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Harald Gröger **Emine Capan**
Anita Barthuber **Klaus-Dieter Vorlop**

Biocatalytic asymmetric hydrocyanation in the presence of (R)- oxynitrilases entrapped in lens-shaped gels

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Biocatalytic asymmetric hydrocyanation in the presence of (R)-oxynitrilases entrapped in lens-shaped gels

Harald Gröger[1], Emine Capan[2], Anita Barthuber[3], and Klaus-Dieter Vorlop[2]

Dedicated to Dr. Stefan Weiss on the occasion of his 60th birthday

Abstract

This contribution contains a brief review of our results on the recent development of a cross-linked and subsequently polyvinylalcohol-entrapped (R)-oxynitrilase and its application in the asymmetric hydrocyanation. In the presence of this novel LentiKat®-entrapped oxynitrilase as a biocatalyst, and benzaldehyde as an aldehyde component, the desired (R)-mandelonitrile was obtained in good yields and with high enantioselectivities. This new type of immobilized lens-shaped biocatalyst has a well-defined macroscopic size in mm-range, and can be re-used efficiently.

1 Introduction

The fine chemicals industry is highly interested in efficient immobilization methods since immobilization offers a broad variety of advantages, e.g., easy catalyst separation and its reuse, simple work up procedures, stabilization of the biocatalyst and so on. Although numerous immobilization methods have been reported so far, only a few of them were applied on technical scale – In spite of the high potential of immobilized biocatalysts, in a United Nations publication (Klyosov 1989), only eight industrial processes have been reported which are based on immobilized enzymes or microbial cells. The manufacture of 7-aminocephalosporanic acid represents (probably) the most important industrial process which is based on the use of an immobilized enzyme. – which indicates the need for more efficient immobilization technologies.

Recently, a promising, industrially feasible immobilization concept for cells was reported by the Vorlop group (Jekel et al. 1998, Wittlich et al. 1999a & b, Welter et al. 1999) This immobilization is based on the entrapment of cells in a hydrogel matrix consisting of polyvinylalcohol. This method, which has been commercialized by geniaLab® (geniaLab 2001), represents a cheap and efficient immobilization technique. In addition, a negligible catalyst leaching is observed, and macroscopically well-defined and flexible lens-shaped particles with a high activity are obtained (type: LentiKats®; diameter: 3 - 5 mm; thickness: 300 - 400 mm). Several successful applications of this concept were already reported by Vorlop and co-workers (Wittlich et al. 1999b, Welter et al. 1999) and Durieux et al. 2000, e.g. for the synthesis of itaconic acid and 1,3-propanediol. Further advantages are that these lens-shaped hydrogels, which can be easily separated, show no abrasion and have a minimized diffusion limitation due to a low thickness of < 0.5 mm. We envisioned that this immobilization concept could be extended to a suitable immobilization method for enzymes in general, and oxynitrilases in particular. Oxynitrilases represent versatile biocatalysts for the preparation of optically active cyanohydrins which find a wide range of pharmaceutical and agrochemical applications (Effenberger 1994a & b, Gregory 1999, Gröger 2001, Hamashima et al. 2000). For example, (R)-mandelic acid - a derivative of (R)-mandelonitrile - represents a fine chemical which is produced on multi-hundred ton scale.

Several substituted optically active (R)-mandelic acids are intermediates in the synthesis of pharmaceuticals. In addition, the (S)-enantiomer of phenoxybenzyladehyde cyanohydrin represents an intermediate in the production of enantiomerically pure pyrethroids which are used as insecticides. A graphical summary of cyanohydrin applications is given in Figure 1.

