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Enzymatic synthesis of optically pure cyanohydrins in microchannels using a crude cell lysate

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Abstract

The synthesis of optically pure cyanohydrins using a crude cell lysate containing the enzyme hydroxynitrile lyase (HNL) was studied in a microreactor in an aqueous–organic biphasic system. Different aldehyde substrates were selected to be converted to their corresponding cyanohydrins. It was successfully demonstrated that this crude cell lysate could readily be applied as a biocatalyst in a microchannel without clogging the channels. The biocatalytic activity toward the different substrates could rapidly be investigated with only small quantities of enzyme needed, compared to batch scale screening. Furthermore, the optimal contact between two immiscible phases in a microreactor resulted in enzymatic reactions with a high initial reaction rate and enantioselectivity, comparable to a batchwise process in which optimized conditions were achieved by vigorous stirring. Thus, performing the selected enzymatic reaction in a microreactor is a facile and cost efficient screening method leading to results which can be directly translated to batchwise processes.

Keywords: Cyanohydrins; Hydroxynitrile lyase; Crude cell lysate; Microreactors

1. Introduction

During the last decades, there has been increased interest in using enzymes for asymmetric synthesis and kinetic resolution to obtain pure enantiomers. A family of enzymes that has been extensively studied are the hydroxynitrile lyases (HNLs). These enzymes catalyse enantioselective C–C-bond formation via the addition of HCN to aldehydes or ketones to yield the corresponding optically active cyanohydrins (Scheme 1), which are synthetically versatile building blocks for the synthesis of fine chemicals, pharmaceuticals and agrochemicals. Both (*R*)- and (*S*)-selective HNLs are widely present in nature, and several genes have been cloned and expressed in microorganisms such as *E. coli* and *P. pastoris* [1].

Traditionally, synthesis of enantiopure cyanohydrins catalysed by HNL was performed in aqueous media (single phase system). However, in several cases less satisfactory results were obtained with respect to enantiopurity and conversion. A significant advancement to overcome this problem was developed by Griengl et al. [2]. They showed that by employing a vigorously stirred biphasic system, cyanohydrins could be prepared in excellent enantiomeric purity and high yield as opposed to the previously described single phase system. Furthermore, the same group concluded that fast formation of a stable emulsion in the reaction mixture was of utmost importance for a successful reaction under these biphasic conditions. In a traditional batch process, such a situation can only be achieved through vigorous stirring. We envisaged that these optimal mixing conditions might well be obtained in suitably designed microchannels.

Various classes of chemical reactions have already been performed in microchannels offering more control over selectivity and suppression of by-product formation due to the high surface-to-volume ratio [3,4]. Furthermore, microreactor technology enables rapid reaction optimisation and screening using relatively small quantities of chemicals, which is of importance for the industry to maintain their rapid development of new chemicals. However, in order for a screening tool to be useful, optimised reaction conditions which have been found using microreactor technology have to be translated to industrial scale processes, which is not trivial since physical properties such as mixing behaviour and mass transport can be markedly different and therefore hamper the comparison between the different scale processes.

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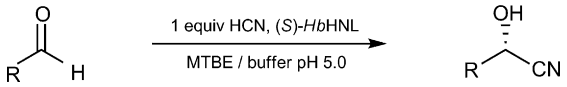


Scheme 1. General enzymatic synthesis of (*S*)-cyanohydrins.

Remarkably, the field of enzymatic synthesis in microreactors is still relatively unexplored. Only a few examples are known, in which either an immobilised enzyme or a purified enzyme is used [5–9], catalysing in most cases hydrolytic reactions. Enzymes that are applied in commercial syntheses, however, are typically crude cell lysates or partially purified preparations to reduce the cost price of the biocatalyst [10]. Due to the high surface-to-volume ratio and small channel dimensions, one can expect that clogging can readily occur when this crude cell lysate will be used; this study is the first report in which this issue will be addressed.

In view of the potential advantageous mixing situation in microreactors and the synthetic viability of C–C-bond formation via HNLs, we set out to investigate the possibility of synthesising cyanohydrins in a microchannel using a crude cell lysate in a biphasic system. Therefore, four commercially available aldehydes **2a–d** were selected (Scheme 2) to be converted into their corresponding cyanohydrins in the microreactor setup, using the crude cell lysate of a HNL.

2. Experimental

2.1. Microreactor (Fig. 1)

The microchannel structure was designed using the software program CleWIN. The actual microreactor was fabricated from borosilicate glass by Micronit Microfluidics BV, Enschede, The Netherlands (HF etched). Chip dimension: length 45 mm, width 15 mm, height 2.2 mm. Channel dimension: width 120 μm, depth 20 μm, total length 70 cm, total internal volume 1.3 μL. A pillar structure was constructed in the middle of the microchannel to stabilise a biphasic laminar flow system, according to a literature procedure [11] (Fig. 1).

