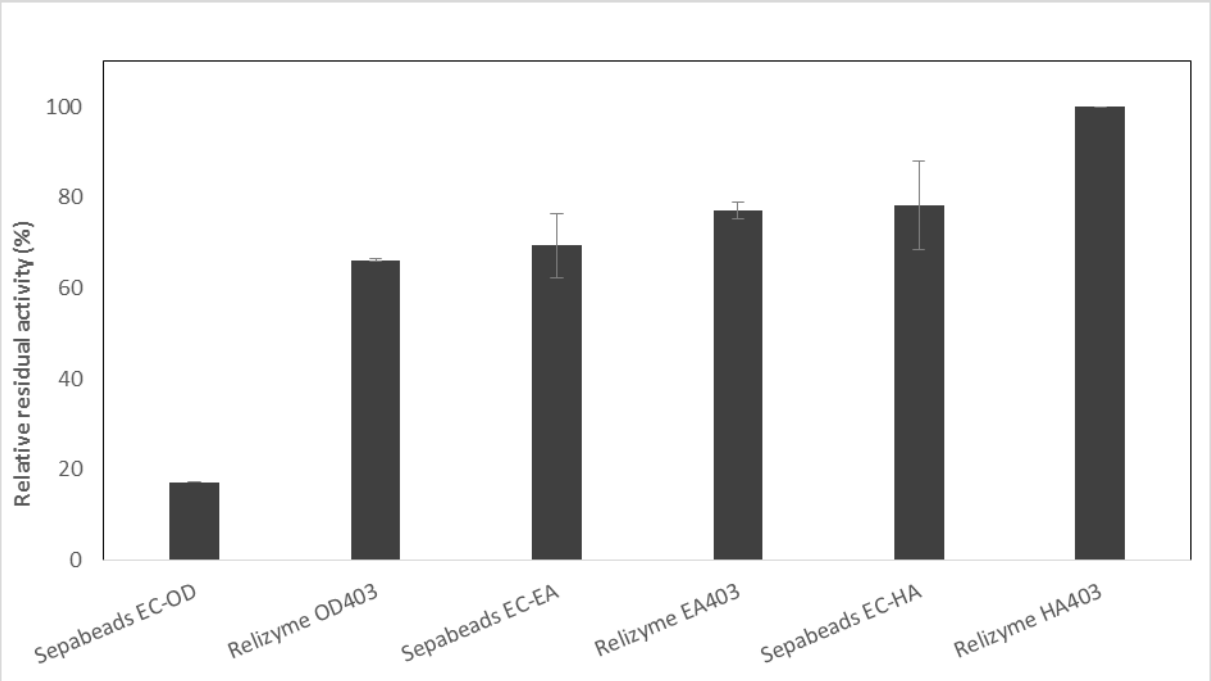


Figure 3 – Residual relative activity for the immobilized preparations. Calculated as *(activity in the immobilised)/(activity of loaded enzyme)×100*. Results based on initial rate measurements for activity.

