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# Enabling Industrial Biocatalytic Processes by Application of Silicone-Coated Enzyme Preparations

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**Abstract:** Composite particles of the commercial lipase preparation NZ435 and silicone (*silcoat*-NZ435) have recently been described as promising biocatalysts for synthetic use. In this study, their actual potential for enhanced performance in industrial applications was evaluated, focusing on scenarios where carrier disintegration and catalyst leaching constitute key limitations. All three investigated model reactions, the syntheses of myristyl myristate, poly(ethylene glycol) 400-coconut fatty acid monoester and ethylene oxide and propylene oxide copolymer (EO/PO)-oleic acid diester, were considerably improved in terms of the maximal number of reaction cycles performed with the same batch of catalyst, and consequently in terms of the obtainable product amount. The total turnover numbers (TTN) increased by a factor up to 50, making the realization of this type of reactions in an industrial process more feasible. The utility of *silcoat*-NZ435 for stereoselective transformations was demonstrated with the enantioselective acylation of 1-phenylethanol with vinyl butyrate, in which full retention of the excellent stereoselectivity of native NZ435 was observed. Moreover, it was demonstrated for the first time that the methodology by which *silcoat*-catalysts are obtained can be successfully transferred to alternative carriers and enzymes (e.g., protease, esterase and laccase), opening a broad field for the implementation and advancement of biocatalysis in industrial processes.

**Keywords:** Novozym 435; polyether ester; process optimization; silicone; stabilization; total turnover number

## Introduction

In recent years, the implementation of biocatalyzed reactions into the production of fine and specialty chemicals has become increasingly important for the chemical industry. This mainly results from the often high chemo-, regio-, and stereoselectivity of biocatalysts under mild reaction conditions (room temperature, neutral pH) yielding high quality products without extensive energy input and downstream processing. Nevertheless, unless very high priced intermediates or active compounds (e.g., for pharmaceuticals) are produced, an economically efficient process often requires low costs for the applied biocatalyst.

A particularly versatile and successful biocatalyst with regard to technical synthesis is Novozym 435 (NZ435; Novozymes, Denmark), a highly active commercial preparation of lipase B from *Candida antarctica* immobilized on a macroporous acrylic polymer resin[1] by adsorption through mainly hydrophobic interactions.[2] It is used for industrial-scale synthesis of various fine chemicals[3] as well as specialty chemicals such as ceramides and emollient esters for cosmetic formulations.[4] Unfortunately, its applicability is currently limited to the conversion and production of compounds with low to medium viscosity and none to only slightly surface-active properties. This obviates the effective production of many cosmetic compounds, for example, when diglycerol[5], polyglycerol or other polyols like *R*-butylglucoside[6] are involved.

Viscosity restricts the application of NZ435 because of the maximum possible pressure drop over the

length of the fixed-bed reactor, which is typically used with this enzyme preparation.[7,8] Transfer of NZ435 to a stirred-tank reactor (STR) as the standard technology for conversion and production of highly viscous compounds, however, is prohibited due to the disintegration of the enzyme carrier by strong shear forces. Alternative reactor concepts such as bubble columns which provide proper mixing while exhibiting only low mechanical forces[8] can prevent this destruction, but at low production volumes, the implementation of a novel technology is usually not justified. Surface-active compounds, on the other hand, promote leaching of enzyme adsorbed on a carrier,[9] a phenomenon frequently observed.[8,10] The applicability of NZ435 can only be extended when these restrictions are overcome.

We recently reported the development of a novel enzyme preparation with considerably improved stability against enzyme leaching and carrier disintegration.[11] This involves the combination of native NZ435 with up to 54% (w/w) silicone to form distinct composite particles with a residual activity of approximately 60% of the initial NZ435 activity towards the lab-scale synthesis of propyl laurate.[12] Promising features of this approach with regard to industrial implementation are the non-toxicity, low price and large-scale availability of silicone as coating material, the established polymer formation *via* hydrosilylation and the possibility to adapt the physicochemical properties of the material to specific needs by varying the polymer chain length, the degree of cross-linking and the number of modifications.[14] Thus, it is clearly advantageous over the reported alternative entrapment of carrier-bound enzymes in sol-gel,[13] which in addition form a brittle material with only low stability against carrier disintegration in standard industrial reactors such as STR.