2.2. Microreactor setup

The microreactor setup that was designed for the experiments is schematically depicted in Scheme 3. In order to perform the experiments, the syringes (brand: SGE Analytical Science; type: 1MDF-LL-GT with Luer Lock) mounted on the syringe pump (brand: Harvard; type: PicoPlus) were connected using Luer adapters, NanoTight nuts and sleeves (brand: Upchurch Scientific; type: P-659, F-331N, F-242) to the capillaries (brand: Polymicro; type: fused silica, i.d. 50–100 μm, o.d. 375 μm).

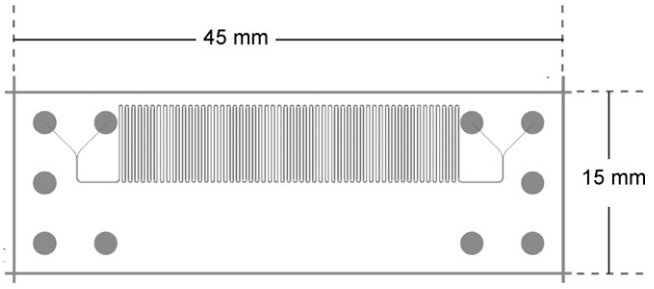


Fig. 1. Schematic representation of the microreactor; total internal volume of the channel is 1.3 μL.

These capillaries were connected using the standard procedure for the chipholder (brand: Micronit Microfluidics BV; type: standard chipholder), to the microreactor using ferrules (brand: Upchurch Scientific; type: N-123-03) to ensure a leak free fluidic connection. For optical inspection of the flow inside the microchannel an inverted brightfield reflected light microscope was used (brand: Zeiss; type: Axiovert).

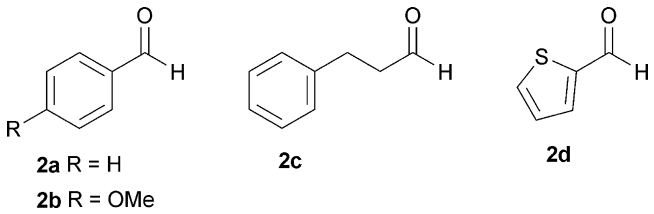
2.3. Chemicals

Unless stated otherwise, all chemicals were obtained from commercial sources and used without further purification: MTBE (Acros, 99%), citric acid (Aldrich, 99.5%), 3-phenyl propionaldehyde (Janssen), benzaldehyde (Acros), 2-thiophene-carboxaldehyde (Acros, 98%), 4-methoxy benzaldehyde (Aldrich, 98%), KCN (Acros, p.a.), anisole (Acros, 99%). All liquid aldehydes were distilled before use.

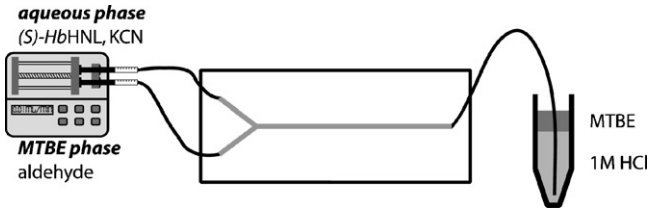
The (*S*)-selective hydroxynitrile lyase, originating from the rubber tree *Hevea brasiliensis* ((*S*)-*Hb*HNL) was applied. The gene encoding for (*S*)-*Hb*HNL was cloned and efficiently expressed in the yeast strain *P. pastoris* as previously described [12]. The enzyme was used as crude cell lysate and was kindly provided by DSM, Geleen, The Netherlands.

2.4. General procedure for the enzyme-catalyzed synthesis of cyanohydrins in microchannels

Solution A (organic phase, MTBE) containing 0.23 M aldehyde and 0.18 M anisole (internal standard) and solution B (aqueous phase, 0.4 M citric acid buffer, pH 5.0) containing 0.23 M KCN and 10% (v/v) crude enzyme solution were prepared.



Scheme 2. Aldehydes subjected to the HNLs.



Scheme 3. Schematic representation of the microreactor setup.

