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Journal of Molecular Catalysis B: Enzymatic 10 (2000) 157–176

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Eupergit® C, a carrier for immobilization of enzymes of industrial potential

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Received 1 July 1999; accepted 27 July 1999

Abstract

Eupergit® C is a carrier consisting of macroporous beads for immobilizing enzymes of industrial potential for the production of fine chemicals and pharmaceuticals. Various enzymes immobilized on Eupergit® C are reviewed in comparison with other carrier materials in terms of the operational stability of the respective biocatalysts at substrate concentrations realistic for industrial production. Other aspects of relevance in that field, such as the demand for purity of enzyme to be immobilized or type of reactor optimal for a given application, are also discussed. An automatic reactor simulating, at laboratory scale, the performance of an industrial stirred tank reactor (STR) is described, and its utilization for evaluating the performance of immobilized enzymes is shown.

Keywords: Eupergit® C; Other carriers; Immobilized enzymes; Biocatalysts; Operational stability; Automatic reactor; Simulation; Industrial performance

1. Introduction

Enzymes in their native form have been used for centuries in the food industry and more recently have found application in the pharmaceutical and chemical industries. Their structure and mode of action are gradually being elucidated by well-known experimental methods, such as the classical X-ray and novel nuclear magnetic resonance (NMR) techniques. Modern genetic engineering techniques make possible the production of enzymes in large quantities and modification of their primary structure, thus altering some of their physico-chemical and biological characteristics. Of great significance are the recent techniques of directed evolution which permit, via DNA shuffling techniques, the preparation of custom-made enzymes, i.e. enzymes working at extreme pHs and temperatures, as well as in the presence of organic solvents.

Sales of enzymes for use in the food, detergent, specialty chemical and diagnostic industries amounted to ~US$700 million in 1992, rose to US$1 billion in 1998 and according to predictions these sales will continue to grow by 10% each year.

Of particular interest is the increasing use of immobilized enzymes, defined by Katchalski-Katzir at the first Enzyme Engineering Confer-

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1381-1177/00/$ - see front matter
PII: S1381-1177(00)00124-7

ence, held at Henniker, NH, USA, in 1971, to denote 'enzyme physically confined or localized in a certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously.'

Enzymes can be immobilized by:

(a) crosslinking, e.g. with glutaraldehyde,
(b) adsorption to a solid carrier matrix,
(c) adsorption to a solid carrier material with subsequent crosslinking,
(d) covalent binding to a solid carrier material,
(e) other techniques, such as entrapment into fibers, microcapsules or membranes, adsorption to membranes, or by compartmenting into membrane reactors,
(f) enzyme crystallization, with or without crosslinking.

The main technical advantage of immobilized enzymes is that they allow heterogeneous catalysis of enzymatic reactions. There are some other advantages, such as economic, ecological and toxicological ones, which will be outlined later in this review.

Since the pioneering work on enzyme immobilization and on immobilized enzymes in the early 1960s [1–3], more than 10,000 papers and patents have been published on this subject, indicating the considerable interest of the scientific community and industry in this field. In a review by Katchalski-Katzir [4] published in 1993 on the industrial applications of immobilized enzymes, flow schemes were presented on the (a) continuous isomerization of glucose and production of high-fructose corn syrup (HFCS) by immobilized glucose isomerase, as developed by Clinton Corn Producing, USA [5–7], and (b) continuous production of L-amino acids by immobilized aminoacylase.

The first industrial application of immobilized enzymes was reported in 1967 by Tosa et al. [3] at the Tanabe Seiyaku in Japan who developed columns of immobilized *Aspergillus oryzae* aminoacylase for the resolution of synthetic, racemic DL-amino acids into the corresponding, optically active enantiomers. Around 1970, two other immobilized systems were launched on a pilot-plant scale. In England, immobilized penicillin acylase, also referred to as penicillin amidase, was used to prepare 6-amino penicillanic acid (6-APA) from penicillin G or V, and in the USA, immobilized glucose isomerase was employed to convert glucose into fructose. These successful industrial applications prompted extensive research in enzyme technology, leading to a steady increase in the number of industrial processes based on sophisticated, immobilized-enzyme reactors.

But apart from such exceptions, only little has been made public on the industrial details of biocatalytic procedures using immobilized enzymes. For example, although it is commonly known that from about 1970 through 1980 the total world production of 6-APA had been switched from chemical methods to biocatalytic methods using immobilized enzymes, even today there are no details available on the respective procedures used by the various companies involved, such as Biochemie Kundl, Beecham, Bristol-Myer-Squibb, Gist-Brocades or Antibioticos. This is, however, understandable, as an industrial producer would never pass on to others his know-how on his catalysts. Consequently, academia can only 'guess' at what is really going on in industry in terms of immobilized enzymes, the methods of immobilization and how these catalysts are being used in the respective production procedures employed.

In this review, we will focus on methods and materials available for the successful industrial scale-up of such immobilized enzymes. Within this context, Eupergit® C has been identified as the carrier suitable for covalent immobilization of enzymes for industrial applications. Eupergit® C was developed between 1974 and 1980 by Röhm, Darmstadt, Germany [8,9]. This company, which was originally named Röhm and Haas, was the first to introduce enzyme technology, approximately 100 years ago, into such practical applications as leather tanning, laundry

detergents and wound healing. Its founder, Otto Röhm, also invented in the 1930s the art of polymerizing methyl methacrylate into what is commonly known today as acrylic glass or plexiglass. Thus, for Röhm, developing a carrier for enzyme immobilization on the basis of methacrylate polymerization was a logical progression of these prior arts.

2. Eupergit® C as a carrier for enzymes

Eupergit® C consists of macroporous beads with a diameter of 100–250 μm, made by copolymerization of *N,N′*-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide (Fig. 1).

The procedure was based on a novel bead-polymerization method, developed by Roehm in 1970 [10], in which two immiscible organic phases were employed. Because of its structure, Eupergit® is stable, both chemically and mechanically, over a pH range from 0 to 14, and does not swell or shrink even upon drastic pH changes in this range. Its mechanical stability is outstanding, as it does not show significant attrition after 650 cycles in stirred tank reactors (STR) with substrate volumes larger than 1000 l (Fig. 2). No change in distribution of its particle size was observed when Eupergit® C was stirred under such conditions.

Eupergit® C is highly ‘‘reactor-compatible’’ since almost any common type of reactor, stirred tank or fixed bed can be used, so long as it is equipped with a bottom sieve to withhold the carrier particles. Special ‘‘bio’’ reactors are not needed for Eupergit®.

Eupergit® C binds proteins via its oxirane-groups which react at neutral and alkaline pH with the amino groups of the protein molecules, e.g. of an enzyme to form covalent bonds which are long-term stable within a pH range of pH 1 to 12 (Fig. 1). Eupergit® C can also bind enzyme molecules via their sulfhydril groups and via their carboxyl groups in the acidic, neutral and alkaline pH range [11]. Thus, Eupergit® C can covalently bind enzyme molecules between pH 1 and 12, i.e. it can bind a given enzyme at the pH range where the enzyme is stable and does not lose activity (Table 1).

Due to the high density of oxirane groups on the surface of the beads (600 μmol/g dry Eupergit® C), enzymes are immobilized at vari-

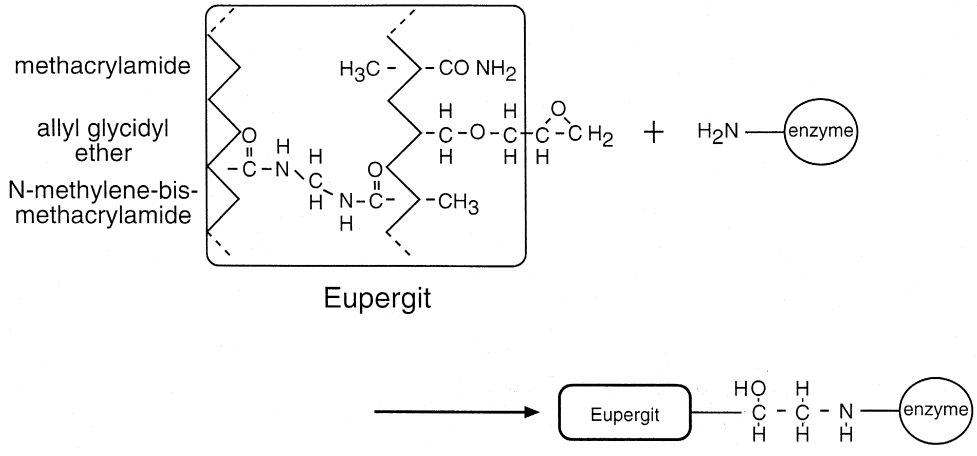

Fig. 1. Structure of Eupergit® C and covalent immobilization of an enzyme.