This study evaluates the actual performance of the composite catalysts, further denoted as *silcoat*-NZ435, in industrial synthesis, with a focus on scenarios where carrier disintegration and catalyst leaching constitute key limitations. As model systems, the syntheses of the emollient ester myristyl myristate and the poly(ether) esters EO/PO-oleic acid diester and poly-(ethylene) glycol 400-coconut fatty acid monoester were investigated with particular regard to recyclability and total turnover number (TTN) of the catalyst under process relevant conditions. Furthermore, the applicability of *silcoat*-NZ435 to stereoselective synthesis was evaluated in the kinetic resolution of *rac*-1-phenylethanol as prevalent model substrate[15,16,17] and finally the transferability of the methodology for *silcoat*-catalyst preparation to alternative carriers with immobilized enzymes is demonstrated for the first time.

## Results and Discussion

Myristyl myristate, poly(ethylene) glycol 400-coconut fatty acid monoester and EO/PO-oleic acid diester are important examples for industrial commodity products with uses in the cosmetic, pharmaceutical and food industries.[18,19,20,21] Their biocatalyzed synthesis can be accomplished with NZ435.[18,22] but suffers from the previously described disintegration of the catalyst carrier in STR or from enzyme leaching in the presence of surface active reactants, respectively.

Here, it was found that both disadvantages can indeed be effectively overcome by implementation of *silcoat*-NZ435 into the production processes in place of native NZ435. In all three systems, this leads to a clear improvement of catalyst recyclability and accordingly to an astonishingly increased total turnover in terms of product formation per mass of catalyst ($m_{product}/m_{NZ435}$). Thus, process performance is considerably advanced.

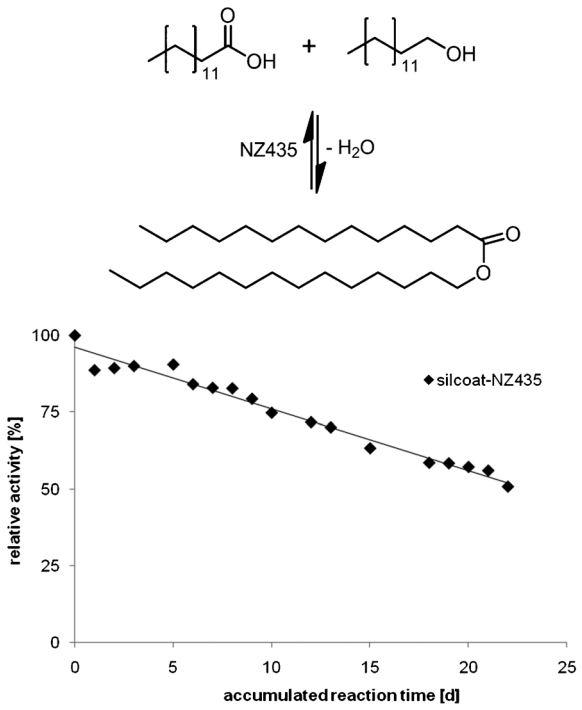
### Increased Stability in STR

Various emollient esters for the cosmetic industry are currently successfully produced on a ton-scale by use of a fixed-bed reactor.[3] With this reactor set-up, the immobilized enzyme can be re-used for many cycles as no mechanical stress is exerted; the lipophilicity of the products prevents desorption of the catalyst from the carrier. However, to allow the syntheses of a larger product variety, multi-purpose reactor set-ups like STR are favorable. The performance of *silcoat*-NZ435 in such a reactor was evaluated at the well studied esterification of myristyl myristate as a model system (Figure 1) and compared to native NZ435.

The esterification was performed in a stirred-flask reactor (SFR) on a lab scale, resembling a small-scale equivalent of a multi-purpose STR. This was operated at a stirring speed of 350 rpm (to mimic the specific power input observed in large-scale STR)[23] under reaction conditions (temperature, catalyst ratio, application of vacuum for water removal) adopted from the large-scale production of myristyl myristate in a fixed-bed reactor.[5,24] Each production cycle covered a reaction time of 24 hours. Within this time, the reaction was run to completion (100% conversion).

