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Communications to the Editor

Enzyme Stabilization by Deposition of Silicone Coatings

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Abstract:

One of the major limitations that prohibit the more frequent use of biocatalysis in the production of specialty and bulk chemicals is the insufficient long-term stability of commercially available enzyme preparations. Especially stability in terms of enzyme leaching and carrier integrity under process conditions is still an issue. Herein, we report on the fabrication of enzyme preparations of superb mechanical stability and outstanding stability towards leaching. For the first time, such immobilisates have been obtained by deposition of silicone coatings, available from cheap silicone building blocks under simple reaction conditions. Using an immobilized lipase (Novozyme 435) as a model compound, the obtained coated particles showed activity yields of more than 92%. The outstanding robustness has been proven by showing the stability towards mechanical stress and towards enzyme desorption by subjection to an assay mimicking harsh leaching conditions.

Ground-breaking developments in enzyme technology have in recent years led to a considerably improved efficiency of biocatalysts in chemical syntheses.[1−4] Thus, an increasing number of industrial production processes can now benefit from the energy savings and sustainability of biocatalyzed reactions. As an example, the lipase-catalyzed synthesis of emollient esters as important ingredients of cosmetics has already reached a multitonne scale. The most prominent catalyst in this and many other processes is Novozyme 435 (NZ435),[5,6] a commercial preparation of lipase B from *Candida antarctica* (CALB) on a granular methacrylate carrier.[7] The adsorption of the enzyme on the carrier considerably increases its already high activity and implies a high molecular stability.[8] Nevertheless, the more frequent use of NZ435 in large-scale application is limited by the insufficient long-term stability under industrial reaction conditions. As the enzyme is only noncovalently adsorbed on the carrier, the use of surfactant-like reaction mixtures or some polar organic solvents such as DMSO[9] provokes enzyme leaching, and furthermore, due to the poor mechanical stability of the carrier, the use in conventional stirred tank reactors causes gradual disintegration of the enzyme preparation.[10] The latter effect is illustrated by stirring and thus grinding NZ435 with a magnetic bar for 90 min in lauric acid. Figure 1 (left picture) shows the formation of a flour-like abrasion even after this short application of relatively low shear forces.

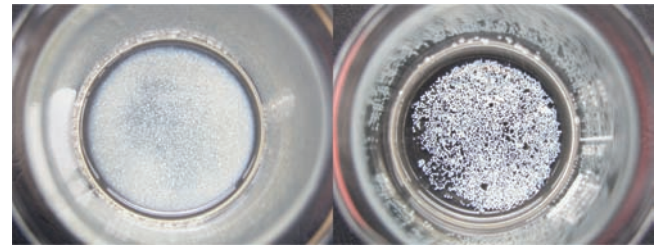

Figure 1. **Native NZ435 (left) and NZ435 with a protective silicone coating (right, NZ435/silicone ratio of 40:60); both after 90 min of strong stirring in lauric acid (60 °C).**

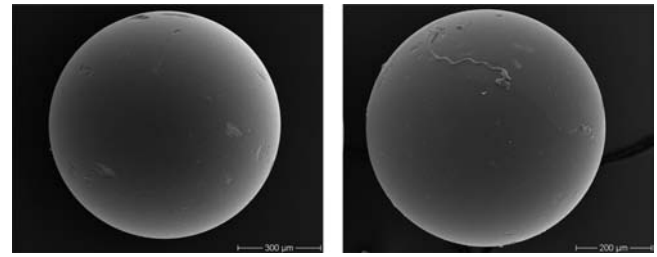

Figure 2. **SEM pictures of native NZ435 (left) and NZ435 with a protective silicone coating (right, NZ435/silicone ratio of 40:60).**

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To overcome leaching problems many people have worked on the stabilization of enzyme immobilisates by covalent attachment of enzymes to the carrier surface[11] or by cross-linking the enzymes with reagents such as glutardialdehyde.[9] Unfortunately, one major drawback of all methods of covalent bonding can be observed quite frequently, which is a significant loss in specific activity. The reason for this is probably a decreased ability to undergo structural changes, necessary to perform induced-fit, on the one hand, and on the other hand a forced disorientation on the carrier surface which restricts the access to the enzyme's active site.