The immobilization of oxynitrilases as biocatalysts for the synthesis of chiral cyanohydrins has been known for a long time. However, many of those immobilization methods for oxynitrilases have practical problems on a large scale – Among the main problems of immobilized methods are the non-satisfied long-term stability, catalyst leaching, abrasion of the immobilisate, and a wide particle distribution (instead of a well-defined macroscopic size) in the case of powdered immobilisates –. In the following, we report a summary of our results on the first development of LentiKat®-entrapped enzymes (here: oxynitrilases)

[1] Harald Gröger, Degussa AG, Project House Biotechnology, Rodenbacher Chaussee 4, 63457 Hanau-Wolfgang, Germany
[2] Emine Capan and Klaus-Dieter Vorlop, Institute of Technology and Biosystems Engineering, Federal Agricultural Research Centre (FAL), Bundesallee 50, 38116 Braunschweig, Germany
[3] Anita Barthuber, Degussa AG, R&D Department FC-AG-RD, Dr.-A.-Frank-Str. 32, 83308 Trostberg, Germany

and their application in the asymmetric biocatalytic synthesis of (R)-mandelonitrile (Gröger et al. 2001a & b, Capan et al. 2001).

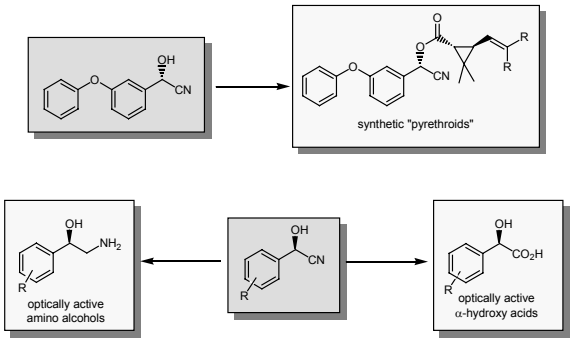

Figure 1:
Graphical summary of cyanohydrin applications

2 Results and Discussion

2.1 The concept for entrapping oxynitrilases

For our studies we used a non-purified (R)-oxynitrilase purchased from ASA Spezialenzyme GmbH, Braunschweig for economic reasons. This oxynitrilase has a specific activity of 13.6 U/mg protein, and an amount of protein of 7.6 mg/mL. In order to prepare an efficiently entrapped oxynitrilase, a two-step procedure was chosen. In a first step, a cross-linking process was carried out which led to an increased molecular weight of the (cross-linked) enzymes. This cross-linking procedure is necessary since enzymes with a molecular weight of max. 50,000 (as in case of oxynitrilases) would not be restrained in the hydrogels. At first the cross-linking process was carried out with glutaraldehyde only, but the resulting cross-linked enzymes showed a drastically decreased activity. Probably the enzyme is deactivated under those conditions. However, a combination of glutaraldehyde and chitosan led to an improved cross-linked enzyme which showed 89 % of its native activity.

In a subsequent step, this cross-linked enzyme was entrapped in a hydrogel matrix which is (mainly) based on polyvinylalcohol. After entrapping the cross-linked oxynitrilase 65 % of its activity remains. The concept of this two-step immobilization method is shown in Figure 2 (Gröger et al. 2001). These lens-shaped hydrogels are highly elastic and flexible towards mechanical treatment.

The general protocol for the preparation of (R)-oxynitrilase-containing, lens-shaped PVAL-hydrogels reads as follows: The cross-linking step of the (R)-oxynitrilase was carried out using chitosan and glutardialdehyde. At first 1.5 g of chitosan are dissolved in 98.5 g acetic acid solution (0.5 %), and 1 M NaOH are added until a pH 5.5 has been obtained. Subsequently 7.89 g of a non-purified oxynitrilase solution (60 mg protein; purchased from ASA Spezialenzyme GmbH, Braunschweig; specific activity: 13.6 U/mg protein; amount of protein: 7.6 mg/mL) are added to 4 g of the chitosan solution. The resulting mixture was treated with 200 µL of a glutardialdehyde solution (50 %; pH 5.5). After stirring for 16 h at 4 °C the cross-linked (R)-oxynitrilase (after centrifugation) is entrapped in LentiKat® by addition of 2.07 g of the chitosan/glutardialdehyde-crosslinked enzyme solution and 7.9 g of water to 74 g of LentiKat®Liquid (a polyvinylalcohol-containing aqueous solution which is commercially available from geniaLab; geniaLab 2001).