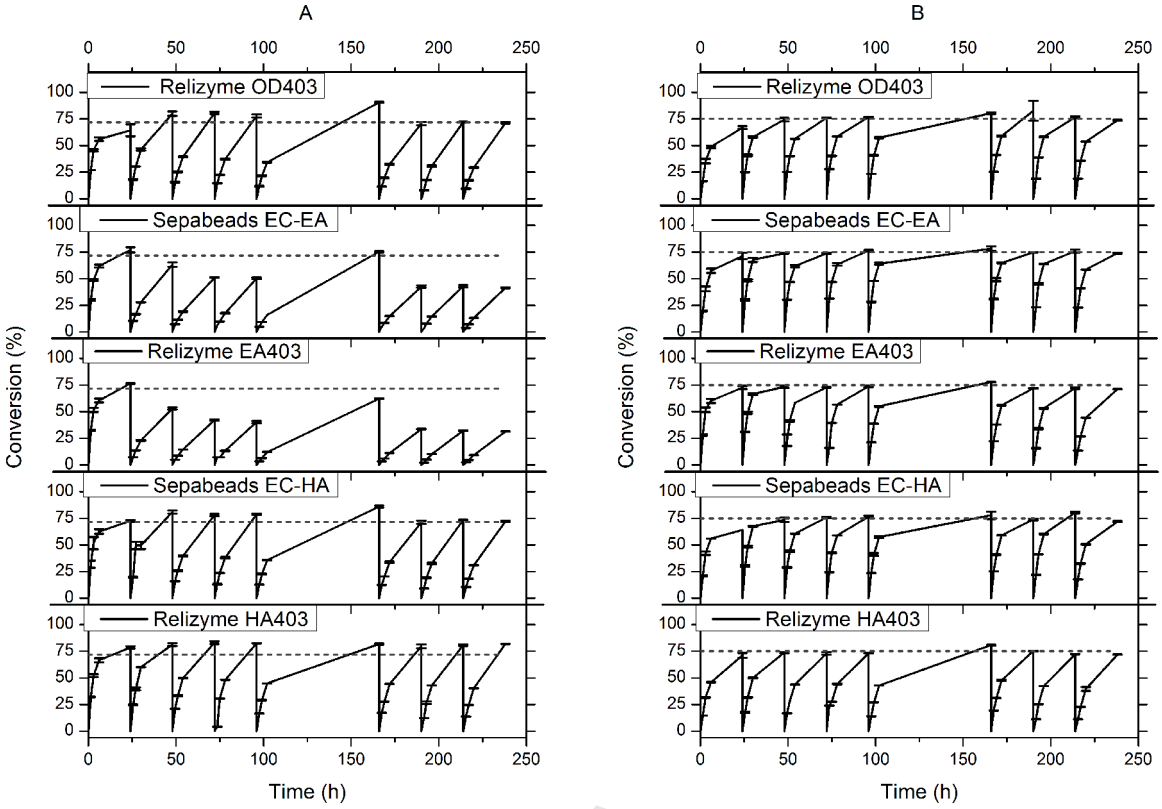


Figure 4: Conversion over time for the different cycles (A: using immobilized (*S*)-ω-TAm; B: using immobilized (*R*)-ω-TAm). Dashed line marks the maximum conversion after 24 hours achieved with free enzyme (used as reference) under similar conditions.

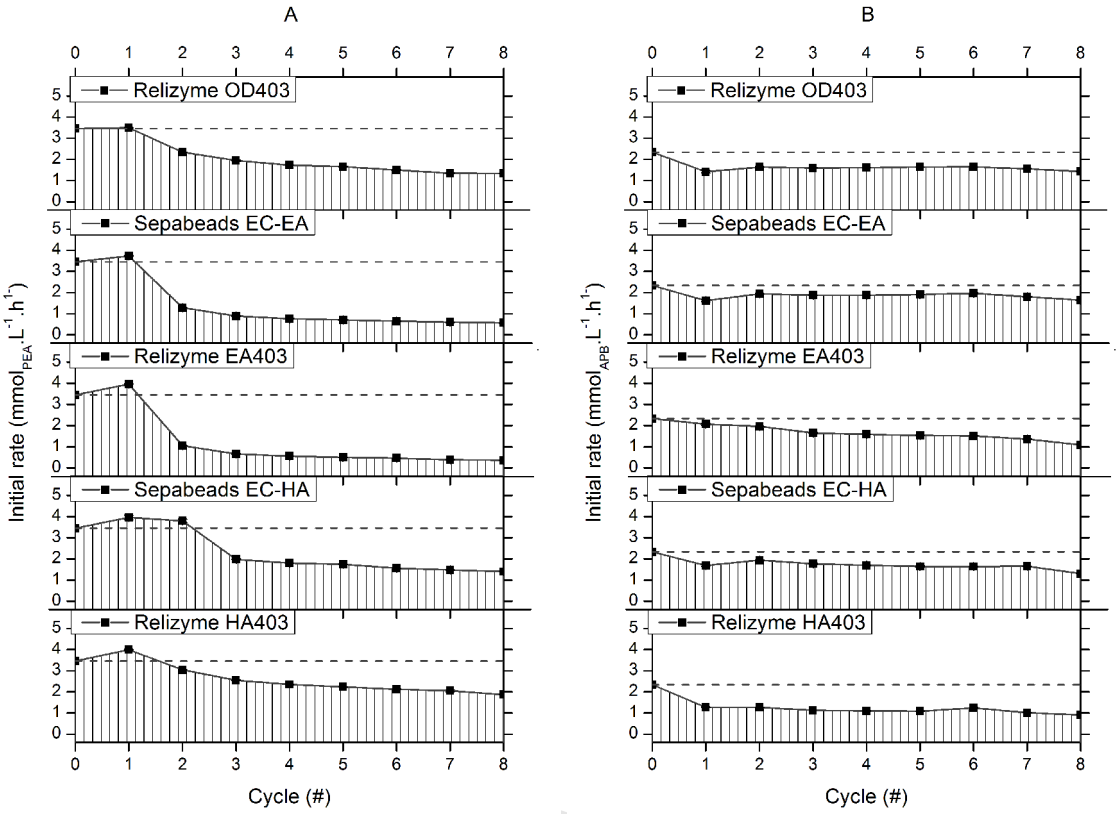


Figure 5: Initial rate measurements for different cycles. A: using immobilized (*S*)-ω-TAm; B: using immobilized (*R*)-ω-TAm). Dashed line marks the maximum conversion after 24 hours achieved for free enzyme (used as reference) under similar conditions.