When native NZ435 was used in this reaction set-up, the catalytic performance of the enzyme was comparable to that in the fixed-bed process[7] and full conversion of substrates within the preset time was easily obtained. This demonstrates the principal suitability of this reactor type for the synthesis. However, severe damage to the enzyme carrier was apparent, which is in agreement with the previously reported disintegration of NZ435 under mechanical stress.[11,12] As a consequence, during catalyst removal the filter was




| | Native NZ435 | *silcoat*-NZ435 |
|---|---|---|
| Half-life time [days] | 1 | 23 |
| TTN [$g_{Produkt}/g_{NZ435}$] | 100 | 2300 |

**Figure 1.** *Top:* Esterification of myristyl alcohol and myristic acid. *Middle:* Initial enzyme activity within the recycling of *silcoat*-NZ435. *Bottom:* Half-life times and TTN of NZ435 preparations.

blocked by NZ435 fragments and abrasion, reducing the filtration efficiency and preventing the recovery of catalyst. In the end, a TTN of 100 $g_{MM}/g_{NZ435}$ could be obtained, a half-life time could not be determined.

In contrast, no abrasion was detectable when *silcoat*-NZ435 (silicone content 45% w/v) was subjected to the same conditions, which is consistent with the previously reported enhanced mechanical stability of this catalyst preparation.[11] Accordingly, product purification, catalyst recovery and recycling were easily accomplished, so proving an excellent applicability of the system from a technical point of view.

The same batch of *silcoat*-NZ435 ran each reaction cycle to completion within 23 days. Therefore, the TTN in this system could be increased by a factor of 23 compared to the set-up with native NZ435, achieving a value of 2300 $g_{MM}/g_{NZ435}$ and a half-life time of 23 days. Due to the very slow deactivation of the catalyst under the investigated conditions ($k_i = 0.037$, Figure 1), the same batch of *silcoat*-NZ435 can catalyze even more reaction cycles to full conversion when the preset reaction time of each cycle is increased.

Thus, the performance of *silcoat*-NZ435 under the harsh conditions exerted in an STR (in terms of mechanical stress), is comparable to native NZ435 in the standard fixed-bed reactor.[25] This shows that *silcoat*-NZ435 offers an economically feasible reaction set-up in multi-purpose STR, which is not only applicable to the synthesis of myristyl myristate as a model product, but to a number of other products with industrial relevance.

## Increased Stability Towards Leaching

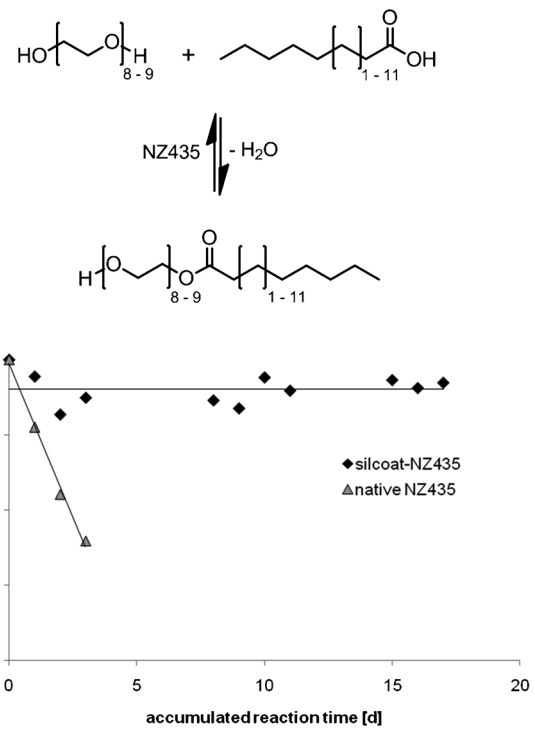
The esterification of PEG 400 with coco fatty acid to the corresponding monoester was chosen as a model system representing a relatively low viscous product, which can easily be handled in a fixed-bed reactor. However, the reaction mixture and the product itself show a strong surface-active behavior, causing protein desorption from the carrier surface. This leads to a loss of enzyme activity in the course of the reaction and particularly during recycling of the catalyst.