Herein we report on the stabilization of immobilized enzyme catalysts such as NZ435 against leaching and carrier disintegration by a novel technique of coating the solid catalysts with a silicone polymer obtained by a hydrosilylation reaction. As proteins usually do not possess chemical functionalities that react under hydrosilylation conditions, the enzymes are only entrapped in a silicone matrix without being covalently linked, thus having all conformational freedom. Indeed outstanding activity yields can be obtained, so that the catalytic activity after this treatment remains in a suitable range for effective industrial application.[12] Materials used for the coating process are cheap, easily available, and can be tailored to the individual needs of the process by varying silicone chain lengths and the number of modifications. Furthermore, the hydrosilylation reaction used for the coating process is also established on industrial scale and can be easily performed in an economically feasible way.

Coating of NZ435 with different amounts of silicone was achieved by mixing a comb-like Si−H-siloxane with a divinyl-terminated polydimethylsiloxane in the presence of Syloff4000, a [Pt]-based heterogeneous catalyst (Karstedt catalyst). Cyclohexane was added to achieve homogeneous mixing of the viscous solution. Polymerization by hydrosilylation was complete after 3 h curing at room temperature (25 °C).

Particles with ratios of NZ435 to silicone polymer of 50:50 or lower appeared fully coated and highly homogeneous. The determination of particle sizes by sieving analysis showed that silicone-coated particles were not significantly larger than native NZ435 (data not shown). A comparison of SEM pictures of native (Figure 2, left) and silicone-coated NZ435 (Figure 2, right) did not exhibit apparent differences in appearance or shape, and it furthermore seems that the silicone polymer was not deposited as an external layer but permeated into the porous carrier as well. This was confirmed by performing element distribution mapping of Si with EDX on cross sections of silicone-coated NZ435. As evident from Figure 3 the silicone polymer is homogenously deposited throughout the core structure of the entire enzyme-support and fills up the complete particle. As negative control, an EDX-scan of native NZ435 was performed, showing no Si-signal.

As the SEM picture in Figure 2 (right) also indicates, it can be assumed that the coating concerned single particles without causing aggregation. In contrast, particles with ratios of NZ435 to silicone polymer higher than 50:50 appeared to be only partly coated.

NZ435 coated with silicone in a 40:60 ratio showed no signs of abrasion or disintegration of the carrier material after stirring in a viscous solution of lauric acid at 60 °C (Figure 1, right picture). Under the same conditions, native NZ435 was successively crushed to small pieces as indicated by the increasing turbidity of the solution (Figure 1, left picture). The determination of particle size distributions (PSD) of native and silicone-coated NZ435 after stirring in lauric acid for different time intervals impressively confirms the aforementioned results; whereas the particle size of native NZ435 clearly decreased (Figure 4, left), silicone-coated NZ435 remained nearly unchanged (Figure 4, right). Obviously, the coating with silicone considerably improved the mechanical strength and preserved the structural integrity of the particles.The catalytic performance of coated and uncoated NZ435 was investigated in terms of esterification activity towards propanol and lauric acid at 60 °C and expressed as PLU (propyl laurate units). This is an accepted standard in the evaluation of a (bio)catalytic system for the industrial synthesis of emollient and similar long-chain fatty acid esters. The apparent esterification activity of the silicone-coated particles with mass related NZ435/silicone ratios of 70:30 to 40:60 varied between 6.2 PLU/mg_{NZ435} and 5.1 PLU/mg_{NZ435} (Figure 5, black bars), thus corresponding to activity yields of 92−111%, related to the used batch of native NZ435 which had a specific activity of 5.6 PLU/mg_{NZ435}. Surprisingly, coating of native NZ435 with silicones in a ratio of up to 50:50 leads to a small, but significant activity increase, whereas further addition of silicones slightly decreases the observed activity. Nevertheless, even those particles obtained by using