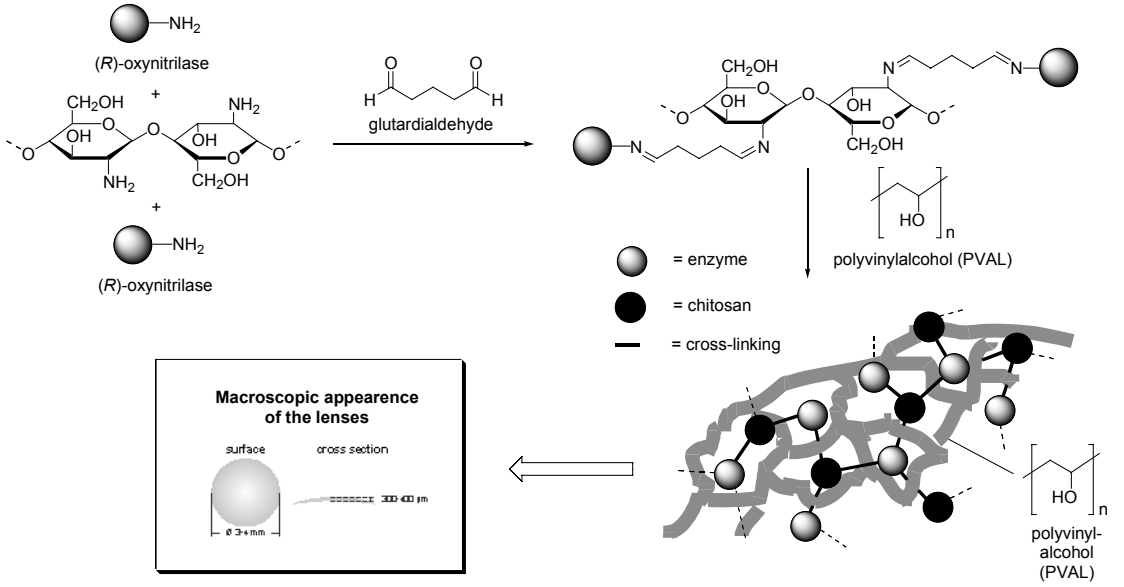

Figure 2:
Scheme of the two-step immobilization method inside LentiKats®

The lens-shaped gels are obtained after dropping the polymeric suspension on a plate using a LentiKat®-printer. For further details on the steps of preparation of the lenses, see Jekel et al. 1998, Wittlich et al. 1999a & b, Welter et al. 1999, Durieux et al. 2000, and geniaLab 2001. The entrapped (R)-oxynitrilases have been obtained on highly elastic, lense-shaped gels with a defined diameter of 3 to 5 mm. The activities of the lenses varied between 8 and 40 U per g lenses. A picture of the shape of those polyvinylalcohol-based lenses is given in Figure 3. The lens-shaped, oxynitrilase-containing catalyst shows a well-defined particle diameter of 3 or 5 mm and a thickness of 0.3-0.4 mm. Further physical properties of such type of lens-shaped hydrogels in general have been described earlier (Jekel et al. 1998, Wittlich et al. 1999a, Welter et al. 1999).

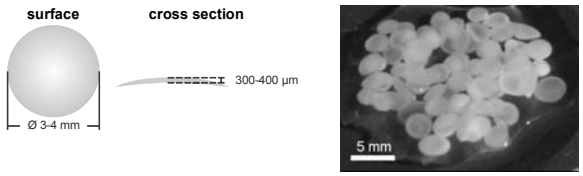

Figure 3:
Scheme and photo of LentiKats®

2.2 Properties of entrapped (R)-oxynitrilases in LentiKats®

A potential catalyst leaching was investigated with respect to the dehydrocyanation reaction of mandelonitrile (in general, the catalytic activity of oxynitrilases is determined via this cleavage reaction of mandelonitrile). A photometrical determination of the reaction course showed that no catalyst leaching occurs after a stirring time of up to 143 h. This conclusion was supported by the (nearly) identical course of the enzyme activities determined from the reactions with "fresh" hydrogels and hydrogels after stirring for 143 h, respectively. For more details regarding this investigation see (Gröger et al. 2001b and Capan et al. 2001). In conclusion, a catalyst leaching or a decrease of the catalyst activity due to a deactivation process was not observed.