The performance of *silcoat*-NZ435 under these conditions was investigated in a lab-scale fixed-bed reactor. As mentioned before, this reactor type offers high productivity at low shear forces, excluding mechanical damage on the NZ435 carrier. An equimolar mixture of PEG400 and fatty acid was pumped through a fixed-bed of NZ435 and *silcoat*-NZ435, respectively. After full conversion within 24 h, the product was removed and replaced by fresh substrate to start consecutive reaction cycles.

As expected, both the carriers of native NZ435 and *silcoat*-NZ435 remained mechanically unaffected throughout all operation cycles. Preliminary experiments on the thermal stability of the enzyme preparations, involving incubation in a non-leaching product and without mechanical forces at different temperatures (60 °C, 90 °C, 110 °C), demonstrated that the composite formation had no stabilizing effect in this regard. Both catalysts, native NZ435 and *silcoat*-NZ435, were stable at 60 °C and lost 10–15% and 50–60% of initial specific activity at 90 °C and 110 °C, respectively (see Supporting Information). Therefore, changes in the activity of the catalyst beyond the magnitude of spontaneous deactivation can be assigned solely to the leaching of the catalyst from the carrier.

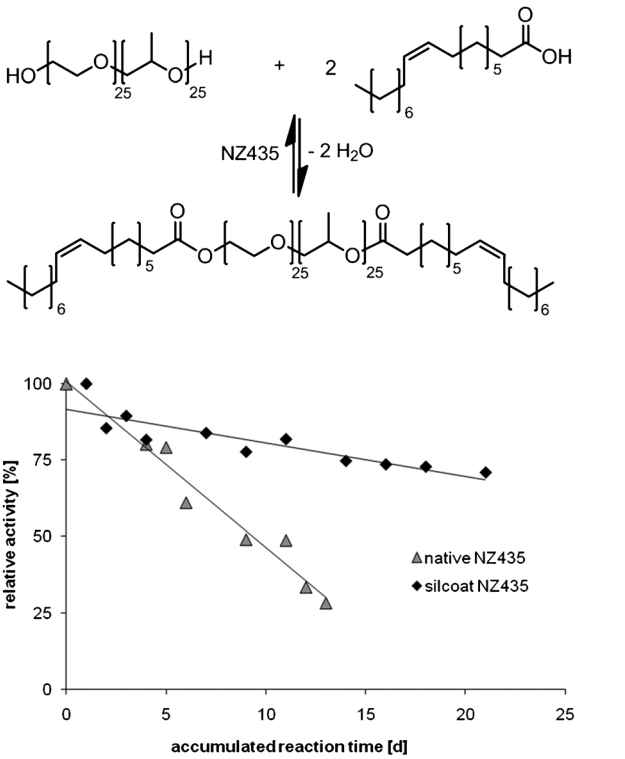
During recycling, native NZ435 rapidly lost activity (Figure 2). In only three production cycles, the initial activity was reduced to 40%, corresponding to a half-life time of 2.2 days. This is in perfect agreement with the distinguished surface-active properties of the in-




| | Native NZ435 | *silcoat*-NZ435 |
|---|---|---|
| Half-life time [days] | 2,2 | > 50 |
| TTN [$g_{Produkt}/g_{NZ435}$] | 189 | >1459 |

**Figure 2.** *Top:* Esterification of PEG 400 with coco fatty acid. *Middle:* Initial enzyme activity during repetitive use in a lab scale fixed bed reactor. *Bottom:* Half-life times and TTN of NZ435 preparations.




| | Native NZ435 | *silcoat*-NZ435 |
|---|---|---|
| Half-life time [days] | 8 | 63 |
| TTN [$g_{Produkt}/g_{NZ435}$] | 329 | 2588 |

**Figure 3.** *Top:* Esterification of EO/PO-polyether and oleic acid. *Middle:* Initial enzyme activity during repetitive use in a lab scale bubble column reactor. *Bottom:* Half-life times and TTN.

vestigated substrate mixture and the produced monoester, both having a strong ability for leaching the enzyme off the carrier. The resulting TTN was about 189 $g_{monoester}/g_{NZ435}$.