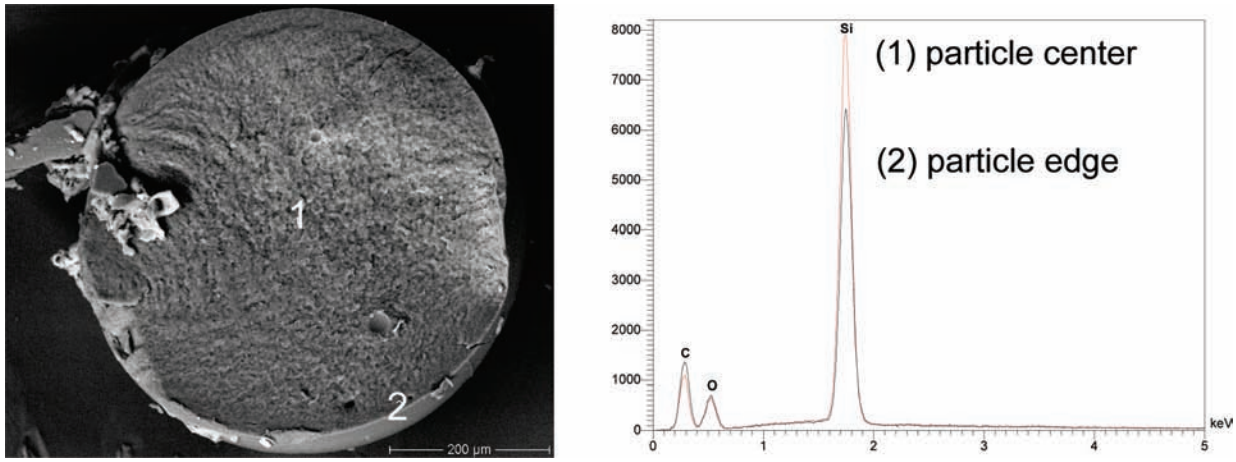

Figure 3. **Element distribution map for silicon (Si) of a cross section of silicone-coated NZ435 (NZ435/silicone ratio 40:60), 1: particle center, 2: particle edge.**

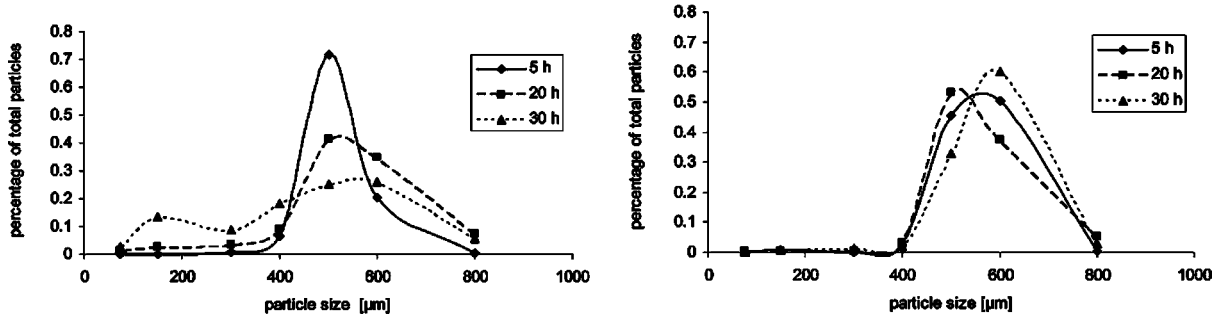

Figure 4. **Particle size distribution (PSD) of native NZ435 (left) and NZ435 with a protective silicone coating (right) after 5, 20 and 30 h of stirring with a magnetic bar in lauric acid at 60 °C.**

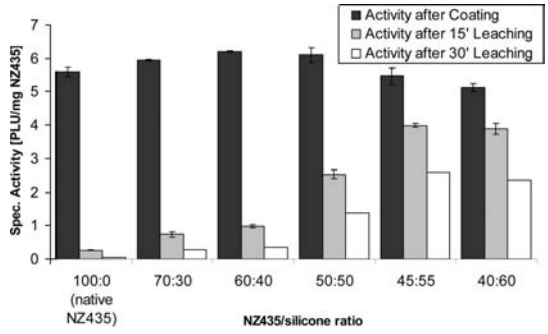

Figure 5. **Activity of native and silicone-coated NZ435 in PLU/mg$_{NZ435}$ of different NZ435/silicone ratios. Activities were determined before (dark grey bars), after 15 min (light grey bars), and after 30 min (white bars), of incubation in 50% ACN/water at 45 °C.**

the higher amounts of silicones yielded a very good activity, thus showing no significant mass transfer limitation by the silicone polymer.

