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Abstract

Whole cells of *Rhodococcus equi* A4, a producer of nitrile hydratase and amidase activities, were immobilized in lens-shaped hydrogel particles, LentiKats®. The immobilized biocatalyst was applied to the biotransformation of benzonitrile, 3-cyanopyridine, *(R,S)*-3-hydroxy-2-methylenebutanenitrile and *(R,S)*-3-hydroxy-2-methylene-3-phenylpropanenitrile. The stability of the nitrile hydratase during the repeated use of the biocatalyst was dependent on the type of the substrate. The enzyme was most stable during the transformation of *(R,S)*-3-hydroxy-2-methylenebutanenitrile. No significant loss of the amidase activity was observed within the course of the biocatalytic reaction.

Keywords: Nitrile hydratase; Amidase; Nitriles; *Rhodococcus equi*; LentiKats®

1. Introduction

Within the past decade, nitrile-transforming biocatalysts became very popular both in laboratory and industry. Attempts to prepare immobilized biocatalysts of this type were mostly based on the entrapment of whole cells in various hydrogels (see Ref. [1] for a review). Some immobilization techniques improved the parameters of the biocatalysts, either by increasing their stability or even broadening their substrate specificity [2–4]. On the other hand, enzyme deactivation and limited mass transfer of the substrate were encountered as the main problems [5,6].

Rhodococcus equi A4 was previously exploited in numerous nitrile biotransformations (see Ref. [1] for a review). The high nitrile hydratase specific activity and the broad substrate specificity are the main benefits of this microorganism. On the other hand, the relatively low nitrile hydratase production per litre of culture is a drawback of this strain. The bacterial growth is weak under the conditions of enzyme induction which is optimal in basal salts broth with acetonitrile as the sole source of nitrogen. This problem would be partly solved when the biocatalyst could be used repeatedly.

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LentiKats® was introduced as a new immobilization method [7]. The lens-shaped particles are prepared from a copolymer of polyvinyl alcohol and polyethylene glycol. They are stable towards mechanical and microbial degradation and, due to their shape, they provide a good substrate and nutrient supply to the cells. Herein, this technique proved effective for the immobilization of *R. equi* A4 cells with nitrile hydratase/amidase activity.

2. Experimental

2.1. Chemicals

(*R,S*)-3-Hydroxy-2-methylenebutanenitrile, (*R,S*)-3-hydroxy-2-methylene-3-phenylpropanenitrile and the corresponding amides were as described previously [8]. (*R,S*)-3-Hydroxy-2-methylenebutanoic acid was prepared by biotransformation using whole cells of *R. equi* A4. Other chemicals were from standard commercial sources.

2.2. Microorganism and cultivation

R. equi A4 (deposited in the Culture Collection of Microorganisms, Masaryk University, Brno, Czech Republic) was grown as described previously [9].

2.3. Immobilization

The immobilization in LentiKats® was performed according to the procedure introduced by geniaLab Bio Technologie [7] with slight modification. Polyethylene glycol (6 g) was dissolved in water (74 ml) and polyvinyl alcohol (10 g) was added at stirring. The mixture was heated to 90 °C until polyvinyl alcohol particles were dissolved completely. After cooling to 25 °C, whole cells were added as a suspension in 50 mM Na/K phosphate buffer, pH 7.5 (10 ml; approximately 0.4 g of dry cell weight). The resulting mixture was dropped on a smooth polystyrene plate and left at room temperature for 90 min to complete gelation. The particles were transferred into 100 mM sodium sulphate for re-swelling. After 12 h, this solution was replaced by 50 mM Na_2HPO_4/KH_2PO_4 buffer, pH 7.5. The biocatalyst was stored in this buffer at 4 °C.

2.4. General procedure for biotransformation

The reaction mixtures (10 ml) consisting of 50 mM Na_2HPO_4/KH_2PO_4 buffer, pH 7.5, 30–35 mM substrate and the immobilized cells (about 2.3 ml of LentiKats®; 9 mg of dry cell weight) or the free cells of the same dry cell weight were incubated at 28 °C and shaking. The biocatalyst was harvested after 1-h reaction, washed with 50 mM Na_2HPO_4/KH_2PO_4 buffer, pH 7.5, and used for the next run under the same conditions.

