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EUPERGIT Oxirane Acrylic Beads: How to Make Enzymes Fit for Biocatalysis

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Abstract:

Enzyme recycling is essential for the development of large-scale enzyme-catalyzed biotransformations. Recycling is most convenient using enzymes immobilized on solid supports. Although immobilization on solid supports has been pursued since the 1950s, there are no general rules for selecting the best support for a given application. The commercial products EUPERGIT C and EUPERGIT C 250 L have been used for a wide variety of different enzymes and reactions. The present review draws up a comprehensive application profile of both EUPERGIT carriers. The reader gets (a) examples of biotransformations using oxidoreductases, transferases, hydrolases and lyases immobilized on EUPERGIT; (b) key data of the biotransformations, i.e., scale, yield, purity, and enantiomeric excess; (c) efficiency of the immobilization (% immobilized activity); (d) where appropriate, operational stability of the immobilized enzyme preparations, that is, number of cycles, residual activity; (e) specific advantages of the immobilized enzyme over the free enzyme apart from enzyme recycling, for example, improved stability and selectivity. Thus, the present review can serve as a guideline when selecting a resin for enzyme immobilization. Literature published between 1985 and 2000 is covered.

Introduction

The fine chemicals industry is increasingly using biocatalytic production processes.[1,2] This development is driven by the growing demand for enantiomerically pure compounds by manufacturers of pharmaceuticals and agrochemicals. In general, biotransformations are more specific and selective than conventional chemical processes, and there is no strongly acidic, alkaline, or heavy metal waste to be disposed of. Biocatalysis is, therefore, regarded as an environmentally friendly method for carrying out synthetic organic chemistry. Moreover, biocatalytic routes are in many instances shorter and thus more cost-effective than their chemical counterparts.

Isolation of novel enzymes from natural sources and the development of tailor-made enzymes by molecular biology techniques will further increase the number of enzymes for synthesis. However, if a specialty enzyme is to be used in an economically feasible industrial process, enzyme recycling is essential. Various strategies for enzyme recycling have been devised, amongst them (a) entrapment in enzyme membrane reactors,[3] (b) encapsulation in polymeric gels,[4] (c) cross-linking,[5] (d) crystallisation with cross-linking,[6] and (e) immobilization on solid supports.[7−9] Although immobilization on solid supports has been pursued since the 1950s, there are still no general rules for selecting the best support for a given application. The commercial products EUPERGIT C and EUPERGIT C 250 L are amongst the most extensively studied matrixes for enzyme immobilization.[10] A comprehensive application profile of EUPERGIT C and EUPERGIT C 250 L has been extracted from publications and patents. Hence, the present review can serve as a guideline when selecting a resin for enzyme immobilization.

Properties of Eupergit

Both EUPERGIT C and EUPERGIT C 250 L are microporous, epoxy-activated acrylic beads with a diameter of 100−250 μm. They differ in the content of oxirane groups and in their porosity. While EUPERGIT C has an average pore size of $r = 10$ nm and an oxirane density of 600 μmol/g dry beads, EUPERGIT C 250 L has larger pores ($r = 100$ nm) and a lower oxirane density (300 μmol/g dry beads). Immobilisation of enzymes on these resins is rapid and easy both at laboratory scale and industrial scale. There is no need for additional reagents or special equipment. As a standard procedure, the enzyme is simply dissolved in aqueous buffer and, after addition of the EUPERGIT beads, left to stand at room temperature or at 4 °C for 12−72 h.

Guisan et al. have proposed a two-step binding mechanism for this process.[11] It is assumed that, in the first step, the enzyme is physically adsorbed on the carrier by hydrophobic interactions. This brings amino and thiol groups on the surface of the enzyme in close proximity to the oxirane groups of the carrier. In the second step they react with the oxirane groups by nucleophilic attack. In this way, very stable C−N and C−S bonds are formed. Lasch and Janowski showed that there was no detectable protein leakage from Azocasein-EUPERGIT C conjugates once residual, noncovalently bound protein had been removed.[12]

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(1) Turner, M. K. *Trends Biotechnol.* **1995**, *13*, 253.
(2) Bommarius, A. S.; Schwarm, M.; Drauz, K. *J. Mol. Catal. B: Enzymol.* **1998**, *5*, 1.
(3) Herrmann, G. F.; Kragl, U.; Wandrey, C. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1342.
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(12) Lasch, J.; Janowski, F. *Enzyme Microb. Technol.* **1988**, *10*, 312.

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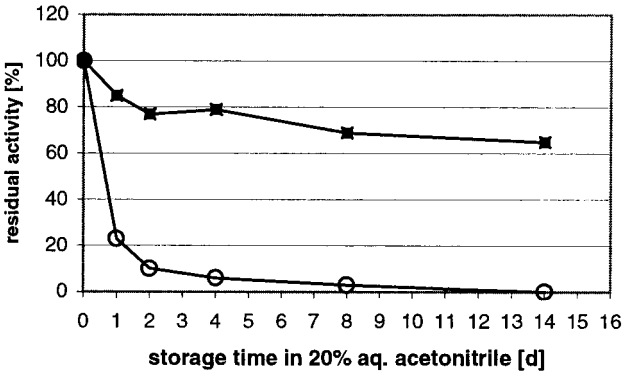
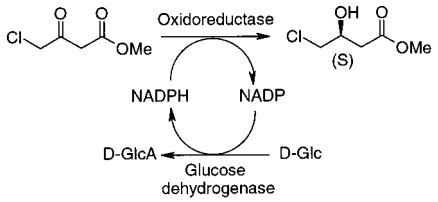


Figure 1. **Residual activity of pig liver esterase after storage in 20% aqueous acetonitrile. ○ free enzyme, ■ enzyme immobilized on EUPERGIT C and stabilized with bovine serum albumine (BSA). Immobilisation: 1 M phosphate buffer pH 7.5, 23 °C, 3 d, then 1 mg BSA per mg enzyme added and mixture stored at 23 °C for another 3 d. Assay: ethyl valerate hydrolysis, pH stat titration (pH 8.0, 25 °C, N_2). Immobilized activity: 60%.**

The high density of oxirane groups on the surface of the support promotes "multipoint attachment". It is assumed that such multipoint attachment leads to an increase in conformational stability of the enzyme and hence improves long-term operational stability of the immobilized biocatalyst.[13] Other advantages of covalent immobilization onto EUPERGIT are increased thermostability,[14] immunisation against aldehydes,[15] and increased stability against denaturing polar organic solvents. The latter is illustrated by Figure 1. In our laboratory, pig liver esterase was immobilized[16] on EUPERGIT C and stabilized by saturation of the remaining oxirane groups with bovine serum albumin.[17] Then the storage stability of the immobilisate in 20% aqueous acetonitrile was determined and compared to that of the native enzyme. After 2 weeks, the residual activity of the immobilized enzyme was still 65%, while the native enzyme had been completely inactivated.

EUPERGIT is a popular resin for the preparation of multiton quantities of biocatalysts for industrial biotransformations.[18] Due to its compatibility with a wide range of different enzymes, it is also frequently used for the immobilization of enzymes in academic and industrial research. Four classes of enzymes are most important for synthetic chemistry: oxidoreductases (E.C.1), transferases (E.C. 2), hydrolases (E.C. 3), and lyases (E.C. 4). All of them have been successfully immobilized on EUPERGIT. The present review describes enzyme-catalyzed biotransformations performed using EUPERGIT for enzyme immobilization. The following information is included: key data of the biotransformations, that is, scale, yield, purity and enantiomeric excess; efficiency of the immobilization (% immobilized activity); where appropriate, operational stability of the immobilized enzyme preparations, that is, number of cycles, residual activity; specific advantages of the immobilized enzyme over the free enzyme apart from enzyme recycling, for example, improved stability and selectivity. Literature published between 1985 and 2000 is covered.

