Synthesis of Chiral Cyanohydrins by Recombinant *Escherichia coli* Cells in a Micro-Aqueous Reaction System

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The synthesis of enantiopure cyanohydrins from aldehydes or ketones and hydrogen cyanide (HCN) catalyzed by hydroxynitrile lyases (HNLs) represents an industrially important biocatalytic route (5) (Fig. 1). Cyanohydrins serve as valuable building blocks in synthetic chemistry and the pharmaceutical industry (10). HNLs are usually applied in aqueous-organic two-phase systems at pH values equal to or below 4.5 in order to suppress the noncatalytic side reaction yielding racemic products (3).

However, such low pH values are not always tolerated by the enzymes. For example, the (R)-selective HNL from *Arabidopsis thaliana* (AtHNL) is unstable at low pH values (9), which severely limits its application in conventional aqueous-organic reaction systems. Application of the HNLs in organic solvent, with minimal water activity, represents an attractive alternative which has recently been explored using precipitated and immobilized enzyme preparations (14). The organic solvent system facilitates the solubility of aromatic substrates and products and suppresses the noncatalytic side reaction due to the low water content. However, the precipitated enzyme cannot be recycled and the preparation of recyclable immobilizates (i.e., enzyme formulations in which the enzyme is covalently linked or noncovalently bound to an inorganic material) requires additional steps and results in a partial loss of enzyme activity. In this respect, the use of whole cells in a monophasic micro-aqueous reaction system (6) would essentially eliminate all the aforementioned problems and thus represent a cost-efficient alternative to the use of purified and/or immobilized enzyme preparations. In order to test the applicability of such a system for the synthesis of chiral cyanohydrins, whole recombinant *Escherichia coli* cells expressing the AtHNL were used here as a whole-cell biocatalyst in a monophasic organic solvent. The cells and solvent can be recycled, and the product can easily be recovered from the solvent. Despite the significant potential of such an approach, only a few studies on the application of whole cells in monophasic organic solvents have so far been reported (6). Most of these studies focus on the stereoselective reduction of ketones by alcohol dehydrogenases using lyophilized yeast cells (6, 15). To the best of our knowledge, only two studies on the use of whole recombinant *E. coli* cells in water-free or micro-aqueous reaction systems have been published. In one study, lyophilized *E. coli* cells overexpressing an alcohol dehydrogenase from *Rhodococcus ruber* were used in a micro-aqueous system with 99% (vol/vol) isopropanol (6). A related strategy has recently been described, with lyophilized *E. coli* cells expressing the *Candida parapsilosis* carbonyl reductase, which were applied in a reaction system containing only neat substrates without the addition of an explicit solvent (11). While *E. coli* has not been used extensively in neat organic solvents, other bacteria such as solvent-tolerant *Pseudomonas* and *Rhodococcus* species have been applied for biotransformations under such conditions (13,16). Classic and more recent examples include the production of cholest-4-ene-3-one by *Nocardiopsis* species in neat carbon tetrachloride (2) and the synthesis of indigo by a *Rhodococcus* strain in pure bis(2-ethylhexyl) phthalate (19). While solvent-tolerant bacteria are promising candidates for nonaqueous whole-cell catalysis (12), they simply cannot rival *E. coli* in terms of easy handling and availability of molecular tools.

The feasibility of a monophasic micro-aqueous whole-cell reaction system for the production of chiral cyanohydrins was initially tested by the synthesis of (R)-mandelonitrile from benzaldehyde and hydrogen cyanide (HCN). The reaction was carried out at 20°C in a reaction system consisting of 1 ml of 2 M HCN solution in buffer-saturated methyl tert-butyl ether (MTBE) containing 350 mg of wild-type AtHNL-expressing *E. coli* BL21(DE3) cells. The reaction was started by the addition of 0.5 mmol benzaldehyde to the reaction system and monitored over 60 min by chiral gas chromatography (GC) (Fig. 2). MTBE is widely used in biocatalysis and has previously been shown to be advantageous for use with free and immobilized AtHNL preparations (14). To recycle the cells, fresh or frozen *E. coli* BL21(DE3) cells expressing wild-type AtHNL were placed in nylon mesh. Thus, the cells can easily be removed from the reaction vessel facilitating easy cell recycling. After each conversion, the cells were washed with MTBE, placed in a new vessel, and MTBE and fresh substrates were added. Both freshly prepared and frozen cells displayed very similar conversion rates and enantioselectivities over as many as three reaction cycles (Fig. 2; see also Fig. S1 in the supplement).