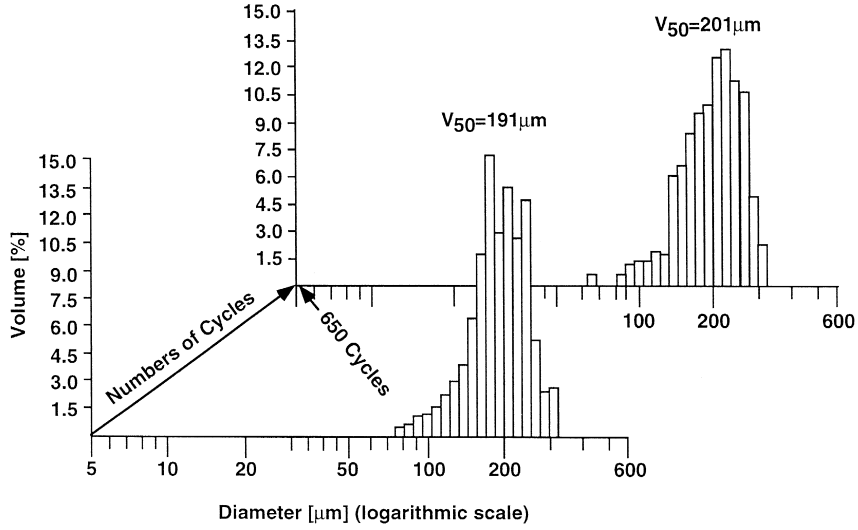

Fig. 2. Particle size distribution of Eupergit®-PcA before and after 650 cycles of industrial penicillin-G splitting (analyzed with a CIS particle analyzer of L.O.T., Israel).

ous sites of their structure. This phenomenon is called ''multi-point-attachment,'' which is considered a major factor for the high operational stability of enzymes bound to Eupergit® C. The procedure of immobilization of enzymes on Eupergit® C is simple: The enzyme is dissolved in buffer, mixed with Eupergit® C and allowed to stand at 20–25°C for 24 to 100 h. The immobilized enzyme is then washed with water and buffer to be used in its subsequent application. The binding capacity of an enzyme is about 100 mg protein/g Eupergit® C (dry weight).

Eupergit® C is commercially available worldwide, and quantities as large as several tons per year have been employed by individual companies for the production of biocatalysts. It has meanwhile also become bench standard for covalent enzyme immobilization in respective R&D activities. Thus, we felt it would not be unjustified to acknowledge these features by placing Eupergit® C into the title of this review,

Table 1
Relative activities of immobilized enzymes on EUPERGIT® C/dependency on pH at binding (from Info EP 3/E, Roehm)

Relative Activities (% of highest activity)						
pH	Pepsin	Trypsin (BAEE)[a]	Phospho-diesterase[b]	Lipase (*C. fluorescens*)	Lipase I (*M. mihei*)	Glucose-Oxidase
4.9	60	80	100	n.d.	100	n.d.
5.3	88	78	92	n.d.	52	n.d.
6.3	100	76	50	61	n.d.	37
7.1	3	100	0	n.d.	46	61
7.7	1	100	0	83	n.d.	81
9.4	0	n.d.	0	100	n.d.	100
10.5	0	n.d.	0	n.d.	n.d.	58

n.d. = not determined.
[a] *N*-Benzoyl-L-arginine ethyl ester hydrochloride.
[b] In Ref. [18].

and thus into the center of our attention. However, other carriers and immobilization methods will also be considered in due course.

In the following we will try to give, via practical examples, an evaluation of the state-of-the-art on:

- Usefulness of Eupergit® as carrier for enzyme immobilization in comparison to other carriers;
- Advantages of immobilized enzymes and immobilized microorganisms as biocatalysts in industry, and the purity of enzymes to be immobilized on Eupergit®;
- An automatic reactor for investigation of biocatalytic reactions, at laboratory scale;
- Insoluble substrates/products compatible with Eupergit® as carriers of immobilized enzymes;
- Measures against microbial contamination;
- Economic considerations, future developments.

Before dealing with these topics, we wish to make a statement on more general aspects. It is commonly accepted that this technology facilitates handling of reactions (e.g. separation of product becomes easy), is in many cases cost saving, and also lowers the ecological burden by minimizing toxic waste problems. There is, however, another aspect, equally or perhaps even more important, namely purity of the product. This aspect may become a key issue in driving product development of pharmaceuticals into increased usage of biocatalysts. Poulsen [12] has shown that chemically produced amoxycillin contains many more impurities than the enzymatically produced material, which is therefore less toxic and less allergenic. Thus, the general belief that products from chemical or enzymatic reactions are identical and of equal quality pertains only to the chemical formula and not to the real facts, namely to impurities resulting from undesired side reactions or solvent residues. For example, even for chemically produced beta lactam antibiotics it was considered a great achievement when chemical synthesis could be carried out in the absence of methylene chloride as solvent. Three years ago Biochemie Kundl launched onto the market an amoxycillin ''Free of Methylene Chloride'' which is a great advance in this field, and certainly of benefit for the patients to be treated with this antibiotic.

3. Eupergit® C in comparison with other carriers

In the Central Research and Development Department of du Pont de Nemours, Seip et al. [13] compared 21 different carriers involving a variety of methods as to their performance in immobilization of glycolate oxidase (Table 2).

When assayed for the stability of the immobilized enzyme in buffer and in reaction, only Eupergit® C and Eupergit® C 250 L qualified as carriers suitable for a stable catalyst. Eupergit® C 250 L has the same chemical structure as Eupergit® C, but has larger pores, while Eupergit® C1Z [14] is a small solid (nonporous) sphere of only 1 μm diameter, showing zero capacity of enzyme binding at the conditions chosen by Seip (see Table 1).

Consequently, Eupergit® C was selected for further studies, namely for co-immobilization of glycolate oxidase and catalase, resulting in a catalyst which was stable under the conditions of the oxidation reaction. In this reaction, glycolic acid and sparged oxygen were reacted in the presence of the coimmobilized enzymes, with flavin mononucleotide as a coenzyme and ethylene diamine (included to protect the product from further oxidation) (Fig. 3). Under optimum conditions 99% yield of glyoxylic acid was obtained.

Seip et al. [13] summarize these findings as follows:

> Many of the deficiencies of the soluble enzymes were eliminated by employing an immobilized enzyme catalyst for the production

Table 2
Immobilization of glycolate oxidase on solid supports (results of du Pont[a])

Immobilization support	Immobilization yield (%)[b]	Immobilized specific activity[c]	Immobilization type
PEI/silica gel (benzylated)	0	0	physical
Amberlite® XAD-4	0	0	physical
Amberlite® XAD-5	4	0.75 IU/g	physical
phenyl agarose	3	0.62 IU/g	physical
Bio-Rex® 70	0	0	ionic
CH-Sepharose® 4B	0	0	ionic
CPG-120 glass beads	16	20.0 IU/g	ionic
CPG-240 glass beads	19	21.0 IU/g	ionic
Celite® R-650	7	9.7 IU/g	ionic
Celite® R-640	6	12.1 IU/g	ionic
CNBr-4% agarose	20	5.0 IU/ml	covalent
CNBr-Sepharose® 4B	25	4.3 IU/ml	covalent
CNBr-Sepharose® 6MB	44	0.68 IU/ml	covalent
PAN-500	19	0.14 IU	covalent
Emphaze™ azlactone beads	10	6.1 IU/ml	covalent
EUPERGIT® C oxirane acrylic beads	17	7.8 IU/g	covalent
EUPERGIT® C 250L	8	5.0 IU/G	covalent
EUPERGIT® C 1 Z	0	0	covalent
epoxy-activated 8% agarose	3	0.14 IU/g	covalent
epoxy-activated Sepharose® 6B	2	0.14 IU/g	covalent
PEI/silica gel/glutaraldehyde	0	0	covalent