Scheme 1. **Stereoselective reduction of a β-keto ester with oxidoreductase from *Geotrichum candidum***



1. oxidoreductases	
alcohol dehydrogenase	E.C. 1.1.1.2
lactate dehydrogenase	E.C. 1.1.1.27
α-hydroxysteroid dehydrogenase	E.C. 1.1.1.50
β-hydroxysteroid dehydrogenase	E.C. 1.1.1.51
pyranose oxidase	E.C. 1.1.3.10
nucleoside oxidase	E.C. 1.1.3.28
phenylalanine dehydrogenase	E.C. 1.4.1.20
D-amino acid oxidase	E.C. 1.4.3.3
formate dehydrogenase	E.C. 1.2.1.2
2. transferases	
transketolase	E.C. 2.2.1.1
3. hydrolases	
carboxylesterase	E.C. 3.1.1.1
triacylglycerol lipase	E.C. 3.1.1.3
β-glucosidase	E.C. 3.2.1.21
β-galactosidase	E.C. 3.2.1.23
trypsin	E.C. 3.4.21.4
thermolysin	E.C. 3.4.24.27
glutaryl-7-ACA acylase	E.C. 3.5.1.4
penicillin amidase	E.C. 3.5.1.11
aminoacylase	E.C. 3.5.1.14
cytidine deaminase	E.C. 3.5.4.5
2-haloacid dehalogenase	E.C. 3.8.1.2
4. lyases	
oxynitrilase	E.C. 4.1.2.10
Neu5ac aldolase	E.C. 4.1.3.3

Biotransformations with Eupergit

Biotransformations using the enzymes listed above are presented in the order of E.C. classification.

1. Oxidoreductases. 1.a. Oxidoreductase from *Geotrichum candidum.* The cell extract of the fungus *G. candidum* contains a single enzyme that catalyses the stereoselective reduction of β-keto esters to enantiomeric alcohols, versatile intermediates for organic synthesis. Patel et al. reported the reduction of the keto ester shown in Scheme 1.[19] Using whole cells as biocatalysts, a chemical yield of 95% and an optical purity of 96% ee was obtained. The optical purity was increased to 99% ee by heat treatment of the cell suspension at 55 °C for 30 min. The enzyme was isolated from *G. candidum* by standard methods, that is, cell lysis, removal of debris and DNA, purification on DEAE cellulose, and then immobilization on EUPERGIT C. The cofactor NADP

(13) Mateo, C.; Abian, O.; Fernandez-Lafuente, R.; Guisan, J. M. *Enzyme Microb. Technol.* **2000**, *26*, 509.
(14) Cabral, J. M. S.; Kennedy, J. F. In *Thermostability of Enzymes*; Gupta, M. N., ed.; Narosa: New Delhi, 1993; pp 162−179.
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(16) Laumen, K.; Reimerdes, E. H.; Schneider, M. *Tetrahedron Lett.* **1985**, *26*, 407.
(17) Torres-Bacete, J.; Arroyo, M.; Torres-Guzman, R.; De la Mata, I.; Pilar Castillon, M.; Acebal, C. *Biotechnol. Lett.* **2000**, *22*, 1011.
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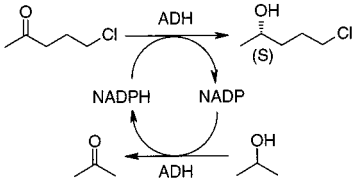
Scheme 2. **Stereoselective reduction of 5-chloropentan-2-one with alcohol dehydrogenase (ADH) from *Thermoanaerobium brockii***

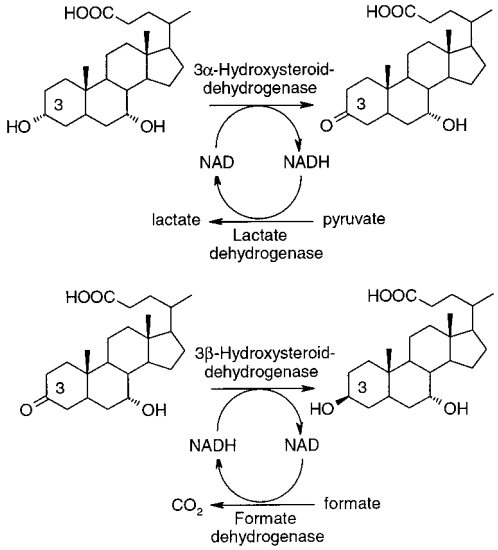


required for these reductions was regenerated by glucose dehydrogenase. The immobilized enzyme gave a slightly lower yield (90%, 300 mg scale), but very high optical purity (>99% ee). Heat treatment of the cells prior to isolation and immobilization of the enzyme was not necessary. Operational stability data are not given. Large-scale syntheses were performed using whole cells, since these cells could be recovered by centrifugation.

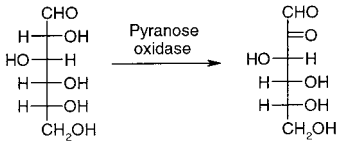
1.b. Alcohol Dehydrogenase E.C. 1.1.1.2. Alcohol dehydrogenase (ADH) from *Thermoanaerobium brockii* is stable at temperatures up to 85 °C and exhibits a high tolerance towards organic solvents. Therefore, it is an attractive catalyst for synthetic organic chemistry. Keinan et al. tried two different resins for immobilization: EUPERGIT C and cyanogen bromide activated CL-Sepharose-4B.[20] Although the immobilized activity on Sepharose was higher than on EUPERGIT C (30% vs 15%), EUPERGIT C was preferred for practical application due to its superior mechanical properties. A column containing ADH on EUPERGIT C was used to reduce aliphatic ketones such as 5-chloropentan-2-one (Scheme 2). The cofactor, NADPH, was regenerated by the coupled substrate approach, in which 2-propanol is oxidized to acetone. Thus, 2-propanol served both as a reducing agent and a cosolvent. The enantiomeric excess of the product, (*S*)-2-chloropentan-2-ol, was 99% ee. Operational stability of immobilized ADH was determined using 2-pentanone as a substrate. The column containing immobilized ADH was operated continuously at 37 °C for 2 weeks with 1% 2-pentanone and 10% 2-propanol. The flow rate was 0.75 mL/min, giving a constant conversion rate of 50%. Next, 2-heptanone was used as a substrate for 1 week. In total, the column was used continuously for 3 weeks. After that period of time, enzymatic activity of the column was still unchanged.

1.c. 3α- and 3*β*-Hydroxysteroid Dehydrogenase E.C. 1.1.1.50, E.C. 1.1.1.51. Riva et al. reported the α → *β* inversion of the C-3 hydroxyl group of bile acids by a two-step procedure.[21] First, the 3α-OH group was oxidized to a keto group with 3α-hydroxysteroid dehydrogenase. Then, the keto group was reduced stereospecifically with 3*β*-hydroxysteroid dehydrogenase to give a 3*β*-OH group. As a representative example, the synthesis of 3*β*,7α-dihydroxy-5*β*-cholan-24-oic acid is shown in Scheme 3. The enzymes were immobilized on EUPERGIT C for multiple use. 3α-hydroxysteroid dehydrogenase was coimmobilized with lactate dehydrogenase (LDH) which was used for cofactor recycling. 3*β*-Hydroxysteroid dehydrogenase was coimmobilized with formate dehydrogenase (FDH). In all cases, immobilized activities were 40−50%. Reactions were performed on an 800-mg scale; yields ranged from 78 to 91%. The reduction of the keto group proceeded with absolute stereospecificity. After three cycles, the residual activities of the four enzymes were as follows: 3α-hydroxysteroid dehydrogenase 70%, LDH 74%, 3*β*-hydroxysteroid dehydrogenase 65%, FDH 75%.