2.5. Analytical HPLC

The reaction mixtures were analyzed as described previously [8].

3. Results and discussion

The immobilized cells retained their full nitrile hydratase activity after immobilization. The operational stability of the immobilized biocatalyst was very good for (*R,S*)-3-hydroxy-2-methylenebutanenitrile within four runs (Fig. 1). However, a drop of the reaction rate was observed during the repeated transformations of benzonitrile (by about 20 and 30% in the second and the third run, respectively, as compared with the first run; Fig. 2). A previous report described cross-linking of polyvinyl alcohol by UV light, using sodium benzoate as a sensitiser [10]. This suggested that LentiKats® might be cross-linked in the presence of daylight and benzoate, which was formed as a minor product from benzonitrile. The cross-linking could cause a diffusional limitation increasing from run to run. However, in an analogous experiment, which employed free cells, the initial rate of benzonitrile hydration decreased even more significantly (by about 30 and 57% in the second and the third run, respectively). Therefore, the drop of the reaction rate of benzonitrile was probably not due to a cross-linking of LentiKats® but due to deactivation of the nitrile hydratase by the substrate and/or the reaction product. In addition, the transformations of 3-cyanopyridine (Fig. 3) and (*R,S*)-3-hydroxy-2-methylene-3-phenylpropanenitrile (Fig. 4) also suggested that the operational stability depended on the substrate used. The biocatalyst was fairly stable in the presence of the former one, while the latter one and/or its reaction product was detrimental to the nitrile hydratase.

Contrary to the nitrile hydratase, the amidase remained fully active during repeated use of the cells. However, during the conversion of *(R,S)*-3-hydroxy-2-methylene-3-phenylpropanenitrile it was probably suppressed by the high substrate concentration (30 mM), as 5 mM of the nitrile was

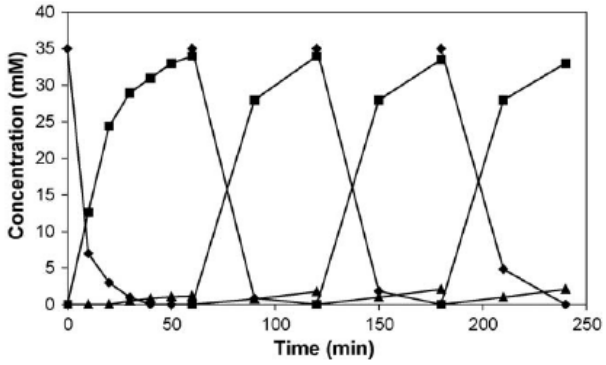

Fig. 1. Biotransformation of (*R,S*)-3-hydroxy-2-methylenebutanenitrile (35 mM; ♦) into (*R,S*)-3-hydroxy-2-methylenebutanamide (■) and (*R,S*)-3-hydroxy-2-methylenebutanoic acid (▲) by immobilized *Rhodococcus equi* A4. See Section 2 for details.

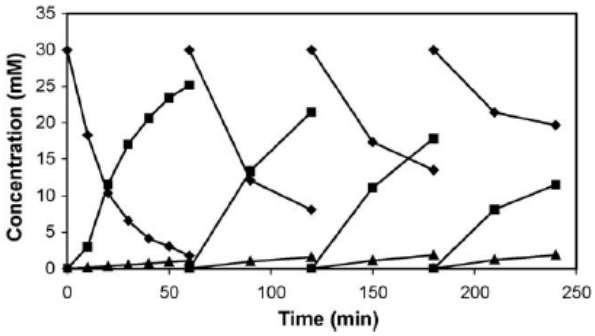

Fig. 2. Biotransformation of benzonitrile (30 mM; ♦) into benzamide (■) and benzoic acid (▲) by immobilized *Rhodococcus equi* A4. See Section 2 for details.