[a] In Ref. [13].
[b] Yields based on percentage of immobilized protein that retains enzymatic activity.
[c] IU (International Unit) is the quantity of enzyme which converts 1 mmol/min of substrate under standard assay conditions; specific activity is equal to IU weight or volume of immobilized enzyme.

of glyoxylic acid. The co-immobilized enzyme catalyst exhibited good stability and attrition-resistance under reaction conditions employing stirring and sparging with oxygen under pressure, and the yield of glyoxylic acid at complete conversion of glycolic acid was typically 98–99%. Recovery and reuse of the co-immobilized enzyme catalyst was easily performed by filtering the catalyst from the reaction mixture and recycling it in consecutive batch reactions; in this manner, a total turnover number for co-immobilized glycolate oxidase of ca. 1×10^7 was obtained after 10 cycles of the enzyme. The ability to sparge oxygen through the reaction mixture without denaturing the enzyme catalyst resulted in a significant improvement in reaction rate compared to reactions run without sparging.

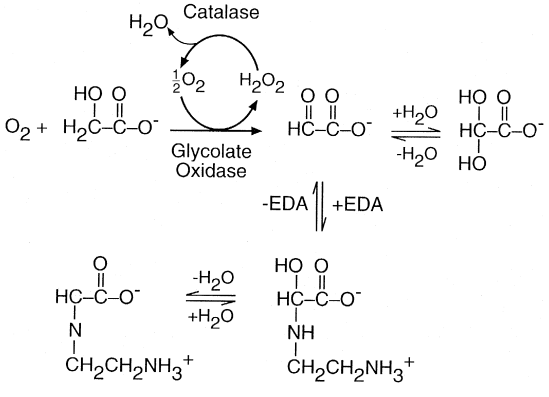

Fig. 3. Enzymatic oxidation of glycolic acid to glyoxylic acid in the presence of a catalase (to destroy the by-product hydrogen peroxide) and ethylenediamine (to limit further oxidation of the glyoxylic acid).

It should be noted that the reaction was carried out at 0.25 M substrate concentration, i.e. at conditions appropriate for industrial production. Furthermore, various reactor types were investigated in this study, with the result that

the STR showed a performance of approximately two orders of magnitude superiority to a fixed bed or a membrane reactor, while the free enzyme was inactivated at 46% conversion after 3 h of reaction time.

Seip's investigation demonstrates the importance of two items for a successful evaluation of the potential of a biocatalyst: First of all, the appropriate reactor type has to be chosen, which in this special case happened to be a STR. And, equally important, the operational stability of the catalyst has to be monitored, at substrate concentrations high enough to allow conclusions for the industrial potential of the system.

A third command to any researcher who wants to claim industrial relevance of his biocatalytic reaction is: Do isolate the product, show the yield in terms of percentage of the theoretical value and of weight, and determine the purity of the product (it is this rule which is most commonly neglected).

A perfectly proper investigation was given by Keinan et al. [15] on asymmetric reduction of ketones with the thermostable dehydrogenase from *Thermo-anaerobium brockii* (TBADH) in which 19 different enantiomeric-pure alcohols were produced from their respective ketones. All these products were isolated and assayed for their purity. For carrier of the enzyme, CNBr-Sepharose showed the higher yield of immobilized activity, but the Eupergit® C-enzyme was more stable and was therefore selected as catalyst for this investigation. For reactor, preference was given to a fixed-bed reactor. The authors state the following advantage in using the immobilized enzyme over the free enzyme:

> The immobilized enzyme approach turned out to be of *crucial* importance in the special cases of unstable substrates or products such as chloro ketones and chloro alcohols, which have limited lifetimes in a homogeneous reaction medium. This stability problem, found for both 5-chloropentan-2-one and its product 5-chloropentan-2-ol was successfully overcome by carrying out the reduction on a column of immobilized TBADH.

For the operational stability, the authors state:

> The stability of immobilized TBADH under operational conditions is indeed remarkable. Following 30 days of continuous operation at 37°C, a TBADH Eupergit® C column used to reduce 2-pentanone suffered no apparent decrease in enzymatic activity. Moreover, changing the influent several times and varying the substrate undergoing reduction likewise had no effect on enzymatic activity.

These reactions were carried out in the presence of 2-propanol serving as a co-solvent and as a regenerator of the coenzyme NADPH for $NADP^+$. It is an attractive feature of TBADH that it can recycle its coenzyme ($NADP^+$) itself (likewise other *sec*-ADHs) (Fig. 4). Thus, *sec*-ADHs were further developed by microbial screening onto a catalyst called BIOCAT 300, which is capable of reducing acetophenone derivatives [16]. This is very important as the major pharmaceuticals with secondary alcohol groups are based around the core structure of *sec*-phenetyl alcohol; e.g. ATENOLOL, used for treatment of cardio-vascular diseases, and ALBUTENOL used for treatment of asthma, with a world market of US$1200 million and US$555 million, respectively in 1995 [16].

Another example, where the stability of the enzyme was considerably increased by immobilization on Eupergit® C, was given by Albrecht et al. [17] for a (*R*)-oxynitrilase. The authors state that the immobilized enzyme could be

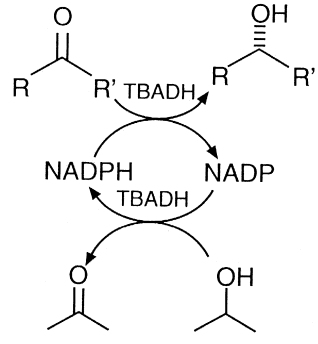

Fig. 4. Reduction of ketones to alcohols catalyzed by TBADH under regeneration of the coenzyme NADPH with 2-propanol.

used repeatedly and without apparent loss of activity for several days. Thus, they used the immobilized enzyme to investigate the substrate range of their reaction, namely the addition of HCN to various ketones, and they produced (*R*)-butanone cyanohydrin on a preparative scale. The reactions were carried out in *t*-butylmethyl-ether.

An even higher operational stability (O-ST) of an enzyme immobilized on Eupergit® C was reported by Keller et al. [18] of Hoechst in 1987. These authors immobilized phosphodiesterase on Eupergit® C at pH 4.5 and used the catalyst in a fixed bed (25 cm diameter, 75 cm depth) for hydrolyzing RNA on a technical scale. They stated that this enzyme column operated at 60°C, and showed no loss of activity after 500 (!) days of continuous operation. This column allowed the production of 10 tons/year of 5′-ribonucleotides, as stated by the authors, who also emphasized the different behavior of the free enzyme. While the free enzyme hydrolyzes both DNA and RNA, the immobilized enzyme hydrolyzes only the latter. Thus, purification of substrate from DNA was not necessary.

Such favorable reports on high O-STs with enzymes immobilized on Eupergit® C are quite common in literature, e.g. for lipases in both aqueous and organic solvent systems, i.e. both in hydrolysis and in synthesis.

Thus, Wirz et al. of Hoffmann La Roche [19] found high stability of a lipase immobilized on Eupergit® C in enantioselective hydrolysis of an ester. Using a small column of only 2.75 g, which they ran for 200 days, they produced more than 12 g product/day, at a substrate concentration of 7%. During these 200 days of continuous operation, they lost only 2% of the enzymatic activity and produced altogether about 2400 g of product, which gives a productivity of 3000 g product/1 g dry Eupergit® C.

Under very tough conditions, namely with triolein for substrate in aqueous 12% triton, a lipase from *Pseudomonas cepacia* (Amano) immobilized on Eupergit® C 250 L, was found to be equivalent, if not slightly superior, to Novo's Lipozyme®, in terms of O-ST (Fig. 5) [20].