2.3 Hydrocyanation with entrapped (R)-oxynitrilases in aqueous media

The lens-shaped oxynitrilases have subsequently been applied as a biocatalyst for the reaction at a low pH of pH 3.75 in aqueous media. The low pH is required in order to prevent the formation of the racemic mandelonitrile which would led to lower enantioselectivities. This effect has been described previously by Kula and co-workers in detail (Niedermeyer and Kula 1990a & b).

In the presence of the cross-linked and subsequently entrapped oxynitrilases, the hydrocyanation in aqueous media at a low pH proceeds well, leading to the desired optically active product (R)-mandelonitrile with an high enantioselectivity of > 99 % ee (Figure 4 and Figure 5, batch 1, see also Capan et al. 2001).

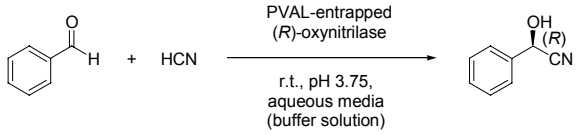

Figure 4:
Reaction scheme for the enzymatic conversion of benzaldehyde to (R)-cyanohydrin in aqueous media

As a next step, the long-term stability and the recycling abilities – a crucial economical criterion – have been investigated. We were pleased to find that the lens-shaped PVAL-entrapped oxynitrilases can be re-used repeatedly in at least 9 batches (Capan et al. 2001). It is noteworthy that the enantioselectivity remained unchanged at high 99 % ee (Figure 5).

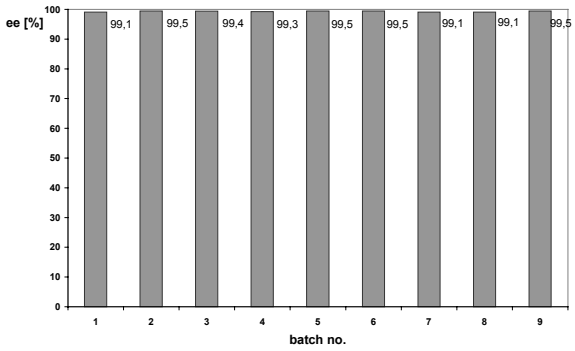

Figure 5:
Long-term stability of the PVAL-entrapped oxynitrilase in aqueous media

2.4 Hydrocyanation with entrapped (R)-oxynitrilases in a biphasic media

Another interesting concept of biocatalytic hydrocyanation reactions is based on a biphasic reaction system, consisting of a water-immiscible organic phase and an aqueous phase, as a reaction media. The presence of an organic solvent guarantees a high enantioselectivity in spite of carrying out the reaction at higher pH values (Effenberger et al. 1987a & b, Griengl et al. 1997, Loos et al. 1995). This beneficial effect of an organic solvents, found by Effenberger in 1987, is due to the fact that the formation of the undesired racemic mandelonitrile is supressed under those conditions (Effenberger et al. 1987a & b). The appli-

cation of biphasic reaction systems for the asymmetric hydrocyanation using oxynitrilases was reported by Griengl et al. 1997 for (S)-oxynitrilases, and Loos et al. 1995 for (R)-oxynitrilases, respectively.

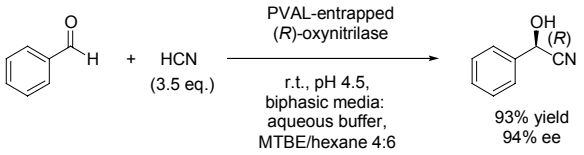

Figure 6:
Reaction scheme for the enzymatic conversion of benzaldehyde to (R)-cyanohydrin in a biphasic media