When recycling *silcoat*-NZ435 under the same conditions, no measurable loss in enzyme activity could be observed enabling the repeated production of monoester with one batch of enzyme for more than 18 days. This increased the TTN by a factor of 8 to TTN > 1459 $g_{monoester}/g_{NZ435}$.

## Application in a More Complex System

The esterification of EO/PO-polyether with oleic acid to the corresponding diester represents a slightly surface-active reaction medium (diester) with a high viscosity (Figure 3). Due to this viscosity, a fixed-bed reactor cannot be used to perform the reaction. Instead, a bubble column reactor (BCR) is applied enabling appropriate mixing, optimal water removal and reduced mechanical stress on the enzyme carriers.[8]

The BCR was filled with the EO/PO-polyether and oleic acid at a molar ratio of 1:2 and was operated at 70 °C. For mixing and removal of process water, a stream of nitrogen was continuously passed through the reactor. Each production cycle covered a reaction time of 24 hours. After each cycle, the catalyst was separated from the product by filtration and was reused in consecutive cycles.

Figure 3 illustrates the performance of native NZ435 and *silcoat*-NZ435 over eight to twelve production cycles with regard to the residual activity at the beginning of every run. Abrasion of the enzyme carrier was neither observed for native NZ435 nor *silcoat*-NZ435 in this reaction set-up. It can therefore be

assumed that changes in the activity of the catalysts beyond the magnitude of spontaneous deactivation are solely induced by catalyst leaching from the carrier.

With native NZ435, a maximum of 8 production cycles could be accomplished before the residual activity decreased below 25%. In contrast, a residual activity of 75% was still measured after 12 production cycles when *silcoat*-NZ435 was introduced to the process. This is in agreement with the previously reported enhanced stability of *silcoat*-NZ435 against enzyme leaching.[11]

While the native enzyme preparation achieved a half-life time of only about 8 days, *silcoat*-NZ435 revealed a half-life time up to 63 days. This corresponds to an increase in TTN from 329 $g_{diester}/g_{NZ435}$ to 2588 $g_{diester}/g_{NZ435}$ for native NZ435 and *silcoat*-NZ435, respectively. Thus, formation of silicone composites can effectively prevent protein leaching from the carrier surface and allows broadening the product portfolio even for surface active and highly viscous reaction media.

## Stereoselective Synthesis and Transferability

Encouraged by the performance of *silcoat*-NZ435 in the synthesis of the specialty esters in the previous sections, further experiments were performed to estimate the potential of silicone coating as a general method for the provision of advanced catalysts for technical synthesis. On the background of the particular importance of enzyme catalysis in the production of chiral intermediates, this involved an examination of the impact of composite formation on the enantioselectivity of NZ435. Since it is known that the properties of the immobilized enzyme strongly depend on the enzyme microenvironment, it should be verified that no significant interactions between the inert hydrophobic silicone matrix and the active site of the enzyme occurs, which is for instance detectable in changes of the enantioselectivity[26]. As a simple and representative model system we have chosen the kinetic resolution of *rac*-1-phenylethanol (**1**) as the alcohol component and vinyl butyrate as the acyl donor (Scheme 1) in a transesterification reaction. It yielded an enantiomeric excess of >99% of *(R)*-α-methylbenzyl butyrate (**2**) at full conversion (50%) and thus exhibited no difference to the native NZ435. Combined with the findings of Hoyos et al.[27] who entrapped Lipase TL (Meito Sangyo, Japan) in pure silicone without affecting the stereoselectivity, this provides a strong hint that composite particles obtained from silicone have no influence on the given stereoselectivity of an enzyme catalyst and can therefore also be effectively applied to the synthesis of chiral compounds.

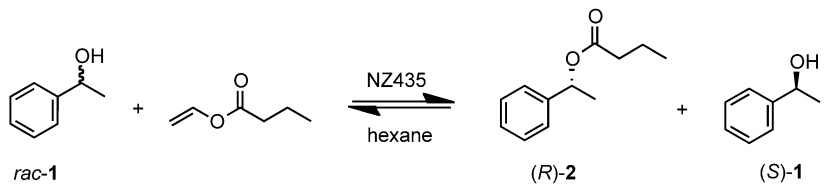
Based on the excellent results achieved with NZ435 as well-established commercial available lipase preparation, a test for transferability of this methodology to other biocatalysts is of great interest. For a proof of principle study, other mostly commercially available carrier-bound enzymes were used with the intention of setting standardized starting positions for following optimizations. In order to cover a variety of industrial relevant enzyme classes, an additional lipase system, a protease,[28] an esterase,[29] and a laccase[30] immobilisate were tested.