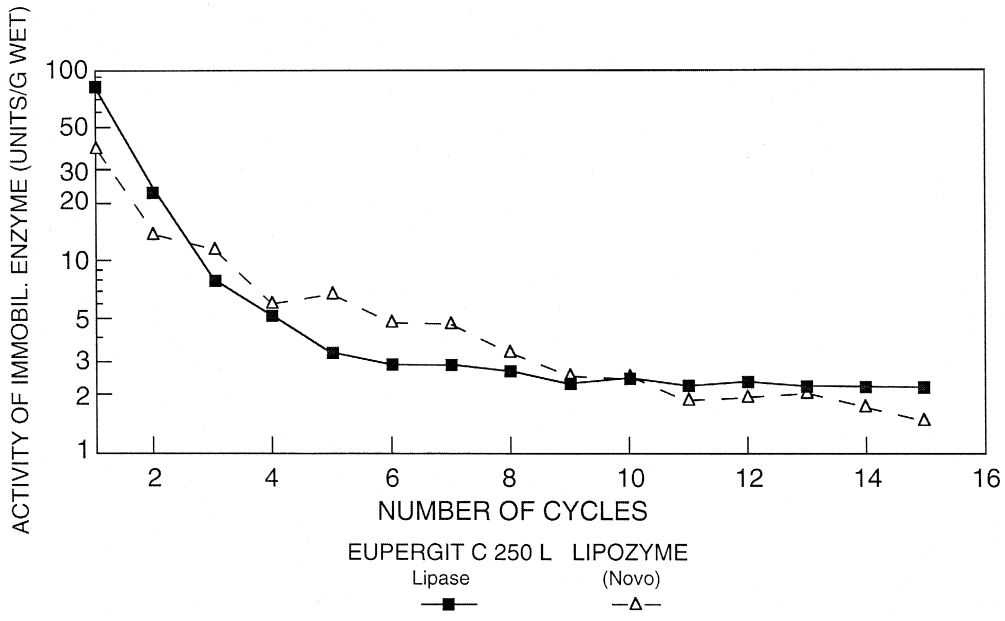

Fig. 5. Enzymatic hydrolysis of triolein in the presence of 12% triton X-100.

Lipozyme® will be presented in greater detail in the following.

Carrea et al. [21] immobilized various lipases on Eupergit® C, and found a lipase from *Aspergillus niger* suitable for hydrolytic removal of the acetyl group in cephalosporin C and two other cephalosporins (Cephalotin and Cefotaxime) which were even faster deacetylated.

The authors state that their Eupergit®-lipase was employed continuously in either a column or a batch reactor for 2 months without appreciable loss of activity.

In ester synthesis in the organic solvent *t*-butylmethyl ether (Fig. 6), Ivanov and Schneider [22] compared the performance of lipase from *P. cepacia* immobilized on five different carriers with respect to activities and operational stabilities. Of these, aminopropylsilica and glutardialdehyde-activated silica showed such low activities that they were excluded from further studies. Celite showed the highest activity, which, however, was lost almost completely after 10 cycles. Octylsilica ($\sim$25% more active than Eupergit®) lost its activity after five cycles, while Eupergit® retained about 30% of its initial activity from cycle #6 through cycle #10. Thus, Eupergit® lipase was again the most stable system in practical application. However, since the initial activity of celite lipase was 20 times higher than that of Eupergit®-lipase and seven times higher than that of the ''free'' enzyme, it would be worthwhile to find a way to stabilize celite-lipase. It is pertinent to note that the ''free'' enzyme in this case was certainly not composed from singular enzyme molecules but was most probably aggregated in its powdered form, insoluble in the organic solvent.

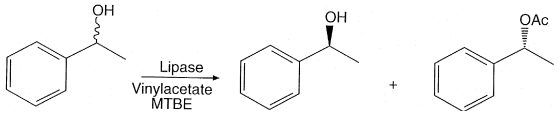

Fig. 6. Stereospecific esterification of 1-phenyl ethanol catalyzed by lipase (*C. fluorescence*).

This high activity of celite-lipase may reflect the advantage of ''singularizing'' the lipase molecules on a large surface area. Perhaps this effect is also the reason for the high activity and favorable performance of a lipase adsorbed to the microporous membrane of hollow fibers in hydrolyzing fish oil in order to remove the saturated fatty acids to obtain glycerides of the dietary beneficial eicosapentaenoic and docosahexanenoic acids [23].

When surveying the literature on enzymatic (trans) esterification, Novozyme® or Lipozyme® are the immobilized enzymes most commonly used. Both of them are marketed by Novo Nordisk. Novozyme® 435 is type B Lipase from *Candida antarctica* adsorbed on an acrylic resin, while Lipozyme® is lipase from *Mucor mihei* immobilized on Duolite A568, which consists of macroporous anionic beads.

For example, Bousquet et al. [24] used Novozyme® for their production of α-butylglycoside lactate via transesterification of the α-butyl-glycoside with a lactate ester in a nonaqueous medium. By removal of the alcohol released during the reaction by vacuum evaporation — by means of which they also kept the water content at a low level — they succeeded in obtaining very high yields (95%) and an acceptable product purity ($>$95%). The relevance for a high yield of product to be obtained by removal of the water which accumulates during an esterification was confirmed by Colombie et al. [25], who used an intermittent airflow system to evaporate water from their reaction mixture which consisted of Lipozyme®, oleic acid and ethanol. We thus realize that besides stable catalysts and proper reactors, measures for in-situ removal of reaction products are of great importance for high conversion rates and good yields.

In the lipase or, generally speaking, in the esterase field, we see a great potential for application of immobilized enzymes, both for research and for industrial development. Millquist-Fureby et al. [26] who used both Novozyme® and Lipozyme® for their studies of re-

gioselective synthesis of ethoxylated glycoside esters using β-glucosidases in supersaturated solutions and lipase in organic solvents give, in this same paper, a review on the use of lipases for the following substrates:

Esters of sugar alcohols, alkyl glycosides and mono- and disaccharides.

For recent reviews, see also Sarney and Vulfson [27] and Riva [28] who reviewed regioselective modifications of carbohydrates using esterases.

According to Secundo et al. [29] *P. cepacia* lipase (PC) can be optimized for catalysis in organic solvents by either immobilizing it covalently on polyethylenglycol (PC-PEG) or use it in the presence of PEG. These preparations display a much higher activity than PC alone, or CLEC-PC [29]. Thus, working with lipase in the field of synthesis needs careful consideration about which form is employed best. Perhaps covalent immobilization on Eupergit® C followed by modification of the Eupergit matrix with PEG might lead to an improved catalyst for synthesis.

4. Advantages of immobilized enzymes and immobilized microorganisms as biocatalysts in industry, in comparison with free enzyme

There is a general rule, found in most reviews and textbooks on enzyme technology, that cheap enzymes must not be used in immobilized form, as this would be too costly for industrial biocatalysis. Instead, the free enzymes should be employed. However, we dare to state here that the time will come when even the cheapest enzyme will be used in immobilized form, simply because of the facile and elegant handling of the reaction, e.g. the almost instant separation of product (which is not contaminated by an enzyme) or the easy automatic control of the reactions within a whole production campaign, if immobilized enzymes of high operational stability are used. Last, but not least, toxicological risks, especially allergic ones, can be minimized by going enzyme-dust-free over many weeks or even several months of production instead of having to prepare the free enzyme component daily, over and over again. Thus, in future, biocatalysis involving soluble substrates and products will be handled via immobilized enzymes, while solid substrates or soluble large substrate molecules, such as cellulose or starch, will be the domain of free enzymes, which will continue to be used in laundry detergents, baking, preparation of fruit juices, tanning of leather and large-scale applications in the food industry.

Immobilized microorganisms with viable or dead cells will continue to be used in procedures dependent on the coenzyme NADH or NADPH. However, most probably they will not enter into many new fields, especially not into those with coenzyme-free applications, as they are basically not clean, and standardization is also a problem. Nevertheless, two industrial applications utilizing immobilized (dead) cells must be mentioned. One of these is the production of approximately 20,000 tons acrylamide/year from acrylonitrile, at the Nitto in Japan by Yamada, utilizing cells of *Rhodococcus rhodochrous* entrapped in polyacrylamide gel [30]. The other process, even much larger in scale, is the production of HFCS by isomerization of glucose to fructose, for which Novo Nordisk (Denmark) had developed the biocatalyst Sweetzyme®. Altogether, an estimated 11 million tons/year of HFCS are being produced by Sweetzyme® and two other biocatalysts.