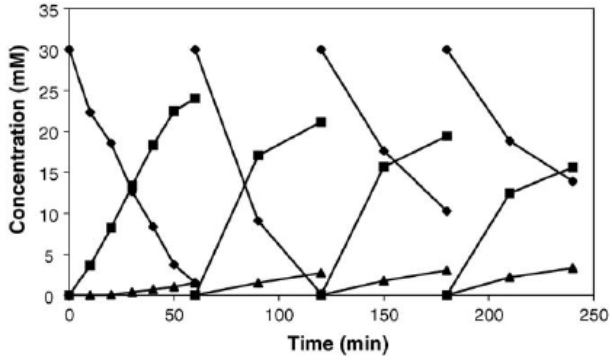

Fig. 3. Biotransformation of 3-cyanopyridine (30 mM; ♦) into nicotinamide (■) and nicotinic acid (▲) by immobilized *Rhodococcus equi* A4. See Section 2 for details.

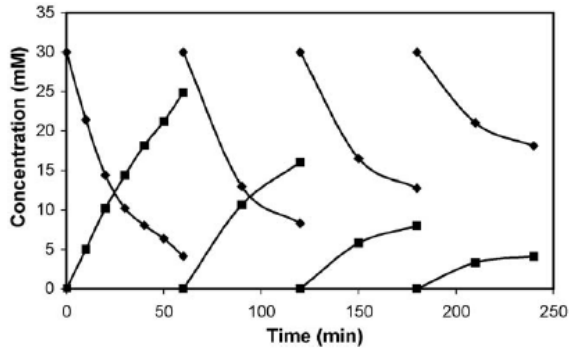

Fig. 4. Biotransformation of (*R*,*S*)-3-hydroxy-2-methylene-3-phenylpropanenitrile (30 mM; ♦) into (*R*,*S*)-3-hydroxy-2-methylene-3-phenylpropanamide (■) by immobilized *Rhodococcus equi* A4. See Section 2 for details.

smoothly converted into the acid by free cells (*unpublished*). The operational stability of the amidase suggests that the immobilized biocatalyst would be an excellent tool for the hydrolysis of amides. Some biocatalytic applications of the amidase produced by strain A4 have been reported previously, such as the hydrolysis of lysergamide [11] and (*R*,*S*)-2-arylpropionamides, precursors of profens [12].

The immobilized biocatalyst showed a very good mechanical stability. No cell release was detected by optical density measurement.

As two of the substrates were chiral compounds, the stereoselectivity of the enzymes is of interest. The conversion of *(R,S)*-3-hydroxy-2-methylene-3-phenylpropanenitrile by *Rhodococcus* sp. AJ270 was reported previously [13]. The amidase showed a low *R*-enantioselectivity ($E = 4.2–4.8$) for the corresponding amide, while no enantioselectivity of the nitrile hydratase could be detected. Chiral HPLC [13] was used to determine the enantioselectivity of the nitrile hydratase and amidase of *R. equi* A4 towards these compounds (*unpublished data*). The behaviour of the amidase of *R. equi* (*R*-selectivity; $E = 3.6–3.9$) was very similar as reported for *Rhodococcus* sp. AJ270. Experiments with the purified nitrile hydratase from *R. equi* A4 proved that this enzyme showed some enantioselectivity towards the nitrile (*S*-selectivity; $E = 2.7–2.8$). It is not surprising that the enantioselectivity of both enzymes was very low towards substrates bearing the stereogenic centre distant from the reactive group. The attempt to separate the enantiomers of 3-hydroxy-2-methylene-butanenitrile by chiral HPLC (Chiral OD-H; [13]) was unsuccesful. However, regarding the above data a significant enantioselectivity of the nitrile hydratase or amidase can be hardly expected for this substrate, as the enzyme would have to discriminate between two substituents of similar size (hydroxyl and methyl).

4. Conclusions

The immobilization in LentiKats® is a prospective method for the preparation of nitrile-converting biocatalysts. Repeated use of the biocatalyst is feasible, though reaction conditions have to be optimized for each substrate. The excellent stability of the biocatalyst during the conversion of 3-hydroxy-2-methylenebutanenitrile suggests that small aliphatic nitriles are the most suitable substrates for this biocatalyst.

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