Scheme 3 **α → *β* inversion of the C-3 hydroxyl group of 3α,7α-dihydroxy-5*β*-cholan-24-oic acid with 3α- and 3*β*-hydroxysteroid dehydrogenase**



Scheme 4. **Regioselective Oxidation of D-glucose with pyranose oxidase from *Peniophora gigantea***

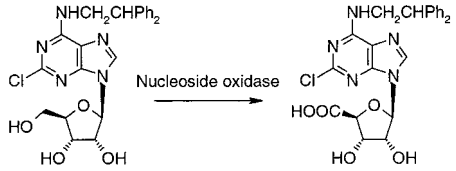


1.d. Lactate Dehydrogenase E.C. 1.1.1.27. The enzyme was coimmobilized together with 3α-hydroxysteroid dehydrogenase (vide supra, 3α- and 3*β*-hydroxysteroid dehydrogenase E.C. 1.1.1.50, E.C. 1.1.1.51).

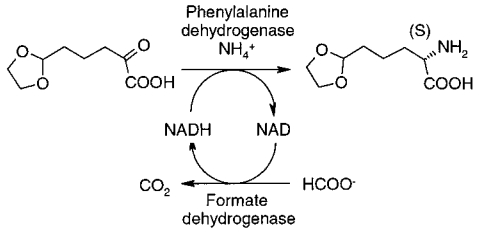
1.e. Pyranose Oxidase E.C. 1.1.3.10. Since carbohydrates are highly functionalized molecules, selective transformations are difficult to accomplish by conventional chemistry. Usually, a multistep protection−deprotection strategy is needed. Not so, using enzyme catalysis. Huwig et al. investigated the regioselective oxidation of D-glucose to 2-keto-D-glucose, catalyzed by pyranose oxidase from *Peniophora gigantea* (Scheme 4).[22] The enzyme was employed immobilized on EUPERGIT C 250 L (immobilized activity 50%). Immobilisation also resulted in a considerably improved operational stability. There was only a 4% loss of activity during one operation cycle (24 h, gram scale). The residual activity after six cycles was 76%. In contrast, the free enzyme lost about 50% of its initial activity during the first cycle. Immobilisation did not change the kinetic parameters, K_M and ν_{max}, or the pH optimum. 2-Keto-D-glucose was isolated in 95−98% yield.

(20) Keinan, E.; Hafeli, E. K.; Seth, K. K.; Lamed, R. *J. Am. Chem. Soc.* **1986**, *108*, 162.
(21) Riva, S.; Bovara, R.; Zetta, L.; Pasta, P.; Ottolina, G.; Carrea, G. *J. Org. Chem.* **1988**, *53*, 88.
(22) Huwig, A.; Danneel, H.-J.; Giffhorn, F. *J. Biotechnol.* **1994**, *32*, 309.

Scheme 5. **Regioselective oxidation of an adenosine derivative with nucleoside oxidase from *Stenotrophomonas maltophila***



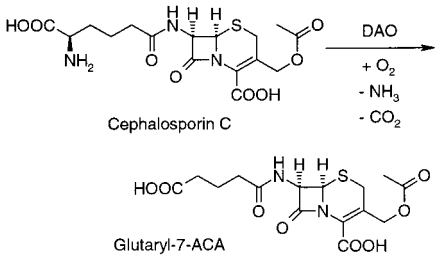
Scheme 6. **Stereoselective reductive amination of an α-keto acid with phenylalanine dehydrogenase from *Thermoactinomyces intermedius***



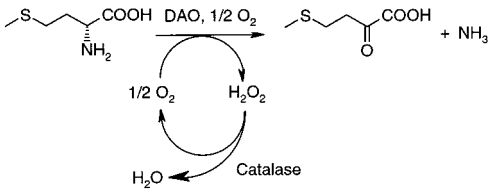
1.f. Nucleoside Oxidase E.C. 1.1.3.28. Another enzymatic carbohydrate oxidation was reported by Mahmoudian.[23] He investigated the oxidation of the adenosine derivative shown in Scheme 5. Chemical oxidation was not entirely regioselective and, therefore, the 2′ and 3′ hydroxyl groups had to be protected. There were also major environmental and handling implications with the use of the chemical oxidant on a large scale. The enzymatic route was more effective and practical. Nucleoside oxidase was immobilized directly from crude homogenates of *Stenotrophomonas maltophilia* (immobilized activity 20−40%, EUPERGIT C). In the presence of quinol, which protects the enzyme against reactive oxygen species, reactions went to completion. The same batch of enzyme could be reused for five cycles. The bioconversion was scaled up successfully to produce multigram quantities of the carboxylic acid.

1.g. Phenylalanine Dehydrogenase E.C. 1.4.1.20. Hanson et al. evaluated the use of phenylalanine dehydrogenase (PDH) from *Thermoactinomyces intermedius* for the reductive amination of the keto acid shown in Scheme 6.[24] The product is a building block for the synthesis of the vasopeptidase inhibitor omapatrilat. The cofactor NAD was recycled via immobilized formate dehydrogenase (FDH) from *Candida boidinii*. While immobilization of FDH proved unsatisfactory (immobilized activity 17%, EUPERGIT C, not optimized), PDH was successfully immobilized on EUPERGIT C 250 L (immobilized activity 41%). Reactions were run in a batch reactor in the presence of dithiothreitol. The enzymes were used for seven cycles, and conversions ranged from 54 to 89% (gram scale). Because the rate of reaction slowed after the sixth cycle, fresh, soluble FDH was added to the seventh batch. After addition of fresh FDH the original reaction rate was restored. From these results it can be concluded that PDH from *Thermoactinomyces intermedius* is suitable for immobilization on EUPERGIT C 250 L and for multiple use.

Scheme 7. **Oxidative deamination of the glutamyl side chain of cephalosporin C with D-amino acid oxidase (DAO)**



Scheme 8. **Oxidative deamination of D-methionine with D-amino acid oxidase (DAO) from pig kidney**



1.h. D-Amino acid Oxidase (DAO) E.C. 1.4.3.3. DAO is employed in the industrial production of 7-amino cephalosporanic acid (7-ACA), a key intermediate for the synthesis of semisynthetic cephalosporin antibiotics. 7-ACA is made from cephalosporin C in a two-stage process. In the first stage, oxidative deamination of the glutamic acid side chain of cephalosporin C by DAO gives rise to glutaryl-7-ACA (Scheme 7). In the second stage (see E.C. 3, hydrolases), the glutarate side chain is cleaved by glutaryl-7-ACA acylase. For the industrial process, utilisation of immobilized DAO is vital for at least two reasons. First, the enzyme has to be easily recoverable and reusable to keep the cost of the biocatalyst as low as possible. Second, after completion of the reaction the crude glutaryl-7-ACA solution should be used directly for the next step without workup. In a patent by Antibioticos S.p.A., immobilization of DAO from *Rhodotorula gracilis* on EUPERGIT C is described.[25] The authors found 28% immobilized activity. The major byproduct of the reaction was ketoadipyl-7-ACA (6%). This could be converted to glutaryl-7-ACA by treatment with a small amount of H_2O_2. The final yield of glutaryl-7-ACA was 91% (30 g scale, 1 h). The enzymatic load of the bioreactor was tested for 100 cycles. The total consumption of immobilized biocatalyst was 32 g/kg product.

DAO can also be used in the synthesis of enantiomerically pure amino acids. The enzyme shows strict enantioselectivity, but low structural specificity. Therefore, it is applicable to the oxidative deamination of the D-enantiomer of alanine, phenylalanine, tryptophan, and methionine (Scheme 8). Nakajima et al. developed a process for L-methionine.[26]

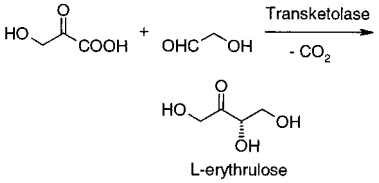
(23) Mahmoudian, M.; Rudd, B. A. M.; Cox, B.; Drake, C. S.; Hall, R. M.; Stead, P.; Dawson, M. J.; Chandler, M.; Livermore, D. G.; Turner, N. J.; Jenkins, G. *Tetrahedron* **1998**, *54*, 8171.