Biocatalytic production of pharmaceuticals and their intermediates is the growing domain of enzymes immobilized on Eupergit® C. One of the highlights in this field is an efficient process for the production of *N*-acetylneuraminic acid, developed by Mahmoudian et al. [31] at Glaxo Wellcome Research in the UK. Here, the respective aldolase from an overexpressing recombinant strain of *E. coli* was used for immobilization on Eupergit® C. This enzyme did not have to be purified. On the con-

trary, the *E. coli* cells had only to be harvested (by centrifugation), resuspended in buffer and disrupted by passing this suspension through a Manton–Gaulin homogenizer. Then, the homogenate was directly, *without clarification*, mixed with Eupergit® C for 5 days, after which the beads were washed. Typically, a 450-l fermentation yielded 20×10^6 units of aldolase which was immobilized to give 70 kg of wet beads, at an activity yield of 30–40%. The authors state that this catalyst and the process were used to produce multi-ton quantities of *N*-acetylneuraminic acid. This work by Mahmoudian et al. shows the beauty and effectiveness of modern biotechnology, or, as the authors put it: it shows the importance of integrated process solutions for the effective scale-up of biotransformation, namely recombinant technique, enzyme immobilization, product isolation. This product (after some derivatization) may be used as an inhibitor of the passage of a virus, e.g. an influenza virus through the mucosa of the respiratory tract, helping to protect us from influenza.

Thus, direct immobilization of enzymes from crude cell homogenates can be easily carried out with Eupergit® C; after enzyme immobilization the cell debris is simply washed off, revealing the ''self-cleaning potential'' of Eupergit® C. Nevertheless, it is obvious that care must be taken that no contaminants leak out from the immobilized system into the product solution.

We can conclude that with Eupergit® C, no extensive purification of intracellular enzyme is needed. On the contrary, there was a substantial loss of operational stability when a highly purified preparation of penicillin amidase from *E. coli* was immobilized on Eupergit® C. In such a case, stabilization of the catalyst can be achieved after enzyme immobilization when the residual oxirane groups are substituted by reaction with Cleland's reagent (dithiothreitol) [32], which shows another unique feature of Eupergit® C. After enzyme immobilization, the bulk of oxirane groups are still present and can be easily modified to achieve the best ''surroundings'' for the enzyme [33]. Eupergit® has > 600 μmol oxirane groups/g dry beads. When ~ 100 mg enzyme are immobilized on 1 g of beads, less than 1% of these groups are involved in covalent attachment to the enzyme molecule. The residual 99% of the oxirane groups slowly hydrolyze into diol groups. However, in some cases, it is advantageous to modify these groups, which can be done easily with thio-compounds, e.g. with 2-mercaptoethanol (Fig. 7).

Using the same technology, Mahmoudian et al. [34] immobilized recombinant cytidine deaminase on Eupergit® C and presented a production procedure for chiral resolution of the two enantiomers of 2′-deoxy-thio-cytidine, in order to obtain the substantially less toxic isomer for studies of its antiviral potential against HIV (Fig. 8).

The authors state: ''the enzyme was immobilized on Eupergit® C, which allowed it to be reused many times. The biotransformation conditions were optimal so that the best use could be made of the catalyst. A robust scaleable product isolation process was developed to yield the crystalline product. Overall yields through the resolution process of 76% were obtained. All aspects of this process are capable of substantial further scale-up with only minor modification.'' This statement reveals another important advantage of Eupergit® C: Scale-up of immobilization and of reactor performance from

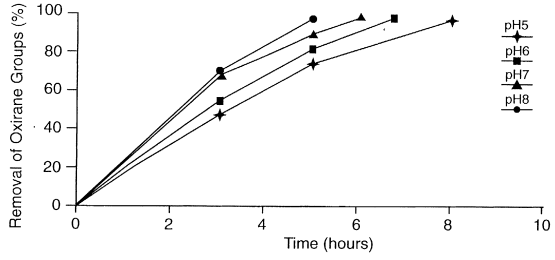

Fig. 7. Removal of excess of oxirane groups in Eupergit® C by reaction with 2-mercapto-ethanol at various pH values (3 g of Eupergit® C ~ 1800 μmol of oxirane groups in 20 ml of 0.1 molar phosphate buffer of the pH indicated in the figure containing 2700 μmol of 2-mercapto-ethanol were shaken at 23°C. Samples were taken for analysis as indicated in the figure).

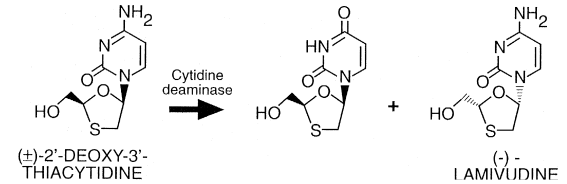

Fig. 8. Production of optically pure Lamivudine using immobilized cytidine deaminase from *E. coli*.

laboratory to industrial scale is very simple, involving only ''minor modifications.''

Extracellular enzymes can also be immobilized without further isolation and purification. All that needs to be done is to remove the biomass by centrifugation, clarify the supernatant by filtration, concentrate it by ultrafiltration, and add 0.1 volume of 1 M potassium-phosphate buffer (of the desired pH), which makes the enzyme solution approximately 0.1 M with respect to this buffer. Enough dry sodium sulfate is added portion-wise to bring its concentration to 1 M. The enzyme is then mixed gently at 23°C for 72–100 h with 10 kg of Eupergit® C in 55 l of this solution. In some cases, additional stabilizing with glutaraldehyde after immobilization can be achieved, but this is optional. The above procedure was patented by Röhm [35] in 1989.

5. An automatic reactor for investigation of biocatalytic reactions on laboratory scale

As an example of the care that must be taken in evaluating the technical performance, we present here, in considerable detail, the procedure adopted for evaluating Penicillinamidase immobilized on Eupergit®.

At the 5th Gordon Conference on Biocatalysis in 1998, Thomas Boller, Andreas Klosendorf and Christian Meier of Röhm presented a poster on a miniaturized automatic STR (Auto-R) for investigation of the performance of biocatalysts. It simulates, in laboratory scale (with 60 ml volume of substrate) an industrial STR with 1000 l of substrate (Fig. 9). This Auto-R was a

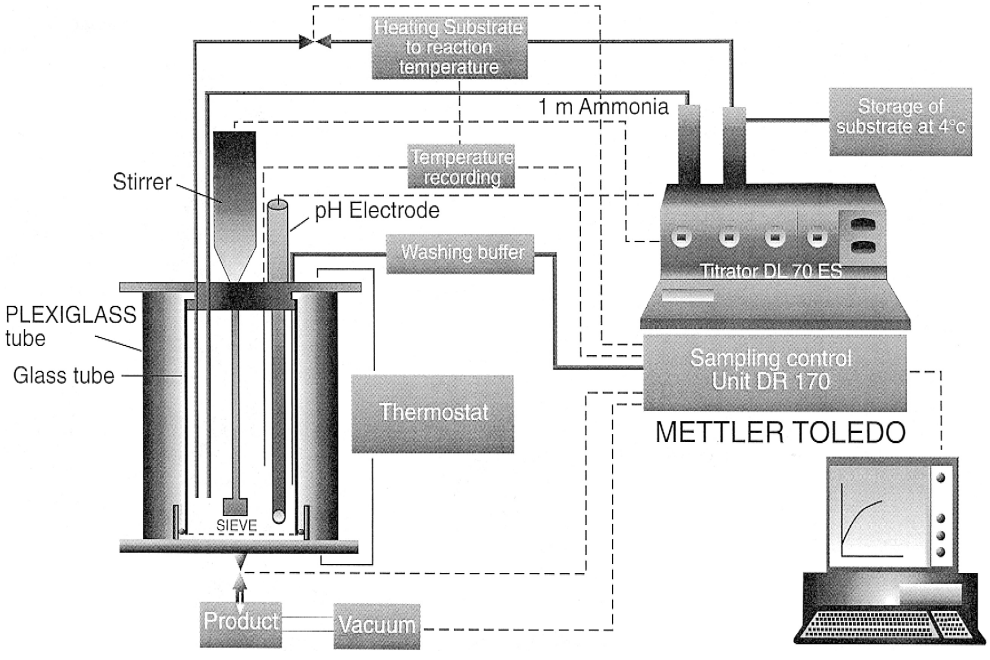

Fig. 9. Automatic reactor (Auto-R) for investigation of the performance of immobilized enzymes (by courtesy of Roehm, Darmstadt, Germany).