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mental material). Although initial reaction rates decreased afterwards, subsequent biotransformations still resulted in nearly complete conversion of benzaldehyde after a reaction time of 60 min. After 5 reaction cycles, AtHNL-expressing cells still converted 85% benzaldehyde with an enantiomeric excess (ee) of 98% within 60 min (data not shown).

Thus, recycling of whole cells resulted in slightly lower initial reaction rates and final yields in subsequent conversion rounds, however, without loss of enantioselectivity. Cyanohydrin synthesis was further studied with different aldehyde substrates in order to evaluate the applicability of our reaction setup in more detail (Table 1). As before, 1 ml of 2 M HCN in MTBE mixed with 0.5 mmol of the respective aldehyde was used as the substrates. All reactions were carried out at 20°C. Fresh (wet) cells showed good conversion rates but gave only moderate ee values for 2-chloromandelonitrile and 2-fluoromandelonitrile (Table 1, reactions 2 and 3). In contrast, 2-furaldehyde (Table 1, reaction 4) was only poorly converted (50%) with low enantioselectivity (ee, 30%).

The difference between enzymatic and nonenzymatic reaction rates for the respective aldehydes may provide a reasonable explanation for the reduced enantioselectivities observed in comparison to those during benzaldehyde conversion. It has previously been demonstrated that, e.g., for 2-chlorobenzaldehyde and 2-fluorobenzaldehyde, the nonenzymatic side reaction in an aqueous reaction system is much faster than for benzaldehyde (1). Consequently, when dry AtHNL immobilizes were used for the conversion of 2-chlorobenzaldehyde and 2-furaldehyde, excellent ee values (>98% (R) enantiomer) could be obtained (14). Therefore, it appears that the water content in the MTBE-washed cell pellet (or in the E. coli cells) compromises the enantioselectivity in our whole-cell reaction system. To elucidate this possibility, the respective E. coli cells were lyophilized and used for the conversion of the same aldehyde substrates. Not surprisingly, conversion was lower than when the same amount of fresh cells was used; however, ee values for all products increased significantly. In particular, 2-furaldehyde could be converted with an ee value of 88% (Table 1, reaction 4). Likewise, 2-chlorobenzaldehyde that was previously converted with a moderate ee of 70% by fresh (wet) cells was now converted with an ee of 90% by lyophilized cells (Table 1, reaction 2).

Bacterial cells are evolved to be stable in an aqueous environ-
for the conversion of aldehydes showing fast noncatalyzed racemic product formation in aqueous systems. Due to its simplicity (use of whole cells, no protein purification, monophasic organic solvent), this novel process for the production of chiral cyanohydrins could also be an interesting alternative for industrial applications. In general, the application of whole-cell biotransformations in pure organic solvents represents a promising alternative to conventional transformations in aqueous media. The use of monophasic microaqueous organic solvents may be particularly interesting when poor substrate or product solubility limits their application or when substrates and/or products are unstable in water. Our approach should be directly transferable to any HNL that can be expressed in E. coli.

Moreover, our microscopic analyses revealed that standard E. coli cells do not lose their cellular integrity when incubated in MTBE. Hence, it can be expected that the transfer of the presented whole-cell biotransformation approach to other enzymatic systems could open up new biocatalytic routes even for the synthesis of novel products which are currently not accessible by biocatalysis.

Likewise, the use of fluorescent reporter techniques in such studies allows an in situ monitoring of the respective biocatalytic system. In this respect, we currently evaluate the potential of FbFPs and other fluorescent proteins as reporters for further biotechnological applications, including solvent screening and localization of enzymes in immobilization carrier materials and micellar systems.

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REFERENCES