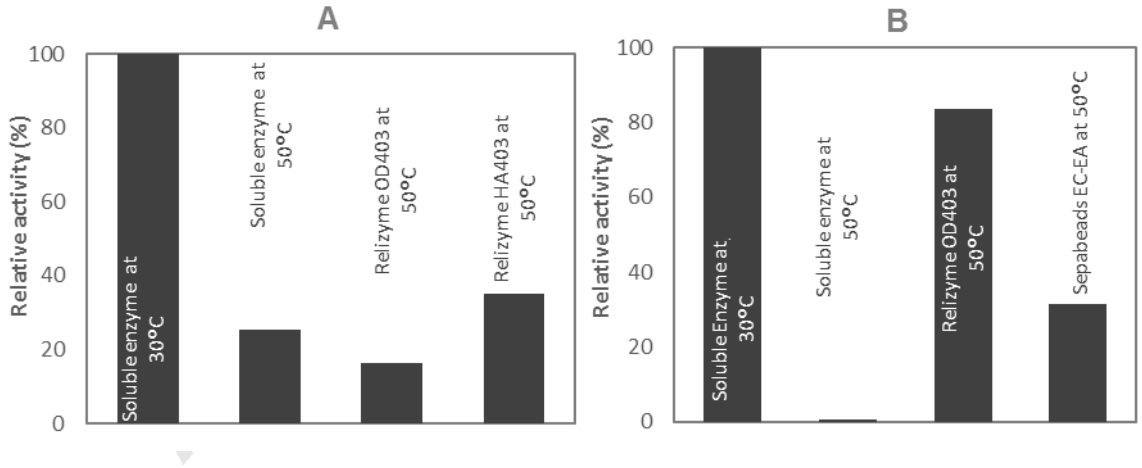


Figure 6: Temperature stability for (*S*)-ω-TAm (A) and (*R*)-ω-TAm preparations (B). *Relative activity (%)=(activity in the immobilised)/(activity of loaded enzyme)×100*. Results based on initial rate measurements for activity.

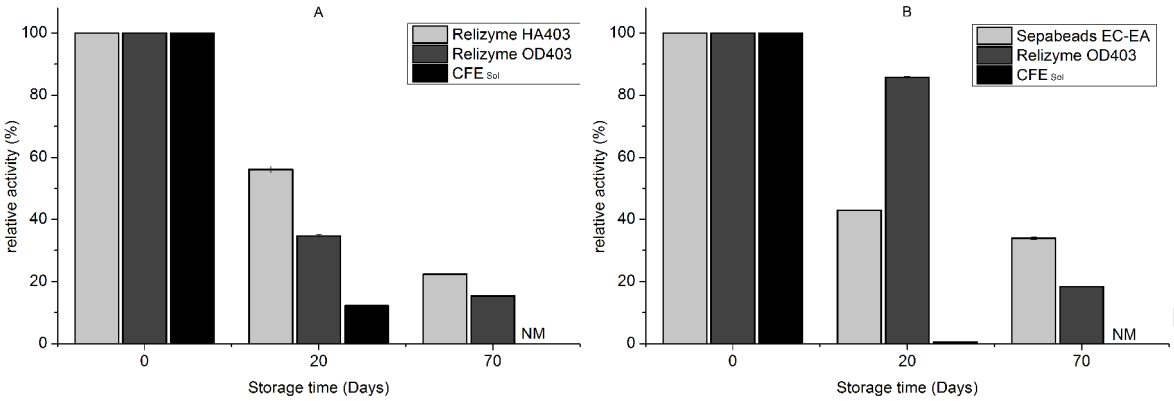
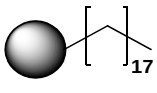
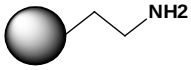
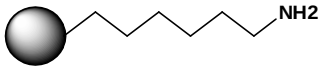


Figure 7: Relative activity after storage at room temperature for (*S*)-ω-TAm (A) and (*R*)-ω-TAm (B). NM – Not measured. *Relative activity (%)=(activity in the immobilised)/(activity of loaded enzyme)×100*. Results based on initial rate measurements for activity.

Table 1: List of resins for enzyme immobilization and their properties.

	Structure and functional group	Resin name [1]	Pore size (nm)	Moisture [2] (%)	Price [3] (€/Kg)
Hydrophobic	 Octadecyl	Sepabeads EC-OD	10-20	55 - 65%	474
		Relizyme OD403	40-60	65 - 75%	576
Ionic interaction	 Ethylamino	Sepabeads EC-EA	10-20	55 - 65%	183
		Relizyme EA403	40-60	60 - 70%	221
	 Hexamethylamino	Sepabeads EC-HA	10-20	50 - 60%	183
		Relizyme HA403	40-60	60 - 70%	221

[1] All resins had a Methacrylate matrix and a particle size in the range of 200-500 µm.. Data provided in Product Technical Data Sheet (PTDS) provided by the supplier, and available at http://www.resindion.com

[2] Data from the PTDS provided by the supplier and available and http://www.resindion.com.

[3] Prices based in packages of 5 or 10 Kg. Prices obtained from Resindion webpage on 3/05/2013.