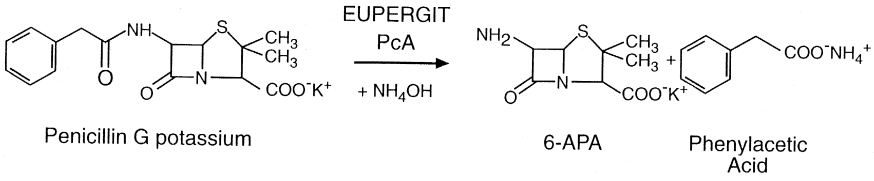

Fig. 10. Enzymatic hydrolysis of penicillin G potassium into 6-aminopenicillanic acid (6-APA) and phenylacetic acid (respectively into their potassium and ammonium compounds).

further development of earlier automatic reactors of Röhm, which had been presented by Kraemer et al. [8] at the European Conferences on Biotechnology in München (1984) and in Florence (1992). Today, the actual Auto-R is commercially available from Nematec [36], while the titrator can be purchased from Mettler-Toledo [37], which also provides technical information. In its core, this reactor consists of a simple glass tube and a bottom sieve, cheap items that can be replaced at almost no cost.

The Auto-R works in the following manner described in the example of the splitting of Penicillin-G (Pen-G) into 6-aminopenicillanic acid (6-APA) catalyzed by the enzyme Penicillin amidase immobilized on Eupergit® C (Fig. 10):

To start an investigation campaign the immobilized enzyme is suspended in buffer and filled into the reactor. Excess buffer is sucked off. Then the automatic program is started. This program transfers the substrate (60 ml of Pen G potassium, at a concentration of 8% in water) from its cold and dark storage place into the pre-warming vessel, and from there into the stirred reactor. At that moment the autotitration system automatically starts to titrate with 1 molar ammonia the phenylacetic acid liberated during the enzymatic hydrolysis of Pen-G, at pH stat control (e.g. at pH 7.8) The amount of ammonia consumed per unit of time is automatically recorded. Thus, a profile of the conversion rate is obtained (Fig. 11). When the conversion has reached approximately 98%, the reaction is stopped automatically (for which the system can be programmed in such a way that it stops a cycle whenever there is no longer a drop of pH, and thus no consumption of ammonia, over a time period of 5 min). Then the product solution is automatically sucked off, while the enzyme remains on the bottom sieve of the reactor. For the next cycle, again 60 ml of substrate are automatically pumped into the pre-warming vessel and then into the reactor. And again the reaction is followed by auto-titration and recording as described for cycle #1. All this is done automatically and all the technician has to do is to prepare fresh substrate every other day.

Thus, 250 consecutive cycles were run continuously over a time period of approx. 300 h or 12 days at exactly the same conditions of pH, temperature, substrate concentration, ratio of enzyme to substrate, as used by Unifar (Turkey)

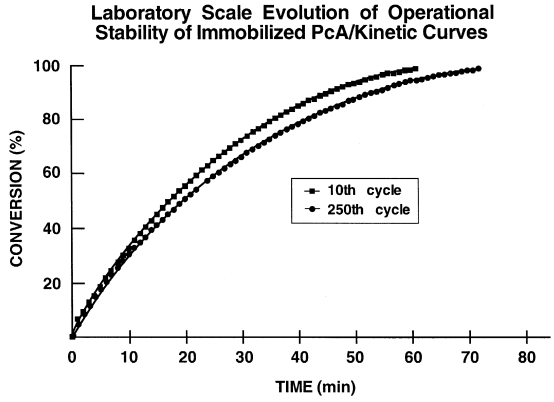

Fig. 11. Conversion rate of Pen-G into 6-APA, as measured in the Automatic Reactor (Auto-R) (by courtesy of Roehm, Darmstadt, Germany).

for industrial Pen-G splitting, at 1000 l scale. The data obtained in the Auto-R were compared with those provided by courtesy of Unifar with the following results (Figs. 12 and 13):

There is considerable similarity between the slopes of the curves obtained for both reactors, when the consumption of ammonia over the first 10 min of the cycles #2–10 is averaged and given a value of ''100,'' and when the averages of the cycles 11–20, 21–30, 31–40 and so forth are plotted against the number of cycles.

Thus, from the results obtained with Auto-R, the performance of an industrial reactor can be predicted with an accuracy of $\pm 10\%$, in terms of its O-ST as to the total number of cycles to be run until 50% of the initial activity is lost. Thus, an estimate can be made of the productivity of the industrial reactor and the number of tons of product it can produce per kg of biocatalyst in one production campaign. The time needed for the industrial campaign is usually longer than the time consumed by the Auto-R, because often in industry there are shut-downs over weekends, and filling and emptying the large volumes of industrial scale takes some time (e.g. at Unifar it takes an additional 20 min/cycle).

Another example for the usefulness of the Auto-R for investigation of reaction conditions is given in Fig. 14a and b, where the effect of increasing the substrate concentration (from 8% to 12% Pen-G K) on the O-ST of the immobilized enzyme and on the time needed for $\sim 98\%$ conversion is shown. Increasing the Pen-G concentration was not advisable, as it resulted in a significant loss of O-ST and a drastic increase of reaction time. Hypothetically, it might have given a higher yield of 6-APA by lowering the loss at its crystallization step (due to its solubility in the mother liquor, which is the final step in its isolation procedure).

O-ST can also be carried out at elevated temperatures by which the number of cycles necessary for a ''production analog'' prediction can be shortened to 25–50 cycles. ''Short-term'' O-ST determination can, for example, be used for evaluation of the quality of a given Peni-

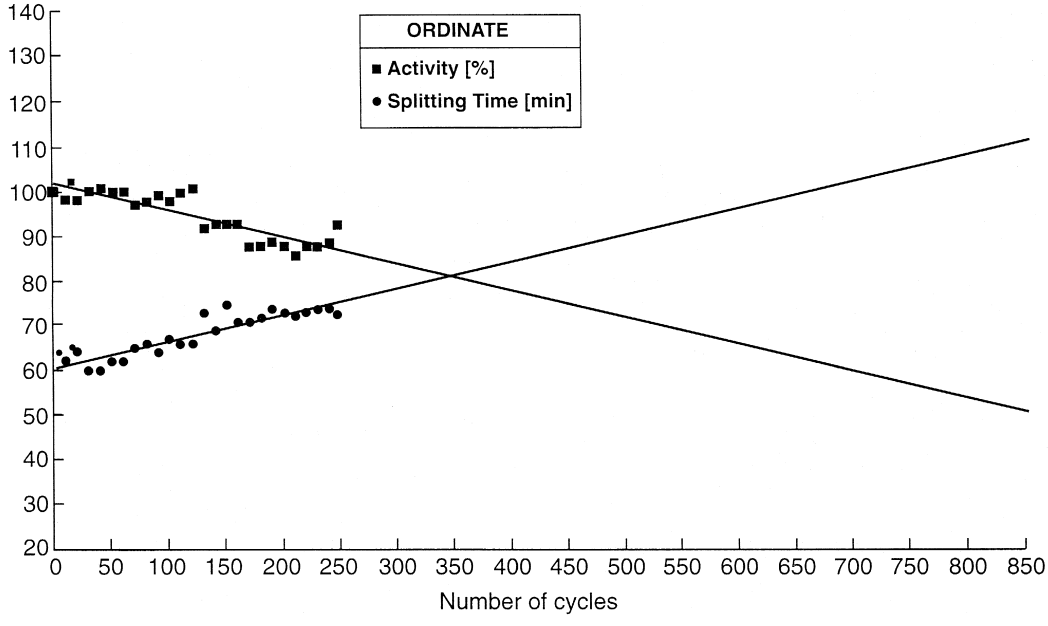

Fig. 12. Splitting campaign of Pen-G into 6-APA catalyzed by Eupergit®-PcA, at laboratory scale carried out in the automatic reactor (Auto-R) showing the profile of activity and reaction time over 250 cycles (data by courtesy of Roehm, Darmstadt, Germany).