(24) Hanson, R. L.; Howell, J. M.; LaPorte, T. L.; Donovan, M. J.; Cazzulino, D. L.; Zannella, V.; Montana, M. A.; Nanduri, V. B.; Schwarz, S. R.; Eiring, R. F.; Durand, S. C.; Wasylyk, J. M.; Parker, W. L.; Liu, M. S.; Okuniewicz, F. J.; Chen, B.-C.; Harris, J. C.; Natalie, K. J.; Ramig, K.; Swaminathan, S.; Rosso, V. W.; Pack, S. K.; Lotz, B. T.; Bernot, P. J.; Rusowicz, A.; Lust, D. A.; Tse, K. S.; Venit, J. J.; Szarka, L. J.; Patel, R. N. *Enzyme Microb. Technol.* **2000**, *26*, 348.

(25) Cambiaghi, S.; Tomaselli, S.; Verga, R. (Antibioticos S.p.A.) Enzymic process for preparing 7-aminocephalosporanic acid and derivatives; Eur. Pat. Appl. EP 496 993 A1, 1992.

(26) Nakajima, N.; Conrad, D.; Sumi, H.; Suzuki, K.; Esaki, N.; Wandrey, C.; Soda, K. *J. Ferment. Bioeng.* **1990**, *70*, 322.

Scheme 9. **Synthesis of L-erythrulose with transketolase from *Escherichia coli***



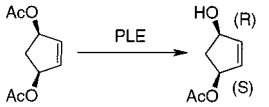
Optically pure L-methionine is widely used as a feed additive, a component of infusion solutions, and a starting material for pharmaceuticals. However, the chemically and enzymatically produced L-methionine often contains a small amount of the D-enantiomer. The D-enantiomer was removed using DAO from pig kidney and catalase from bovine liver in a continuously operated slurry reactor. Immobilisation markedly increased the stability of the two enzymes. When free DAO was left to stand in 10 mM phosphate buffer, containing 50 μM FAD, at 25 °C in the dark without stirring, it was fully inactivated within 15 days. In contrast, DAO immobilized on EUPERGIT C was stable for at least 20 days. The stability of catalase was markedly increased, too. The slurry reactor could be operated for 7 days without any addition of fresh enzyme and coenzyme. Using a 10-mL reactor and a mixture of 99.5% L-methionine and 0.5% D-methionine, product output was 6 mmol/day (L-Met, >99% ee). This corresponds to a space time yield of about 90 g/L/day.

1.i. Formate Dehydrogenase E.C. 1.2.1.2. The enzyme was coimmobilized together with 3β-hydroxysteroid dehydrogenase (vide supra, 3α- and 3β-hydroxysteroid dehydrogenase E.C. 1.1.1.50, E.C. 1.1.1.51).

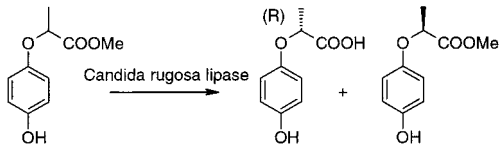
2. Transferases. 2.a. Transketolase E.C. 2.2.1.1. Transketolase catalyses the stereospecific transfer of a two-carbon ketol group to an aldehyde acceptor. The reactants do not need to be phosphorylated, thereby obviating the necessity of subsequent dephosphorylation of the product. The reaction leads to an asymmetric carbon−carbon bond formation, making the enzyme of particular interest for the production of synthetic carbohydrates. Brocklebank et al. used immobilized transketolase (EUPERGIT C, immobilized activity 20%) for the synthesis of L-erythrulose from β-hydroxypyruvic acid and glycol aldehyde (Scheme 9).[27,28] The free enzyme suffers from inactivation by glycol aldehyde. Presumably the aldehyde forms a Schiff base with amino groups that are essential for enzyme activity. When incubated with 0.5 M glycol aldehyde, the enzyme lost 90% of its initial activity within 4 h. In contrast, the immobilized enzyme lost only 5% within 6 h. Since the generation of CO_2 makes the reaction irreversible, complete conversion can be achieved. Reactions were run on an analytical scale only. Operational stability improved with extended immobilization times (data not shown).

3. Hydrolases. 3.a. Carboxylesterase E.C. 3.1.1.1. Pig Liver Esterase (PLE). PLE has found extensive use in the enantioselective hydrolysis of prochiral and racemic esters. Laumen, Reimerdes, and Schneider were able to show that PLE can be conveniently immobilized on EUPERGIT C.[29] The immobilized enzyme proved highly active (immobilized activity 68%) and stable and could be reused many times. Enantioselective hydrolysis of *cis*-1,4-diacetoxycyclopentene (Scheme 10) with immobilized PLE furnished the (1*S*,4*R*)-monoacetate in 59% yield and >98% ee after one recrystallisation (92-g scale, 14 h).

Scheme 10. **Enantioselective deacetylation of *cis*-1,4-diacetoxycyclopentene with pig liver esterase (PLE)**



Scheme 11. **Enantioselective ester hydrolysis with lipase from *Candida rugosa***



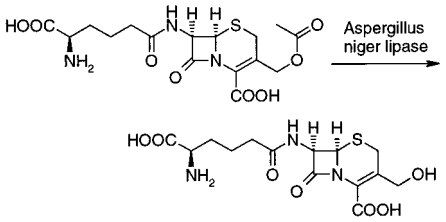
3.b. Triacylglycerol Lipase E.C. 3.1.1.3. *Candida rugosa* Lipase. The lipase isoenzymes CSC-1 and CSC-2 from *C. rugosa* are useful catalysts for stereoselective hydrolyses and syntheses of carboxylic acid esters. A patent by Rhone-Poulenc describes the kinetic resolution of racemic methyl-2-(4-hydroxyphenoxy)-propionate with these enzymes (Scheme 11).[30] Isoenzyme CSC-1 was immobilized on EUPERGIT C (immobilized activity 39%). Using native CSC-1, an enantiomeric excess of 94% ee (R configured acid) was obtained at 46% conversion. Using the immobilized CSC-1, the enantiomeric excess was 94% ee (*R*-configured acid) at 42% conversion (analytical scale). Hence, it can be concluded that the enantioselectivity of CSC-1 is not affected by immobilization. The operational stability was not determined.

3.c. *Aspergillus niger* Lipase. Due to the high reactivity of the β-lactam ring, cephalosporin C is an ideal compound for mild and selective deacylation. Carrea et al. screened 16 lipases for the 3-deacetylation of cephalosporin C (Scheme 12).[31] Lipase from *A. niger* proved most efficient. The lipase was selected for immobilization on EUPERGIT C. Long-term operational stability of the immobilisate was determined using two different reactors, a batch reactor and a packed bed reactor. For each cycle, 50 mg of cephalosporin C was dissolved in 12 mL of phosphate buffer. Deacetylation took approximately 5 days. In the batch reactor, 12 cycles were run without observing appreciable loss of activity. The packed bed reactor, a small column of 1 cm × 4.5 cm, was used continuously for 12 weeks.

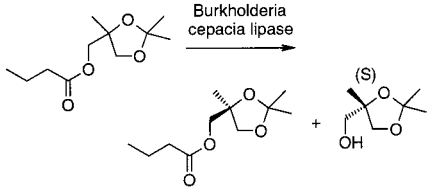
3.d. *Burkholderia cepacia* Lipase. Wirz, Barner, and Huebscher prepared the synthetically valuable chiral synthon

(27) Brocklebank, S.; Woodley, J. M.; Lilly, M. D. *J. Mol. Catal. B: Enzymol.* **1999**, *7*, 223.
(28) Brocklebank, S. P.; Mitra, R. K.; Woodley, J. M.; Lilly, M. D. *Ann. N.Y. Acad. Sci.* **1996**, *799*, 729.
(29) See ref 16.
(30) Cobbs, C. S.; Barton, M. J.; Peng, L.; Goswami, A.; Malick, A. P.; Hamman, J. P.; Calton, G. J. (Rhone-Poulenc Inc.) Lipase isozymes for stereoselective hydrolysis of esters or transesterification. PCT Int. Appl. WO 90 15146, 1990.
(31) Carrea, G.; Corcelli, A.; Palmisano, G.; Riva, S. *Biotechnol. Bioeng.* **1996**, *52*, 648.