The formation of catalytically active silicone composites was attempted with LCAHN, a commercial alternative to NZ435, in which lipase B from *C. antarctica* is adsorbed on a polystyrene carrier, a commercial protease (alcalase, IMMALC350) on an acrylic polymer, a commercial esterase (Esterase ERO) on Eupergit and a laccase on poly(methyl methacrylate) ("Flavostar"). Importantly, this was successful for all the investigated materials, albeit with a rather diverse ability for silicone uptake and large varieties in the

**Table 1.** Residual activities of different silicone-deposited enzyme preparations.

| Enzyme | Carrier | Silicone [%] | Residual activity [%] |
|---|---|---|---|
| Lipase[a] | Polystyrene | 40 | 66 |
| Protease[b] | Acrylic polymer[e] | 40 | 65 |
| Esterase[c] | Eupergit | 50 | 26 |
| Laccase[d] | PMMA | 44 | 5.4 |

[a] Esterification of lauric acid and 1-propanol.
[b] Hydrolysis of ethyl *N*-acetylglycinate.
[c] Hydrolysis of ethyl pentanoate.
[d] Oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS).




**Scheme 1.** Kinetic resolution of *rac*-1-phenylethanol by NZ435.

residual catalytic performance being observed (Table 1).

For lipase B and alcalase a residual activity above 60 % was observed, which is in the same range as for NZ435[12] and therefore offers good prospects for a future application to technical synthesis. In contrast, esterase and laccase revealed only low residual activity after transfer to a *silcoat*-catalyst. However, at a closer look, this behavior can most probably be assigned to the rather hydrophilic substrates converted by these enzymes, as their uptake must be considerably restricted by the hydrophobic silicone matrix. Thus, the results do not actually restrict the transferability of *silcoat*-catalyst formation to a broad range of enzymes. For application to a broad range of reactions, however, matrix design towards an increased hydrophilicity must be considered and is currently under investigation.

## Conclusions

The use of *silcoat*-NZ435 overcomes some of the most important limitations faced in the application of biocatalysis under industrially relevant conditions. The easily applicable silicone deposition opens the way to use high-performance and therefore high-priced biocatalyst preparations in a cost-effective and highly economical manner. In addition, the possibility to use these improved biocatalysts in an STR makes accessible a broad range of potential products, such as emulsifiers or rheological additives, which are currently inaccessibly due to their high viscosities and surface activity and the associated stability problems of NZ435 or comparable enzyme preparations.

## Experimental Section

### Materials

All reactants were obtained from Evonik Goldschmidt GmbH (Germany) and Sigma Aldrich (Germany) and were used without further purification. Novozym 435 and "Flavostar" Laccase were received from Novozymes A/S (Denmark), LCAHN from Sprin Technologies (Italy), Esterase ERO from Fluka (Germany) and IMMALC350 from Chiral Vision (Netherlands). *silcoat*-Catalysts were prepared as described in literature.[11] .

### General Set-Up for Repetitive Synthesis

In comparative studies between native and *silcoat*-NZ435, the same amount of catalyst related to NZ435 was applied. All calculations are referred to the amount of native biocatalyst included in the preparation.

***Esterification of myristyl alcohol with myristic acid (MM) in STR:*** Equimolar amounts of myristyl alcohol and myristic acid (1.035 mol) were melted and filled in a three-neck, round-bottom flask. The solution was stirred with an Intermig mixer at a speed of 350 rpm. The reaction was started by addition of biocatalyst (1% w/w) at 60 °C. Vacuum was applied for water removal. Samples were withdrawn from the round flask, acid values were measured to follow the initial enzyme activity and the conversion of the esterification. After 24 h and full conversion, the product was removed by filtration and fresh substrate mixture was added to the enzyme to start consecutive cycles.