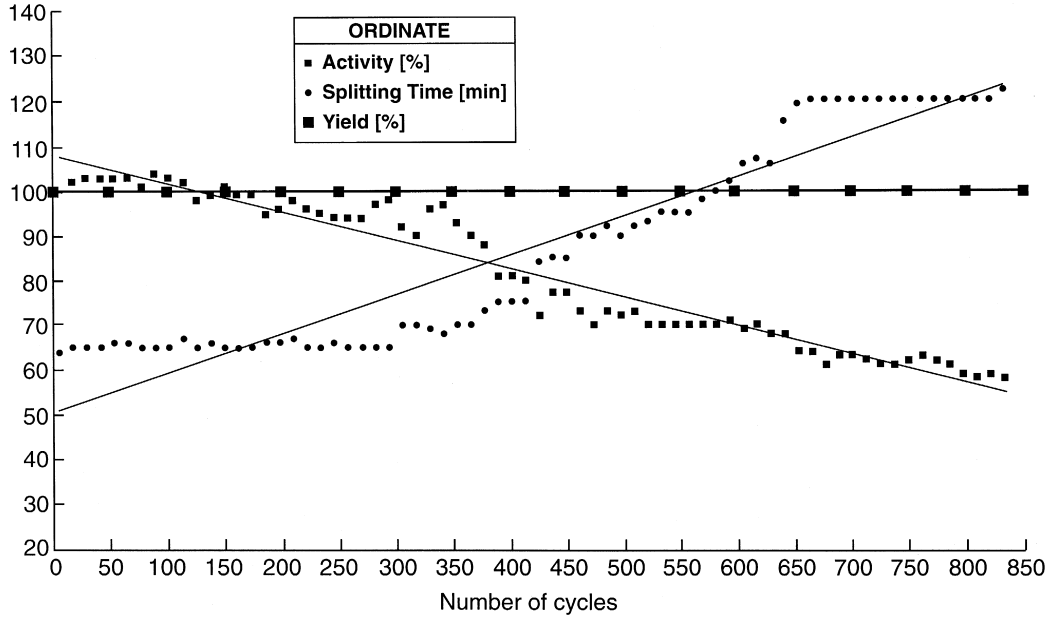

Fig. 13. Industrial splitting campaign showing profile of activity of Eupergit®-PcA, reaction time and yield over 850 cycles (activity and yield from the average of cycles 2–10 having been given the value of ''100%'') (data by courtesy of UNIFAR, Istanbul, Turkey).

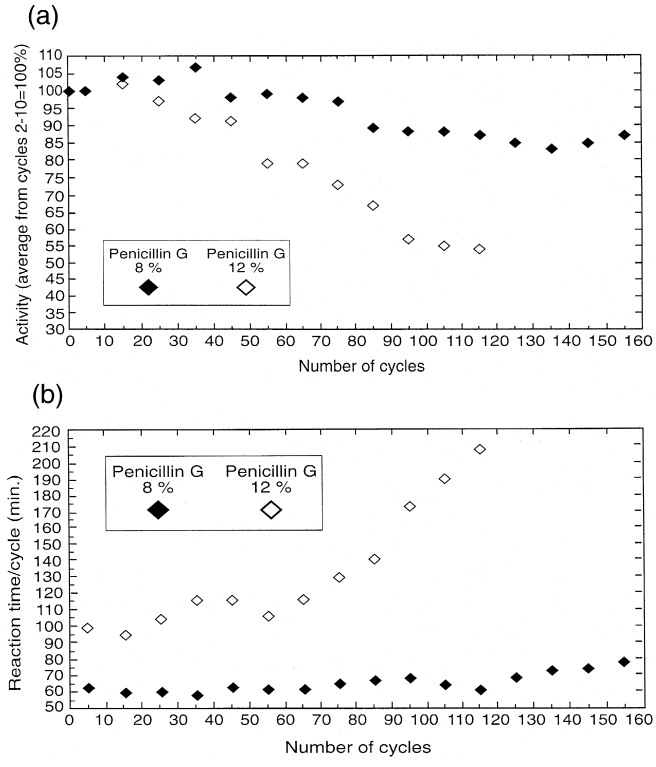

Fig. 14. (a) Dependency of the operational stability (O-ST) of Eupergit®-PcA at splitting of Pen-G K into 6-APA at 8% and 12% substrate concentration, as investigated in the Auto-R (data by courtesy of Roehm, Darmstadt, Germany). (b) Dependency of the reaction time of splitting of Pen-G into 6-APA from the concentration of substrate, as investigated in the Auto-R (same splitting campaign as in (a), data by courtesy of Roehm, Darmstadt, Germany).

cillin lot for 6-APA production. For example, Pen-G (crude) though standardized in terms of its antibiotic activity may contain up to 4% ''odd'' side chain penicillins, which are usually aliphatic acids (instead of phenyl acetic acid). These penicillins would contribute to the antibiotic activity, but they would *not* be split by the immobilized enzyme, which would result in a lower yield of 6-APA and, in a prolonged splitting time; also inhibit the enzyme. Altogether, this would lead to an inferior performance in industrial production and to considerable financial loss. This risk can be avoided if the respective Pen-G is submitted to a short-term O-ST evaluation, and, in addition, to NMR analysis, which detects the odd-side chains easily and instantly.

To summarize: an Auto-R, as presented here, is a useful item for anyone seriously involved in enzymatic splitting of Pen-G, cephalosporin-G or cephalosporin C. Beyond that, the reactor can be used in its present form for investigation of other biocatalytic reactions where acidic or basic products result. However, with some modification of the detection system, which means replacing the pH-electrode with a biosensor measuring directly the products formed, the Auto-R could become very useful for the investigation of the catalytic performance of any immobilized enzyme.

6. Insoluble substrates and products compatible with Eupergit®

According to the findings of Kasche and Galunsky [38], enzyme catalyzed reactions can be carried out ''easily and very rapidly'' in aqueous suspension with up to 25% (w/w) of insoluble substrates and/or precipitate (insoluble) products. Within that context ''insoluble'' means slightly soluble, or sufficiently soluble so that the rate of solubilization of the crystals is equal to or higher than that of the catalytic turnover. The authors emphasize that the addition of organic solvents would not increase the reaction rate to the extent observed for ''insoluble'' substrates.

As an example of an insoluble substrate, the authors presented their synthesis of the protected dipeptide *N*-Acetyl-Tyr-Arg-NH_2 with the insoluble substrate *N*-Acetyl-L-Tyr-ethyl ester, using chymotrypsin for enzyme.

As an example of an insoluble product, the authors chose the hydrolysis of D-Phenylglycinamide using the enzyme penicillin amidase immobilized on Eupergit® C and on other carriers.

They came to the conclusion that immobilized enzymes were generally not suited for this technique, with the exception of Eupergit® C. They argue that the volume of the pores of Eupergit® C is small enough to prevent crystallization of product inside the pores, as the saturation-concentration is never reached while the transport of product molecules into the external liquid phase outside is fast enough. Thus, the Eupergit® C enzyme could be used repeatedly many times without apparent loss of activity, while carriers with larger pore volume such as Eupergit® C250L, or CH-Sepharose (4B) or the former experimental product of Röhm, prep 2918 (coated core) were not feasible for this technology. Prep 2918 consists of a solid spherical core of approx. 300 μm on which Eupergit® C1Z is coated, using a special technique patented by Röhm [39]. For details see Figs. 3–5 of Kasche's original work.

Separation of an insoluble product can be achieved by using a sieve with pores small enough to retain the catalyst, yet large enough to let the product crystals through. In the patent application WO 96/02663 by Gist-Brocades, this principle was utilized for the enzymatic synthesis of amoxycillin. The product crystals of amoxycillin are continuously removed by filtration and subsequent centrifugation (thus shifting the equilibrium of the reaction into the direction of synthesis), while the supernatant (mother liquor) is being recirculated into the reaction tank. The supernatant is re-saturated with substrate by being passed through a feed

tank filled with a slurry of the substrate molecules D-*p*-hydroxy phenyl glycinamide (D-HPGA) and 6-APA. According to the authors' claim, this technique allows reduction of molar excess of side chain substrate (D-HPGA) versus 6-APA from 3:1 to 1.5:1, and leads to amoxycillin of high purity.