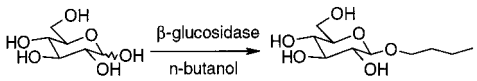
Scheme 12. **Mild hydrolysis of the 3-O-acetate of cephalosporin C with *Aspergillus niger* lipase**



Scheme 13. **Enantioselective hydrolysis of a butyrate protected 2-methyl glycerol derivative with *Burkholderia cepacia* lipase**



Scheme 14. **Synthesis of *n*-butyl-*β*-D-glucopyranoside using *β*-glucosidase from almonds**



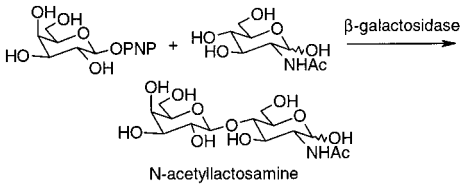
shown in Scheme 13 by lipase-catalyzed kinetic resolution of the respective butyrate.[32] Immobilisation of the lipase on EUPERGIT C turned out to be almost quantitative. However, only 3% immobilized activity was found. Remarkably, this disappointing result was more than compensated by the excellent operational stability. Thus, a column packed with 2.8 g of immobilisate was run continuously for 6 months, and abandoned only for lack of substrate. Product output was >12 g per day, with an enantiomeric excess of 99% ee at 50% conversion.

Similar experiments with lipase from *B. cepacia* were reported by Ivanov and Schneider.[33] They immobilized lipase from *Pseudomonas fluorescens* (identical to the enzyme from *B. cepacia*) on several carriers including EUPERGIT C 250 L. The immobilisates were evaluated using as assay the hydrolysis of tributyrin; 60% of the initial activity of the EUPERGIT conjugate was lost after five esterification cycles, but the residual, moderate activity remained constant. During the following five cycles, no more activity losses were observed.

3.e. *β*-Glucosidase E.C. 3.2.1.21. Otto et al. immobilized *β*-glucosidase from almonds on EUPERGIT C for the glycosylation of *n*-butanol by reverse hydrolysis (immobilized activity 40%, Scheme 14).[34] Reactions were conducted in butanol, and product yields varied according to the water content of the medium. Using the free enzyme, product yield was 8% at 10% water content, but no product was formed at 1% water content. Using the immobilized enzyme, product yield at 10% water content was only 2.5%, but at 1% water content 4% of product was obtained (analytical scale).

(32) Wirz, B.; Barner, R.; Huebscher, J. *J. Org. Chem.* **1993**, *58*, 3980.
(33) Ivanov, A. E.; Schneider, M. P. *J. Mol. Catal. B: Enzymol.* **1997**, *3*, 303.
(34) Otto, R. T.; Bornscheuer, U. T.; Syldatk, C.; Schmid, R. D. *Biotechnol. Lett.* **1998**, *20*, 437.

Scheme 15. **Regioselective *β*-galactosylation of *N*-acetyl-D-glucosamine with *β*-galactosidase from *Bacillus circulans* (PNP = *p*-nitrophenyl)**



Obviously, the immobilization reduces the amount of water needed for enzyme activity in organic solvents. This is clearly an advantage when using the reverse action of hydrolases in synthesis. Operational stability of the immobilisate was not determined.

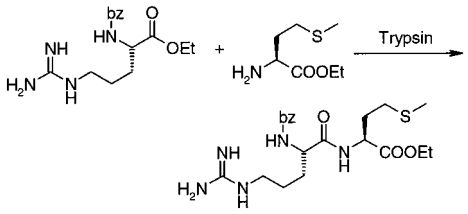
3.f. *β*-Galactosidase E.C. 3.2.1.23. Glycosidases are attractive catalysts for oligosaccharide synthesis. They are readily available and accept a wide range of substrates. Moreover, they are more robust than glycosyl transferases and do not need expensive nucleotide sugars as glycosyl donors. Their major drawback is, however, a lack of regioselectivity. Since regioisomeric oligosaccharides are generally difficult to separate, this hampers extensive use of glycosidases on a larger scale. Several attempts have been made to overcome this problem: (a) variation of the anomeric substituent,[35] (b) cleavage of undesired regioisomers using a second glycosidase,[36] and (c) glycosylation of partially protected glycosyl acceptors.[37] The strategy applied by Hernaiz and Crout to the regioselective synthesis of *N*-acetyl lactosamine is perhaps the most elegant one.[38] Glycosylation of GlcNAc with *p*-nitrophenyl-*β*-D-Gal and *β*-galactosidase from *Bacillus circulans* afforded 30% of the 1,4-disaccharide and 3% of the undesired 1,6-disaccharide (Scheme 15). When using *β*-galactosidase immobilized on EUPERGIT C (immobilized activity 84%), 36% of the 1,4-disaccharide was obtained, and no 1,6-linked product was detected (mmol scale, 2 h). Thus, the immobilized enzyme showed a markedly improved regioselectivity. After four cycles, the residual activity of immobilized *β*-galactosidase was 80%.

In a separate paper, the same group reported on the thermostability of the immobilized *β*-galactosidase.[39] The immobilisate was stored in 50 mM citrate buffer pH 5 at 50 °C. After 250 min, the residual activity was determined in the *p*-nitrophenyl-*β*-D-Gal hydrolysis assay. The immobilized enzyme had lost 25% of its initial activity, while the free enzyme had already lost 85%.

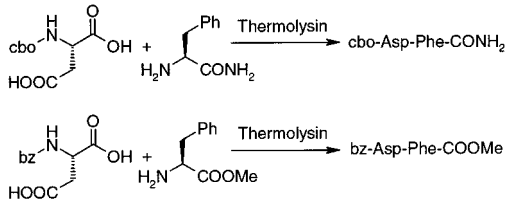
Improved thermostability was also reported for *β*-galactosidase from *Aspergillus oryzae*.[40] The immobilisate was stored in 100 mM acetate buffer pH 5 at 65 °C. After 1 h, the free enzyme was completely inactive (*o*-nitrophenyl-*β*-

(35) Nilsson, K. G. I. *Carbohydr. Res.* **1987**, *167*, 95.
(36) Hedbys, L.; Johansson, E.; Mosbach, K.; Larsson, P.-O.; Gunnarsson, A.; Svensson, S. *Carbohydr. Res.* **1989**, *186*, 217.
(37) Menzler, S.; Seker, H.; Gschrey, M.; Wiessler, M. *Biotechnol. Lett.* **1997**, *19*, 269.
(38) Hernaiz, M. J.; Crout, D. H. G. *J. Mol. Catal. B: Enzymol.* **2000**, *10*, 403.
(39) Hernaiz, M. J.; Crout, D. H. G. *Enzyme Microb. Technol.* **2000**, *27*, 26.
(40) Huzjak, D.; Huzjak, J.; Krizanic, J. *Prehrambeno-Tehnol. Biotehnol. Rev.* **1994**, *32*, 177.

Scheme 16. **Synthesis of a protected Arg-Met dipeptide with trypsin, bz = benzoyl**



Scheme 17. **Thermolysin catalyzed synthesis of aspartame precursors; bz: benzoyl, cbo: carbobenzyloxy**



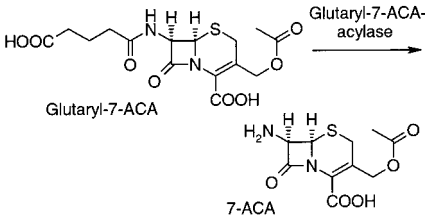
D-Gal assay). In contrast, the immobilisate had a residual activity of 35%. Apart from the increased thermal stability, the immobilized enzyme was also more resistant to denaturation by highly concentrated solutions of urea. After 2 h in 8 M urea at 30 °C, the residual activities of the free and immobilized enzyme were 10 and 70%, respectively.