In the presence of the cross-linked and subsequently entrapped oxynitrilase-catalyst (with an activity of 8.16 U per g lenses) at pH 4.5 in a biphasic system, the desired product (R)-mandelonitrile was obtained in 93 % yield and with 94 % ee (Figure 6, Gröger et al. 2001a & b). The general procedure for the asymmetric hydrocyanation using oxynitrilases entrapped in lens-shaped gels reads as follows: To a solution of the PVAL-entrapped (R)-oxynitrilases (lens-shaped capsules; g lenses per mmol substrate: entries 2 and 4-5, 1.84 g; entry 3, 3.67 g; entry 6, 2.47 g) in 4 mL of a citric buffer, subsequently 4 mL of the organic solvent, 1 mmol of benzaldehyde, and 3.5 mmol of HCN (as an 20 % aqueous solution) are added. After stirring the reaction mixture for two hours, the organic layer is separated, and the aqueous layer is washed with 2 x 20 mL MTBE (subsequently, the aqueous layer is treated with NaOCl solution in order to decompose HCN which has been used in excess amount). The organic phases are dried over magnesium sulfate, and after filtration the volatile materials are removed in vacuo. The product (R)-mandelonitrile is obtained with a purity of > 90-95 % (in some cases of up to 99 %).

It is noteworthy that this result is comparable to the one obtained with "free" oxynitrilases. Thus, in spite of modifying the enzyme by cross-linking and entrapping a comparable synthetic, results with respect to enantioselectivity and yield were obtained with "free" and PVAL-entrapped oxynitrilases, respectively (Gröger et al. 2001a & b).

The "catalyst loading" of the entrapped oxynitrilase has an influence on the reaction course of the asymmetric hydrocyanation. When using an entrapped oxynitrilases with a somewhat higher catalyst loading of 40 U per g lens, a yield of 74 % yield and slightly decreased 91 % ee were obtained. In addition, the organic solvent plays an important role. Under optimized conditions an enantioselectivity of 99 % ee can be obtained when carrying out the reaction in a biphasic media with diisopropylether as an organic solvent (Gröger et al. 2001b).

As in case of the study in an aqueous media, investigation with respect to the recycling abilities of the entrapped oxynitrilases were carried out in a biphasic media. In total, the lens-shaped hydrogels were recycled 20 times without a decrease of enantioselectivity (Figure 7, Gröger et al. 2001b). In contrast, the ee slightly increased from 91 % ee to 95 % ee which might be due to an increased stabilization of the enzyme within the hydrogel matrix. The yield remains in the same range of ca. 80 %. It is further noteworthy that the oxynitrilase-containing hydrogels do not change their elasticity, size and flexibility.

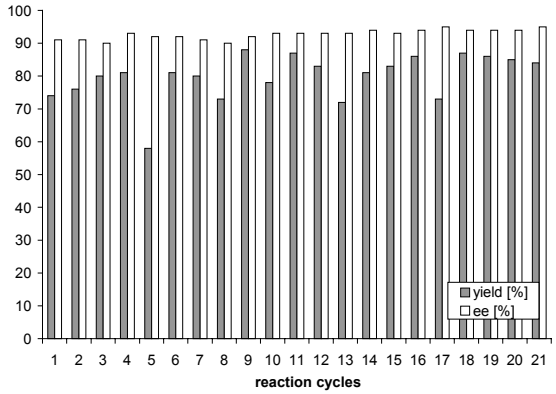

Figure 7:
Long-term stability of the PVAL-entrapped oxynitrilase in a biphasic system

3 Conclusions

In conclusion, a cross-linked and subsequently PVAL-entrapped oxynitrilase catalyst was developed and applied successfully for the asymmetric hydrocyanation of benzaldehyde. These new LentiKat®-entrapped oxynitrilases show the following properties:

- Macroscopic well-defined size (diameter in mm-range)
- High yields, and high enantioselectivity of up to 99 %
- High synthetic efficiency
- Recyclable without loss of enzymatic activity
- Suitable for technical reactors due to high elasticity
- Leaching of the oxynitrilase has not been observed
- Encapsulation materials are cheap and non-toxic
- Simple encapsulation protocol.

Thus, the asymmetric biocatalytic hydrocyanation via LentiKat®-entrapped oxynitrilases represents an attractive access to the commercially important optically active mandelonitrile.

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