

# Cross-Linked Aggregates of (*R*)-Oxynitrilase: A Stable, Recyclable Biocatalyst for Enantioselective Hydrocyanation

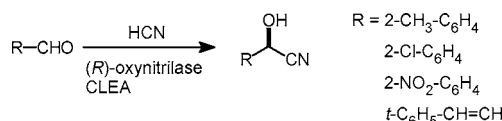
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## ABSTRACT



The (*R*)-oxynitrilase from almonds was immobilized as a cross-linked enzyme aggregate (CLEA) via precipitation with 1,2-dimethoxyethane and subsequent cross-linking using glutaraldehyde. The resulting preparation was a highly effective hydrocyanation catalyst under microaqueous conditions, which suppress the nonenzymatic background reaction. The beneficial effect of these latter conditions on the hydrocyanation of slow-reacting aldehydes is demonstrated. The oxynitrilase CLEA was recycled 10 times without loss of activity.

The (*R*)-specific oxynitrilase from almonds (hydroxynitrile lyase *Prunus amygdalus*, PaHnL, E.C. 4.1.2.10), which mediates the hydrocyanation of a wide range of aldehydes with high (>98%) enantioselectivity,<sup>1–3</sup> is an emerging industrial biocatalyst. It is usually applied in an aqueous–organic two-phase system, in which the enzyme resides in the aqueous (working) phase and the reactants and products reside in the organic (extractive) phase. The resulting low reactant concentration in the aqueous phase suppresses the uncatalyzed background reaction that otherwise would erode the ee (see Figure 1).<sup>4</sup> The nonenzymatic reaction is further suppressed by maintaining the pH in the acidic range.<sup>5</sup>

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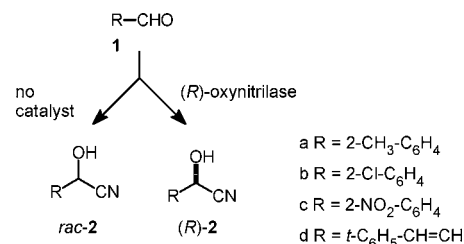
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Satisfactory results have been achieved in such systems with a wide range of aldehydes, and a mathematical model that predicts the final conversion and ee has been drawn up.<sup>6,7</sup> It is obvious that increasing the enzyme loading and reducing the aqueous phase volume will increase the competitiveness of the enzymatic reaction and improve the enantiomeric purity of the product.

Accordingly, enzymatic hydrocyanations have been carried out in a microaqueous organic medium, in combination with

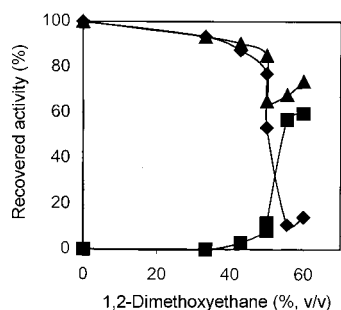


**Figure 1.** Competition of enzymatic and uncatalyzed hydrocyanation.

an immobilized enzyme, such as defatted almond meal.<sup>8–10</sup> We have found that such a microaqueous medium is particularly advantageous in the hydrocyanation of the sluggishly reacting 2-chlorobenzaldehyde, but the use of almond meal imposed a lower limit on the water contents of 4–8% (v/v).<sup>11</sup> Highly concentrated aqueous enzyme solutions have been used,<sup>12</sup> but recycling these is problematic.

The efficient enzymatic hydrocyanation of slow-reacting aldehydes evidently requires a recyclable, anhydrous enzyme preparation with a high volumetric activity that allows high catalyst loadings without introducing extra water into the system. An immobilization methodology that meets these requirements is the preparation of cross-linked enzyme aggregates (CLEAs).<sup>13</sup> Further advantages of the latter methodology are its simplicity, low cost, and fast optimization.<sup>14</sup> We will show that a CLEA of PaHnL is an efficient and readily recyclable hydrocyanation catalyst.

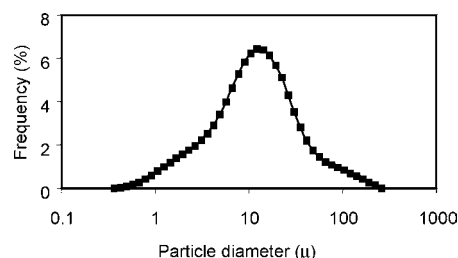
We first investigated the precipitation of semipurified (*R*)-oxynitrilase from a 50% glycerol solution. The best result, as regards activity loss, was obtained with 1,2-dimethoxyethane. Upon addition of the latter to a final concentration of 60% (v/v),<sup>13</sup> precipitation was nearly complete (see Figure 2) and approximately 60% of the original activity was recovered from the precipitate upon redissolution.



**Figure 2.** Precipitation study of (*R*)-oxynitrilase from almonds with 1,2-dimethoxyethane; activity recovery from the precipitate upon redissolution (■) and from the supernatant (◆) and total activity (▲) vs the precipitant concentration. The starting activity corresponds to 100%.

Subsequent cross-linking of the precipitate with glutaraldehyde afforded a CLEA that did not leach any enzyme

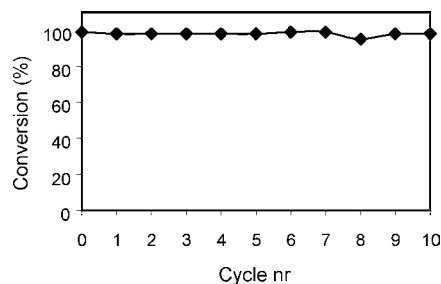
upon washing. A dry powder was obtained upon rinsing with acetonitrile and diethyl ether, followed by vacuum-drying. The particle size dispersion, as measured by laser diffraction, was very wide, from 0.5 to 200  $\mu$  (average 12  $\mu$ , see Figure 3). The activity recovery was disappointingly low when we



**Figure 3.** Particle size distribution of the (*R*)-oxynitrilase CLEA.

subjected the CLEA to the standard oxynitrilase activity assay, the cleavage of mandelonitrile. The recovery amounted to 5% at room temperature and increased to 9.6% when the assay was done at 0 °C, indicating that there is an effect of diffusional limitation in the CLEA particles.<sup>15</sup> This issue will be further discussed later.