3.g. Trypsin E.C. 3.4.21.4. Eckstein and Renner employed trypsin and papain in enzyme-catalyzed peptide syntheses.[41] Both enzymes were immobilized on EUPERGIT C and VA-Epoxy Biosynth, the latter being no longer commercially available. For papain, VA Epoxy was more suitable. For trypsin, EUPERGIT C was superior to VA-Epoxy in terms of immobilized activity (exact values not given). The immobilisate could be lyophilized three times without significant loss of activity and maintained its full activity even when being stored at room temperature for three weeks. The dipeptide shown in Scheme 16 was synthesized on a 0.25 mmol scale. Purification was much easier than in the case of the chemically synthesized sample, since the enzymatic approach produced fewer side-products. Operational stability data are not provided.

3.h. Thermolysin E.C. 3.4.24.27. Another useful enzyme for peptide synthesis is thermolysin (TLN). It was immobilized on EUPERGIT C and on aminosilica carriers by Eckstein and co-workers.[42] Due to its superior handling properties, EUPERGIT C was chosen for preparative synthesis. The authors note that the activity of the immobilisate almost doubled when thermolysin was immobilized in the presence of one of the substrates, Cbo-Ala or Phe-COOMe. Possibly the substrates serve to protect the enzyme's active site. The enzyme was used in the synthesis of protected aspartame precursors (Scheme 17). Synthesis with the native enzyme was performed in aqueous medium, since precipitation of the product, which is necessary for reversal of the natural hydrolysis reaction, does not occur in organic solvents. In contrast, synthesis with the immobilized enzyme

(41) Eckstein, H.; Renner, H.-J. *Chem. Pept. Proteins* **1993**, *5/6 (Pt. A)*, 211.
(42) Eckstein, H.; Huttner, D.; Zheng, X. In *Peptides 1998*, Proceedings of the 25th European Peptide Symposium; Bajusz, S., Hudecz, F., Eds.; Akademiai Kiado: Budapest, 1999; pp 130−131.

Scheme 18. **Cleavage of glutaryl-7-aminocephalosporanic acid with glutaryl-7-ACA acylase**



cannot be conducted in an aqueous phase, because the precipitate clogs the pores of the resin. Instead, immobilized thermolysin is stable enough to be used in organic solvents. While the free enzyme was better suited for the synthesis of Cbo-Asp-Phe-$CONH_2$ (free TLN in water: 95% yield, EUPERGIT-TLN in ethyl acetate: 50%), the immobilized enzyme gave higher yields of Bz-Asp-Phe-COOMe (free TLN in water: 50%, EUPERGIT-TLN in ethyl acetate: >95%, scale not reported). EUPERGIT-TLN lost 23% of its initial activity during the first three cycles. Thereafter, the activity remained constant. In total, seven cycles were recorded.

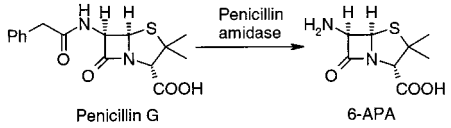
3.i. Glutaryl-7-ACA acylase E.C. 3.5.1.4. As mentioned above, glutaryl acylase catalyses the cleavage of the glutaryl side chain of glutaryl-7-aminocephalosporanic acid (glutaryl-7-ACA, Scheme 18). Nikolov and Danielsson immobilized glutaryl acylase from *P. syringae* on four commercial resins: EUPERGIT C (covalent immobilization via epoxy groups), CPG-10 (covalent immobilization via glutaraldehyde spacer), Amberlite IRA-904 (cross-linking with glutaraldehyde after physical adsorption) and DEAE-sepharose FF (cross-linking with glutaraldehyde after physical adsorption).[43] The highest immobilized activity was obtained with EUPERGIT C (66−72% compared to 20−57% in the case of the other three resins). Reactions were run at 37 °C and pH 7.5 (pH-stat) in a batch reactor equipped with a mechanical stirrer. Each cycle of glutaryl-7-ACA hydrolysis took 80−90 min (scale not given). After six cycles, the immobilized enzyme (EUPERGIT C) had a residual activity of 65%. CPG-10 and Amberlite gave similar activities, Sepharose gave lower residual activities.

Binder, Brown, and Romancik published the immobilization of glutaryl acylase from *Pseudomonas* strain BL072 on EUPERGIT C (immobilized activity 63%).[44] Glutaryl-7-ACA was hydrolyzed in a packed bed reactor at pH 8 (pH-stat control). The substrate solution was recirculated until completion of the reaction (10 g of substrate input, approximately 20 h per cycle). Washing the column after each cycle was found to be necessary to preserve maximum catalyst activity. After 20 cycles (400 h), the residual activity was 60%.

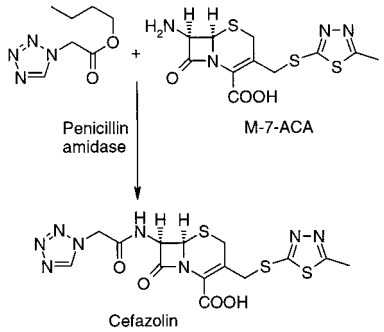
3.j. Penicillin amidase E.C. 3.5.1.11. Penicillin amidase (PcA) is used in the industrial hydrolysis of penicillin G and penicillin V. Several thousand tons of 6-aminopenicillanic acid (6-APA, Scheme 19) are produced each year using this

(43) Nikolov, A.; Danielsson, B. *Enzyme Microb. Technol.* **1994**, *16*, 1031.
(44) Binder, R.; Brown, J.; Romancik, G. *Appl. Environ. Microbiol.* **1994**, *60*, 1805.

Scheme 19. **Hydrolysis of penicillin G using penicillin amidase**



Scheme 20. **Synthesis of cefazolin with penicillin acylase**

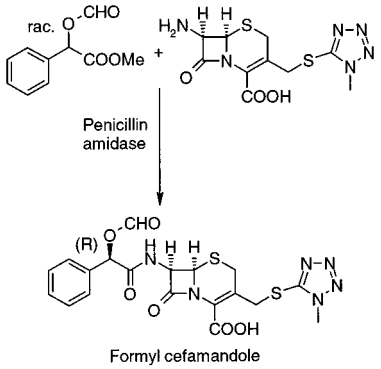


enzyme.[45] Torres-Bacete et al. investigated the immobilization of penicillin acylase from *Streptomyces lavendulae* on EUPERGIT C.[46] It is noteworthy that saturation of the remaining oxirane groups with bovine serum albumin led to a dramatic enhancement in activity. Immobilized activity without BSA was 87%, with BSA, 114%. The BSA-modified immobilisate could be recycled for at least 50 consecutive batch reactions without loss of catalytic activity. Substrate input was 20 mg of penicillin V potassium salt per cycle. The optimal temperature of the enzyme changed after immobilization from 50 to 60 °C.[47]

More recently, penicillin amidase has been applied to the synthesis of semisynthetic penicillin and cephalosporin derivatives. This is accomplished by the reverse action of the enzyme. Basically, the amino group of the β-lactam nucleus, for example, 6-APA or 7-ACA, is reacted with novel carboxylic acid derivatives. Kostadinov et al. published the synthesis of cefazolin from 3-[5-methyl-1,3,4-thiadiazol-2-yl]-7-ACA (M-7-ACA) and *n*-butyl tetrazole-1-acetic acid ester (BTAA, Scheme 20).[48] Reactions were run with PcA immobilized on EUPERGIT C in a batch reactor (35 °C, pH 6.8, pH-stat, 4 equiv BTAA, water as solvent, mg scale). Each cycle took approximately 165 min. The yield of cefazolin was 56% based on M-7-ACA. The same batch of enzyme was reused for more than 40 batches. After 40 batches, residual activity of EUPERGIT−PcA was 93−95%.