7. Coenzyme dependent dehydrogenases / oxidases immobilized on Eupergit® C

Flavinotype coenzymes are usually tightly bound (dissociation constants $< 10^{-8}$) or even covalently linked to their respective enzymes. Thus, it is sufficient to add small amounts of these coenzymes to the substrate. For example, in the case of immobilized glycolate oxidase/catalase (as described by Du Pont researchers) 10^{-5} mol/l flavinmononucleotide (FMN) is added via the substrate to compensate for losses of FMN caused by separation of product solution [13].

NADH or NADPH Coenzymes are usually loosely bound to their enzymes (dissociation constant $> 10^{-4}$) to allow them to quickly switch (oscillate) between their target enzyme molecules and their recharging enzyme molecules. Thus, in the case of a biocatalytic reaction in vitro, considerable amounts of coenzyme would be lost when the production solution is filtered off the immobilized enzyme. To avoid such loss, Kula and Wandrey [40] developed a NADH which is covalently immobilized on polyethylene glycol to be used with formate dehydrogenase (FDH) as the recharging enzyme in membrane reactors. Possibly, Wandrey's NH_2-PEG-NADH could be immobilized on Eupergit® C 250 L following the procedure of Fleminger et al. for NH_2-PEG [41] after immobilization of the target enzyme and the FDH. Perhaps this would result in an efficient NAD^+ regenerating system. An alternate way to solve this NAD^+-regeneration problem may be the development of enzymes that recycle their NAD^+ themselves.

8. Membrane reactor / Eupergit® C1Z

Membrane reactors have been developed and introduced mainly by Kragl et al. [42]. This technology claims that it is not necessary to use solid carriers (such as Eupergit®) for enzyme immobilization, as enzyme molecules can be ''immobilized'' in tanks equipped with membranes of porosities too small to let the enzyme molecules pass; but large enough to let the substrate (or product) molecules enter and leave the reactor. On this basis, Degussa (Germany) has developed an industrial procedure for the production of amino acids in large scale by chiral resolution using DL-acylated amino acid as substrates and L-aminoacylase as enzyme.

Later, Wandrey used Eupergit® C1Z for immobilization of an aldolase to increase the size of this catalyst to the extent that membranes with larger pore size could be used, thus allowing ''unlimited mass transport'' in the catalytic reaction (Fig. 15). A membrane reactor of this kind was operated continuously for 140 h to produce 25 g product/day/l at an enzyme consumption of 12,300 U/kg product [43].

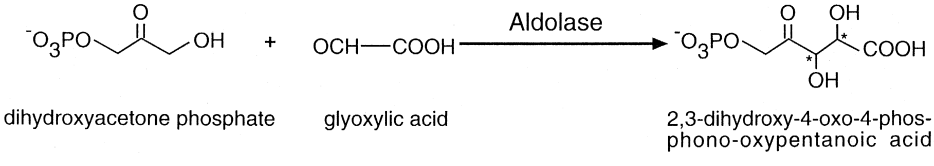

Fig. 15. Enzymatic production of 2,3-dihydroxy-4-oxo-5-phosphono-oxypentanoic acid using aldolase (from rabbit muscle) immobilized on Eupergit® C1Z.

9. Measures against microbial contamination

The matrix of Eupergit® C shows resistance against microbial attacks, as synthetic polymers usually do. In the 15 years since Eupergit has been on the market, no such incident of microbial destruction of the beads occurred. However, biomolecules, in particular proteins, which are immobilized on a solid matrix are feeding grounds for all kinds of microorganisms. Protection against microbes therefore has a high priority in order to maintain the maximum efficiency of any immobilized system. Following are some guidelines for this.

(a) Personnel engaged in preparing enzymes immobilized on Eupergit® must wear sterile gloves and facemasks. Eupergit® C itself comes sterile from its production and is packed to maintain a level of less than 1000 counts of fungi and bacteria per gram of dry material.

(b) Water and buffers used for dissolving the enzyme to be immobilized must be sterile, and should contain an antimicrobial agent, e.g. ethyl *p*-hydroxy-benzoate or any other antibacterial and antimycotic agent compatible with the enzyme to be immobilized and the binding mechanism, which means that agents which would react with oxirane groups must be avoided.

(c) For storage and shipping, preservation conditions must be compatible with the immobilized enzyme, e.g. 60%–80% glycerol slurries, or 30% NaCl, or 500 ppm ethyl *p*-hydroxybenzoate can be used for storage of various enzymes. Sodium azide must *not* be used, as it is much too dangerous an agent and is not even very effective against various microorganisms. Alternative measures, if compatible with the enzyme, are: antibiotics of all kinds, gamma-irradiation or storage in a deep freeze at minus 20°C.

(d) In case of a microbial contamination, most, if not all, microbes can be efficiently killed by the addition of formaldehyde at a final concentration of 0.1%–0.9%. This measure is compatible with most enzymes, as well as with Eupergit®, so long as strictly handled in terms of duration of such treatment, pH and temperature [44]. Safety instructions on handling formaldehyde must be followed.

10. Economic considerations, future development

The productivity of 6-APA in an industrial production campaign, as shown in Fig. 13, was 720 kg 6-APA/kg wet Eupergit®-Penicillin amidase (E-PcA), as this campaign was actually run for 900 cycles, at 1000 l substrate/cycle, containing 8% Pen-G potassium and yielding 40 kg of 6-APA/cycle. The load of E-PcA was 50 kg (wet weight), containing 10 million units of enzyme activity. Thus, 2000 kg of 6-APA/kg of dry Eupergit® C were produced. The cost of the Eupergit® C share is therefore US$0.21/kg 6-APA, which is significantly less than 1% of the value of this product, presently priced at US$40/kg.

In the other examples given in this review, covering a range of hydrolytic and synthetic reactions both in aqueous and in organic solvents, the productivity of the respective Eupergit®-enzymes was usually higher than 1000 kg product/1 kg dry Eupergit® C. Thus, in many cases the cost of Eupergit® C is less than US$0.42/kg product, which is less than 1% of the cost for products priced at US$42/kg, and less than 10% for such products priced at US$4.20/kg. To this, the costs for the enzyme and its immobilization must be added, which should be less than US$2.00/kg of product.

As fine chemical makers are increasingly using enzymatic methods to produce chiral intermediates [45], biocatalysis will grow in the future, as will the use of Eupergit® C as a carrier for enzymes of potential for industrial production, because of its advantageous technical properties. Of course, it has to be kept in mind that to start biocatalytic methods a steep investment must be made in equipment and technology. But, as Alan Shaw of ChiroTech put it in the above review [45], at the end of the day, if

biotransformation works, it will usually deliver the most economic route. We wish to add: not only the most economic, but also the ecologically cleanest and the least toxic route, for the sake of both consumer and maker.

This review has focused on practical applications of enzymes immobilized on Eupergit® C and other carriers for the production of fine chemicals and pharmaceuticals. Aspects of enzyme kinetics and mass transfer effects were neglected. However, they are of great importance, as they may result in substrate or pH gradients which may reduce rate and yield of a reaction, as Tischer and Kasche [46], as well as Spiess et al. [47] have pointed out most recently. These authors also give advice for counter-measures against such undesired effects. Furthermore, an interesting comparison of the kinetic properties of crosslinked crystals and enzymes immobilized on porous beads is also given [46].

Acknowledgements

We wish to thank Dr. Ferhat Ilhan of Unifar (Istanbul); Dr. Christian Meier, Andreas Klosendorf, and Thomas Boller of Roehm (Darmstadt); Dr. Steen Riisgaard, Soren Jepsen and Hans Christian Holm of Novo Nordisk (Bagsvaerd), as well as Professors Maria-Regina Kula (Dusseldorf), Volker Kasche (Hamburg-Harburg), Manfred Schneider (Wuppertal), Christian Wandrey (Juelich), Nathan Sharon (Weizmann Institute, Israel), Gidi Fleminger (Tel Aviv University) for their helpful comments and information.

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