***Esterification of PEG 400 and coco fatty acid (monoester) in fixed-bed reactor:*** Equimolar amounts of polyethylene glycol 400 and coco fatty acid C8-18 (700 mmol) were mixed at 60 °C in a round flask connected to a fixed bed column filled with 1.5% NZ435 (weight% related to substrates). The process was started by pumping the substrate mixture through the fixed enzyme bed with a pump rate of approx. 10 mL/min. Reaction water was continuously removed by vacuum. Samples were withdrawn from the round flask, acid values were measured to follow the initial enzyme activity and the conversion of the esterification. After 24 h, the reaction was stopped at full conversion, the product was removed and consecutive cycles were started with fresh substrate mixture.

***Esterification of EO/PO polyether with oleic acid (diester) in bubble-column reactor:*** EO/PO and oleic acid were mixed at a molar ratio of 1:2 (0.038 mol:0.076 mol) and transferred to a bubble-column reactor having a diameter of 3 cm. Mixing of the substrates and biocatalyst as well as the removal of reaction water was performed with a continuous stream of 0.6 L/min nitrogen. The reaction was started by addition of biocatalyst (2% w/w). Samples were withdrawn from the round flask; acid values were measured to follow the initial enzyme activity and the conversion of the esterification. After 24 h and full conversion, the product was removed by filtration and fresh substrate mixture was added to the enzyme to start consecutive cycles.

### Analysis of Catalytic Activity

***Acid number (MM, PEDE, PEME):*** Acid numbers were determined by titration using a 100 mM solution of KOH in EtOH and phenolphthalein as indicator. Samples (0.5 g) were dissolved in ethanol (50 mL) containing 1% (w/v) of indicator.

***Kinetic resolution of 1-phenylethanol:*** Lipase activity in kinetic resolution was determined using racemic 1-phenylethanol (0.15 mmol) in hexane (1.5 mL) and vinyl butyrate as acyl donor (0.15 mmol). NZ435 (25 mg) and *silcoat*-NZ435 (50 mg) was added and the mixture was stirred at room temperature. Samples were taken after 2 h and diluted in *n*-hexane. Conversion and enantiomeric excess were determined by GC analysis using the chiral column FS Cyclodex β I/P. The detailled GC program is given in the Supporting Information. Retention times were determined by using the commercially available racemic alcohol and the corresponding ester as references. The retention time for (*R*)-α-methylbenzyl butyrate was determined by using enantiopure (*R*)-1-phenylethanol as substrate in the transesterification reaction with vinyl butyrate as acyl donor.

***Esterification:*** Esterification activity of lipase was determined at the formation of propyl laurate. The reactions were performed in sealed glass vessels containing lipase

preparation (20 mg) and a solvent-free equimolar mixture of lauric acid and 1-propanol (16 mmol) at 60 °C. Samples (50 μL) were taken every 5 min over a time range of 25 min and were diluted with decane (950 μL). The product was analyzed by gas chromatography (Shimadzu 2010 equipped with a BTX column from SGE) as described previously.[11]

***Hydrolysis of ethyl N-acetylglycinate:*** For determination of protease (alcalase) activity ethyl *N*-acetylglycinate (3.4 mmol) was dissolved in phosphate-buffer (25 mM, pH 7.5, 25 mL) containing 1% (w/w) Tween 80. The reaction was started by adding the catalyst (100 mg) and performed under stirring at 40 °C. Formation of acetic acid was determined by titration using a 100 mM solution of NaOH.

***Hydrolysis of methyl pentanoate:*** Esterase activity was determined with a solution of methyl pentanoate (3.8 mmol) in phosphate-buffer (1 mM, pH 8, 25 mL). The reaction was started by adding the catalyst (10 mg) and was performed under stirring at room temperature. The formation of pentanoic acid was determined by titration with a 100 mM solution of NaOH.

***Oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS):*** For determination of laccase activity, ABTS (1.8 μmol) was dissolved in phosphate buffer (100 mM, pH 6, 19 mL). The reaction was started by adding the catalyst and was performed under stirring at 37 °C. Changes in absorption were followed spectrophotometrically at 405 nm.

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