The newly prepared CLEA was subjected to an operational stability test in the hydrocyanation of 2-methylbenzaldehyde (**1a**), using a microaqueous 2% diisopropyl ether (DIPE) medium. The CLEA particles could be easily separated from the reaction mixture when the reaction was complete. The biocatalyst was washed with water and reused 10 times without performance loss (see Figure 4). Rapid deactivation



**Figure 4.** Effect of recycling on the performance of the (*R*)-oxynitrilase CLEA in the hydrocyanation of **1a**.

took place when the washing step was omitted, presumably due to accumulation of 2-methylbenzoic acid, which is a powerful inhibitor of the oxynitrilase.

The performance of the PaHnL CLEA was assessed in the hydrocyanation of three aldehydes that are known to react

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quite sluggishly. 2-Chlorobenzaldehyde (**1b**) was selected, as it is a chiral intermediate in the industrial synthesis of the antithrombotic agent clopidogrel. 2-Nitrobenzaldehyde (**1c**) is an interesting reactant due to the electronegativity and steric demands of the nitro substituent. We investigated the hydrocyanation of *trans*-cinnamaldehyde (**1d**) because the adduct (**2d**) may be a useful intermediate in the synthesis of ACE inhibitors.<sup>16</sup> Three reaction systems were employed: the CLEA in microaqueous (2%, v/v) DIPE and the CLEA, as well as free enzyme in a biphasic DIPE–H<sub>2</sub>O (1:1) medium (see Table 1).

**Table 1.** Hydrocyanation of Aldehydes **1b–d** in the Presence of (*R*)-Oxynitrilase<sup>a</sup>

reactant	bio-catalyst	temp (°C)	medium <sup>b</sup>	time (min)	conversion (%)	ee (%)
<b>1b</b>	CLEA	25	microaqueous	60	99	92
<b>1b</b>	CLEA	0	microaqueous	120	98	95
<b>1b</b>	CLEA	25	2-phase	120	99	56
<b>1b</b>	CLEA	0	2-phase	90	99	76
<b>1b</b>	free	25	2-phase	60	99	52
<b>1b</b>	free	0	2-phase	60	99	77
<b>1c</b>	CLEA	25	microaqueous	60	99	62
<b>1c</b>	CLEA	0	microaqueous	210	99	65
<b>1c</b>	CLEA	0	2-phase	120	99	38
<b>1c</b>	free	0	2-phase	130	100	40
<b>1d</b>	CLEA	25	microaqueous	120	90	99
<b>1d</b>	CLEA	0	microaqueous	240	96	99
<b>1d</b>	CLEA	25	2-phase	180	80	84
<b>1d</b>	free	25	2-phase	150	86	92

<sup>a</sup> Reaction conditions: 0.2 M aldehyde and 0.6 M HCN (1.2 M with **1d**) in DIPE (1 mL), 90 U of free enzyme, or 12 mg of CLEA.

<sup>b</sup> Microaqueous: 20  $\mu$ L of 20 mM citrate buffer pH 5.5 added to the above solution. 2-Phase: 1 mL of citrate buffer.

The hydrocyanation of **1b** took place with full conversion in our reaction systems. A comparison of the activities of the free enzyme and the CLEA in 50% aqueous DIPE showed that the CLEA was somewhat less active than the free enzyme (see later). The biocatalyst formulation had little effect on the enantiomeric purity of the product, which was only a modest 56 and 77% at 25 and 0 °C, respectively. Hence, there is a considerable contribution by the uncatalyzed background reaction in 50% aqueous DIPE. A further reduction in rate was observed when the reaction was

performed in microaqueous medium, but the increase in the product ee shows that the nonenzymatic reaction is almost completely suppressed under these conditions. **2b** with 95% ee was obtained from the reaction in microaqueous medium at 0 °C, which compares well with the 91% ee that we achieved in the presence of 8% water<sup>11</sup> and is one of the highest ever measured.<sup>17</sup>

Similar trends were observed in the hydrocyanation of **1c**, but the product ee was considerably lower in all reaction systems and could not be improved beyond 65%. It would seem that this mediocre enantioselectivity is inherent to the enzyme and is not caused by a background reaction. The hydrocyanation of **1d**, besides being slow, also suffered from an unfavorable equilibrium,<sup>18</sup> which was remedied by increasing the excess of HCN to 6-fold. The product ee was high and little affected by the reaction temperature.

A comparison of the reactions with the CLEA and the free enzyme shows that the latter is 1.5–2 times more active; hence, at least 60% of the active sites in the CLEA are catalytically competent. The question arises why the activity recovery, as determined by photometric assay, is only 5%. We ascribe this latter result to the low (0.2 mM) reactant concentration in the assay, which provides only a small driving force for diffusion and makes the latter rate-limiting. The increase in apparent activity recovery at 0 °C also indicates that diffusion limitation is involved, as the reaction rate tends to be more temperature-dependent than the diffusion rate.<sup>15</sup>

In conclusion, we have shown that the PaHnL CLEA is a stable and recyclable biocatalyst. The application of the CLEA in microaqueous medium clearly is a superior biocatalyst for the enantioselective hydrocyanation of slow-reacting aldehydes.

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**Supporting Information Available:** Detailed descriptions of experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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