One of the challenges of kinetically controlled enzymatic syntheses is terminating the reaction at the optimal point of conversion, i.e., before the hydrolysis of the product becomes faster than its formation. Park, Lee, and Ryu found that, for the above synthesis of cefazolin with EUPERGIT−PcA, product hydrolysis can be slowed considerably by using a two-phase medium consisting of ethyl acetate and buffer (4 equiv of BTAA).[49] The maximum yield of cefazolin (45%, mg scale) was obtained after 12 h. Between 12 and 23 h the yield remained essentially unchanged. When the reaction was conducted in water, the highest yield was observed after at 2.5 h. After 5 h, half of the cefazolin formed had been rehydrolyzed.

Scheme 21. **Diastereoselective synthesis of formyl cefamandole with penicillin acylase**



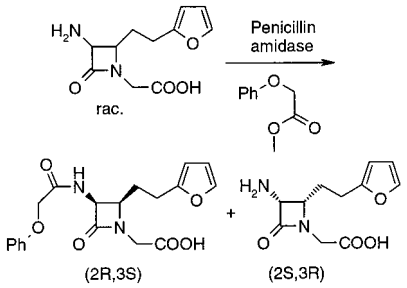
Penicillin amidase can distinguish between the enantiomers of racemic carboxylic acid derivatives. This can be exploited in the synthesis of diastereomerically pure cephalosporin derivatives from racemic carboxylic acids. Fuganti et al. reported the synthesis of formyl cefamandole, the therapeutically employed form of cefamandole.[50] EUPERGIT−PcA-catalyzed synthesis from methyl-*O*-formyl-D,L-mandelate and the appropriate cephalosporin nucleus affords exclusively formyl cefamandole containing the *R*-configured side chain (30% yield, 30 min, batch process, 5 equiv of methyl-*O*-formyl-D,L-mandelate, Scheme 21). Reactions were run on a 1 mmol scale, operational stability was not determined.

Other cephalosporins that have been synthesized using penicillin amidase on EUPERGIT C are cefadroxil and cephalexin. The process is described in a patent held by Novo Nordisk.[51]

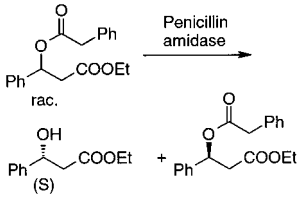
To obtain high optical purities, it is important that the resolution process can be terminated rapidly and easily when the desired degree of conversion has been reached. Immobilized penicillin acylase can be removed instantly by filtration. Therefore, the enzyme has been used for the resolution of a variety of different racemic mixtures. One example is the cis racemic [(2*R*,3*S*),(2*S*,3*R*)]azetidinone shown in Scheme 22, a key intermediate in the synthesis of the antibiotic loracarbef.[52] Enantioselective acylation with EUPERGIT−PcA and methyl phenoxyacetate as the acyl donor furnished the (2*R*,3*S*) amide in 45% yield and with excellent optical purity (100% ee, scale not reported). Operational stability data were not given.

(45) Bianchi, D.; Bortolo, R.; Golini, P.; Cesti, P. *Chim. Ind. (Milan)* **1998**, *80*, 879.
(46) see ref 17.
(47) Torres-Bacete, J.; Arroyo, M.; Torres-Guzman, R.; De la Mata, I.; Castillon, M. P.; Acebal, C. *Biotechnol. Appl. Biochem.* **2000**, *32*, 173.
(48) Kostadinov, M.; Nikolov, A.; Tsoneva, N.; Petkov, N. *Appl. Biochem. Biotechnol.* **1992**, *33*, 177.
(49) Park, C. B.; Lee, S. B.; Ryu, D. D. Y. *J. Mol. Catal. B: Enzymol.* **2000**, *9*, 275.
(50) Fuganti, C.; Rosell, C. M.; Rigoni, R.; Servi, S.; Tagliani, A.; Terreni, M. *Biotechnol. Lett.* **1992**, *14*, 543.
(51) Clausen, K. (Novo Nordisk A/S). Preparation of β-lactam antibiotics by enzymic acylation. PCT Int. Appl. WO 93 12250, 1993.
(52) Zmijewski, M. J.; Briggs, B. S.; Thompson, A. R.; Wright, I. G. *Tetrahedron Lett.* **1991**, *32*, 1621.

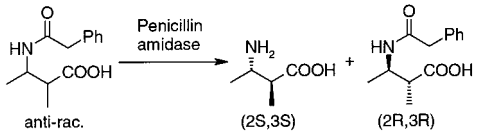
Scheme 22. **Enantioselective acylation of an azetidinone with penicillin amidase**



Scheme 23. **Enantio- and chemoselective hydrolysis of a phenylacetyl ester with penicillin amidase**



Scheme 24. **Enantioselective deacylation of an aliphatic α-alkyl-*β*-amino acid with penicillin amidase**

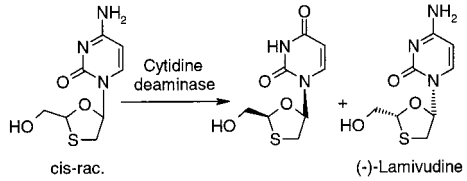


Baldaro et al. resolved secondary alcohols such as the one in Scheme 23 using EUPERGIT−PcA.[53] The racemic phenylacetyl ester was suspended in a mixture of acetonitrile and phosphate buffer (pH 8, pH-stat control, 28 °C). The enzyme was added and the mixture stirred at 200 rpm. When approaching 50% conversion, the reaction was terminated by filtration of the beads, giving rise to the (*S*)-alcohol (enantiomeric excess 98% ee, mg scale). The (*S*)-alcohol is a key intermediate in the synthesis of the antidepressant (*R*)-fluoxetine. Operational stability data were not given.

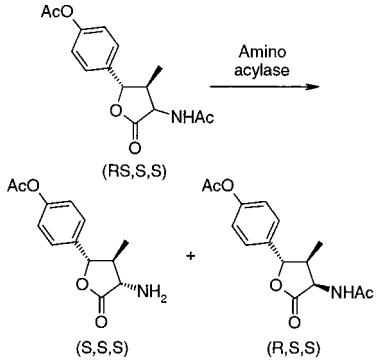
Cardillo, Tolomelli, and Tomasini investigated the enzymatic resolution of *β*-amino acids.[54] The racemic substrate shown in Scheme 24, a small and thus extremely versatile chiral building block, was resolved using EUPERGIT−PcA in a 10:1 mixture of phosphate buffer and ethanol under pH-stat control (pH 7, 30 °C, approximately 5 h). The reaction was stopped at 50% conversion, yielding the (2*S*,3*S*)-*β*-amino acid with high optical purity (100% ee, 100-mg scale). The enantiomeric excess was determined after derivatisation to the corresponding acetamide methyl ester. Operational stability data were not given.

3.k. Cytidine Deaminase E.C. 3.5.4.5. Lamivudine (Scheme 25) was developed as a therapeutic drug for the treatment of HIV.[55] Although both isomers are equipotent, (−)-lamivudine is substantially less toxic than (+)-lamivudine. Research quantities of lamivudine were resolved by

Scheme 25. **Enantioselective deamination of 2′-deoxy-3′-thiacytidine with cytidine deaminase from *Escherichia coli***



Scheme 26. **Concomitant diastereo- and chemoselective deacetylation with aminoacylase from *Aspergillus***



chiral HPLC, but this was not amenable to scale-up. Therefore, Mahmoudian et al. resolved the compound by enantioselective deamination of the unwanted isomer. Initial experiments with free and immobilized cytidine deaminase from *Escherichia coli* showed that enantioselectivity was high (>99% ee). Enzyme immobilized on EUPERGIT C was used for scale-up. Typically, a 500-L batch with 3 kg of racemic lamivudine gave (−)-lamivudine in 76% isolated yield (97% pure, 99.8% ee). The same batch of enzyme was used for at least 15 cycles. The total consumption of immobilized biocatalyst was 111 g/kg product.