As a fast assay for the determination of enzyme leaching, a simple procedure that causes quantitative desorption of the enzyme from the carrier within short time was used.[14] The enzyme preparations were incubated in 50% ACN/water at 45 °C for 15 and 30 min. This caused an almost complete activity loss when using native NZ435. FT-IR spectrometry showed that the enzyme was indeed leached off the carrier and not only deactivated (data not shown). In contrast, NZ435 particles coated with silicone retained significant activity under the same conditions (Figure 5). At a ratio of NZ435:silicone of 45:55 or lower a residual activity of up to 76% was observed. The applied test conditions, which were chosen to yield a fast and significant result, must be considered as very harsh compared to the average leaching stress in industrial ester syntheses. It can therefore be assumed that these silicone-coated enzyme preparations show a significantly increased half-life time when exposed to reaction conditions of industrial esterification processes. This is currently investigated in our laboratories.

In conclusion, the deposition of fine silicone coatings on immobilized enzymes such as NZ435 allows the generation of catalysts with high mechanical strength, excellent leaching stability, and considerable catalytic performance in industrially relevant esterification reactions. It thus overcomes some of the major restrictions of applying NZ435 to the synthesis of bulk and specialty chemicals in simple stirred-tank reactors. Moreover, the coating is effortlessly achieved, based on cheap, safe and easily available raw materials. It can be expected to be applicable to a whole range of other carrier-bound enzyme systems.

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Experimental Section

Materials. Novozyme 435 (NZ435), *Candida antarctica* lipase B immobilized on a macroporous acrylic resin, was obtained from Novozymes A/S (Bagsvaerd, Denmark); Syloff 4000 was obtained from Ebalta-Kunststoff GmbH (Rothenburg/Tbr., Germany). Silicone building blocks were produced by known procedures.[13] All other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and used as obtained.

V-siloxane:

$$CH_2{=}CH{-}SiMe_2O{-}(SiMe_2O)_{98}{-}SiMe_2O{-}CH{=}CH_2$$

Si−H-siloxane:

$$Me_3SiO{-}(SiMe_3O)_{43}{-}(SiHMeO)_5{-}SiMe_3$$

Coating Procedure. V-siloxane and Si−H-siloxane were mixed in sealable plastic vessels in a molar ratio of 1.1:1 related to the number of reactive groups (Si−H-groups for Si−H-siloxanes and vinyl-groups for V-siloxanes). NZ435, cyclohexane and Syloff 4000 were added consecutively and shaken for 2 min. The mixture was transferred into a bowl of stainless steel and agitated for 20 min. Particles were cured at ambient temperature for 2−3 h, dried, and stored at 4 °C. A more detailed coating protocol is described elsewhere.[12]

Mechanical Stability under Process Conditions. One hundred milligrams of particles was magnetically stirred in a beaker-glass containing 5 mL of lauric acid at 60 °C. The particles were recovered after 5, 20 or 30 h, thoroughly rinsed with acetone, and then used to determine the particle size distributions by sieving analysis. Size exclusions in sieve analysis were 75, 150, 300, 400, 500, 600 and 800 μm.

Determination of Activity and Leaching. The catalytic activity of enzyme preparations was expressed in terms of propyl laurate units (PLU) at 60 °C. Reactions were performed in closed round-bottom flasks, containing about 500 mg of particles and about 30 g of a solvent-free solution of substrates (lauric acid and 1-propanol) at equimolar concentrations. The reaction time was 60 min. Samples of about 500 mg were withdrawn from the supernatant every 5 min, transferred into Erlenmeyer flasks, and diluted with toluene. A few drops of

phenolphthalein were added, and the content of free lauric acid was determined by titration with 0.1 M NaOH. One PLU corresponds to the amount of enzyme that catalyses the production of 1 μmol propyl laurate per minute. The specific activity in this work is related to the amount of NZ435. The leaching stability of enzyme preparations was determined by stirring the particles in 50% ACN/water at 45 °C for 15 and 30 min, respectively. The as-treated particles were recovered, thoroughly rinsed with water and immediately used for determination of remaining activity in terms of PLUs as described above. All measurements were performed in triplicate.

Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDX). SEM was conducted using a Hitachi S-2700 instrument at an acceleration voltage of 20 kV. Samples were fixed on a double-sided adhesive foil. Water was evaporated before samples were sputtered with thin gold films. Element distribution maps were performed by EDX using a Röntec-XFlash detector at beam currents of 20 nA. Cross sections of silicone-coated NZ435 were prepared by quick-freezing under liquid nitrogen and grinding in a mortar. Water was evaporated before samples were coated with a thin layer of carbon.

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