3.l. Aminoacylase E.C. 3.5.1.14. Kapeller et al. used aminoacylase from *Aspergillus* sp. immobilized on EUPERGIT C to prepare an optically pure building block for the total synthesis of the nucleoside antibiotic Nikkomycin B.[56] Starting from the [(*RS*),(*S*,*S*)] mixture (Scheme 26), the enzyme carried out two tasks in one step, that is, diastereomeric separation and chemoselective deprotection of the amino group of the desired (*S*,*S*,*S*)-diastereomer (500-mg scale, 48 h). The product was purified by flash chromatography (48% isolated yield, optically pure). Operational stability of immobilized aminoacylase was not determined.

Aminoacylase is capable of cleaving not only amide bonds but also ester bonds. In a comparative study by Liljeblad et al., the enantioselective butanolysis of α-substituted dimethyl succinates and -glutarates by aminoacylase (acylase I) from *Aspergillus* genus (free enzyme), *A. melleus* (free enzyme), and *Aspergillus* sp. (immobilized on EUPERGIT C) was investigated.[57] All biotransformations were performed in neat butanol. The immobilized enzyme proved superior to the native enzymes in terms of activity and enantioselectivity. Gram-scale resolution of the dimethyl ester allowed the

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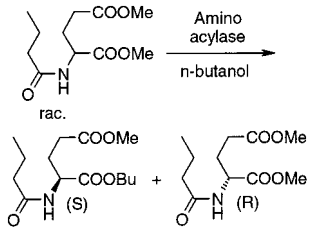
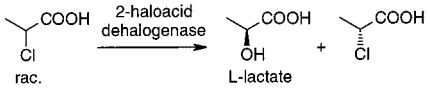
Scheme 27. **Enantio- and chemoselective butanolysis with aminoacylase from *Aspergillus***



Scheme 28. **Enantioselective dehalogenation of 2-chloropropionic acid with L-2-haloacid dehalogenase from *Pseudomonas* strain**

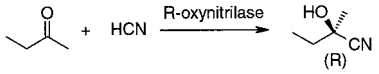


simultaneous preparation of both the unreacted (*R*)-dimethyl ester (42% isolated yield, 91% ee) and the (*S*)-butyl-methyl ester (44% isolated yield, 99% ee, Scheme 27). The operational stability was not determined.

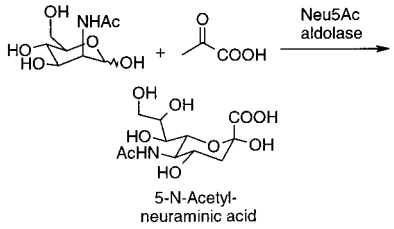
3.m. 2-Haloacid dehalogenase E.C. 3.8.1.2. Ordaz et al. tagged L-2-haloacid dehalogenase from *Pseudomonas* CBS3 with a peptide tail containing six histidines and overexpressed the enzyme in *E. coli*.[58] The His-tagged protein was purified by single-step affinity chromatography on Zn^{2+}-chelating sepharose. The enzyme was immobilized on chelating sepharose and EUPERGIT C, respectively (immobilized activities not reported). The binding capacity of sepharose was limited to 1.2 mg of protein per gram of support. EUPERGIT C was able to bind 20 mg of protein per gram (higher loadings were not attempted). The immobilized enzymes were used for the stereoselective dehalogenation of racemic 2-chloropropionic acid on an analytical scale (Scheme 28). As expected, only L-2-chloropropionic acid was converted, affording L-lactate and D-2-chloropropionic acid. Next, the operational stability of the immobilisates was determined. After 10 cycles the enzyme on EUPERGIT had a residual activity of 70%. On chelating sepharose, the residual activity was only 20%. In addition, covalent immobilization on EUPERGIT C increased the stability of L-2-haloacid dehalogenase at both high temperature and pH. Considering that His-tagging is now an established method in molecular biology to simplify isolation of recombinant proteins, the good results for covalent immobilization of modified L-2-haloacid dehalogenase are very promising indeed.

4. Lyases. 4.a. Oxynitrilase E.C. 4.1.2.10. Albrecht, Jansen, and Kula applied (*R*)-oxynitrilase from *Linum usitatissimum* to the synthesis of enantiomerically enriched cyanohydrins.[59] Cyanohydrins are versatile intermediates for the synthesis of α-hydroxy acids, α-hydroxy ketones, *β*-ethanolamines, and α-hydroxy-α-methylcarboxylic acids. The enzyme was obtained from seedlings and immobilized on EUPERGIT C. The cyanohydrin of 2-butanone was synthesized on a gram scale (Scheme 29). Reactions were conducted in *tert*-butyl-methyl ether saturated with citrate phosphate buffer (50 mM, pH 4, 75% yield, 77% ee). Working at pH 4 is essential for high optical purity, because at neutral or alkaline pH the enzymatic reaction competes with chemical cyanohydrin formation. The half-life of the native enzyme at pH 4 is only approximately 1 h. Immobilisation on EUPERGIT C improved the stability at pH 4 considerably. The enzyme could be reused without apparent loss of activity over a period of several days.

Scheme 29. **Enantioselective synthesis of the cyanohydrin of 2-butanone with (*R*)-oxynitrilase from *Linum usitatissimum***



Scheme 30. **Synthesis of *N*-acetyl neuraminic acid from *N*-acetyl mannosamin and pyruvate using Neu5Ac aldolase from *Escherichia coli***



4.b. Neu5Ac Aldolase E.C. 4.1.3.3. 5-Acetyl-neuraminic acid (Neu5Ac) is a major representative of natural amino sugars. It is incorporated at the terminal position of many glycoproteins and glycolipids and plays an important role in a wide range of biological recognition processes.[60] Mahmoudian et al. published the first enzymatic production of Neu5Ac on a large scale, using the enzyme Neu5Ac aldolase (Scheme 30).[61] To allow reuse of the enzyme, the aldolase was immobilized directly from crude *E. coli* homogenate onto EUPERGIT C (immobilized activity 30−40%). Reactions were performed on a 2-L scale with 40 g of *N*-acetyl-mannosamin (ManNAc). Typically, reactions were complete within 5 h, and the same batch of immobilized enzyme was reused for at least nine further cycles without any significant loss of activity. The process has been used to produce multiton quantities of Neu5Ac for the production of the antiinfluenza drug, Zanamivir.

Conclusions and Outlook

In most of the above examples, oxidoreductases (E.C. 1) and hydrolases (E.C. 3) were used. This reflects the outstanding synthetic potential of these particular enzymes, making them the hottest candidates for industrial application. However, Nature has not made enzymes for use in chemical processes. To fulfill their role in synthesis, they have to be optimized and formulated. The more robust and easy to handle an enzyme preparation, the better the acceptance by organic chemists and the suitability for industrial use. Immobilisation is a proven strategy to improve both handling

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and stability. As can be seen from the above examples, operational stability of enzymes immobilized on EUPERGIT is generally very high. This is most important, for a high operational stability minimises both enzyme and carrier costs. Indeed, the high operational stability makes EUPERGIT an attractive carrier for industrial use. Industrial scale applications of EUPERGIT have so far been reported for penicillin amidase, D-amino acid oxidase, glutaryl-7-ACA acylase, Neu5Ac aldolase and cytidine deaminase. We hope that this review will give an insight into the properties of EUPERGIT and encourage the scientific community to immobilise novel enzymes with interesting properties for fine chemicals manufacturing.

Acknowledgment

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