Both solutions were filtrated before use through a nylon filter (Acrodisc 13, pore size: 0.2 μm). Syringe I filled with solution A and syringe II filled with solution B were mounted on the syringe pump and connected to the microchip. To initiate the reactions, the flow rate was set to the desired flow speed (i.e. 4 μL/min) and stabilised for 30 min. After this stabilisation period, the end of the outlet capillary of the microchip was placed into a microvessel (quench vessel) for 10 min (collection time), which contained 100 μL 1 M HCl and 200 μL MTBE. One hundred microliters of the quenched MTBE layer of the microvessel was transferred into a HPLC vial containing 900 μL hexane:isopropyl alcohol (1:1). Five microliters of this solution was injected into a Shimadzu VP chiral HPLC system, equipped with a Chiralpak AD-H (250 mm × 4.6 mm) column and a UV-detector (λ = 215 and 254 nm) for analysis of enantiomeric excesses and conversions. As eluent different ratios of hexane and isopropyl alcohol were used. Flow rate was 1 mL min^{-1}. Subsequently, the flow speed of the syringe pump was decreased (i.e. 2 μL/min), and the system was stabilised again for 30 min. Then the end of the outlet capillary of the microchip was placed into a new quench vessel for 10 min and analysed afterwards as described before. This procedure was repeated until all flow rates were screened. All reactions were performed using a 1:1 flow rate of the aqueous and organic phase, thereby maintaining equimolar amounts of HCN (0.23 M, aqueous phase) to aldehyde (0.23 M, organic phase). Flow rates investigated were 4, 2, 1, 0.5, 0.25 and 0.1 μL/min. The reaction time corresponded to the residence time in the microchannel, which could be calculated by dividing the total internal volume of the microreactor (including the outlet capillary) by the total flow rate of both syringe pumps. In this way, reaction times ranging from approximately 1 to 30 min could be achieved. The total screening per aldehydes was carried out within 4 h and consumed less than 400 μL of aqueous and organic phase.

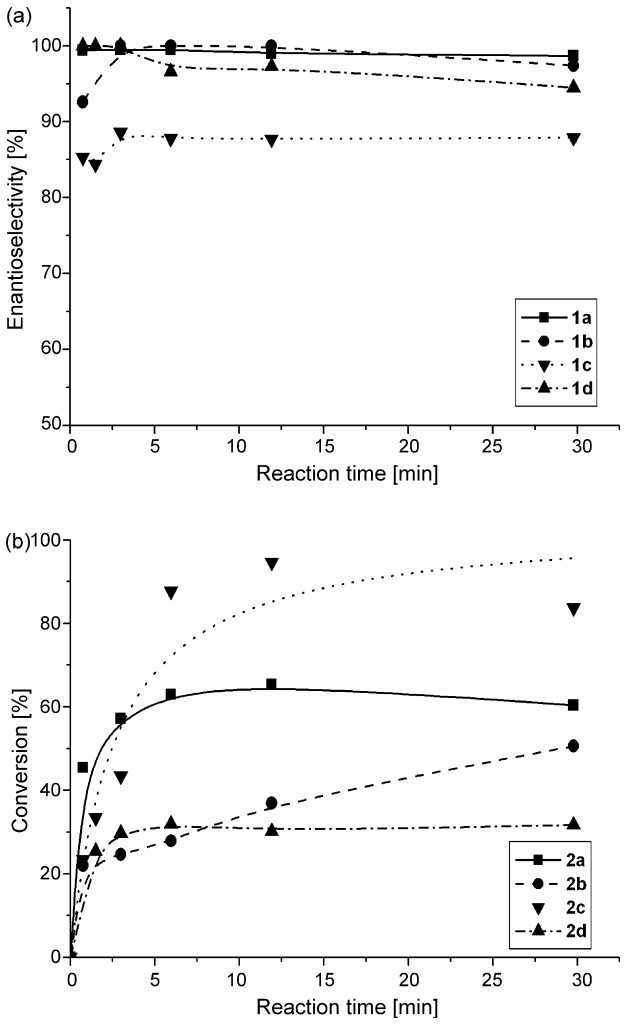


Fig. 2. (a) Conversion of aldehydes **2a–d**. (b) Enantioselectivity of aldehydes **2a–d**. All reactions were performed with equimolar concentrations of aldehyde and HCN (0.23 M). Reaction times were varied by changing the flow speed of the organic and aqueous phase.

3. Results and discussion

The selected aldehyde substrates (**2a–d**) were converted into their corresponding cyanohydrins in the microreactor, using the crude cell lysate containing the HNL enzyme. The progress (conversion and enantioselectivity) of these reactions was analysed by chiral HPLC and is depicted in Fig. 2a and b. Fig. 2a clearly shows that the reactions readily proceed in the microreactors. Interestingly, in most cases (**2a**, **2c–e**) a rapid equilibrium was reached within 5 min. This is probably due to the fast mass transport, which at this small scale is not diffusion limited. Substrate **2b** did not show a rapid equilibrium, due to the deactivating properties of the *para*-methoxy group, which slows down the enzymatic reaction. The final conversion lies for benzaldehyde (**2a**) around 65% as a consequence of the equimolar ratio of aldehyde and HCN and can be shifted by applying larger amounts of the cyanide source or using more enzyme. A similar trend was observed for the three other aldehydes, with an equilibrium depending on the reactivity of the aldehyde involved.

The enantioselectivity for the aromatic substrates (**2a**, **2b**, **2d**) was generally higher than 95%. The aliphatic substrate (**2c**) showed a somewhat lower enantioselectivity of approximately 85%. This might be explained by the higher reactivity of aliphatic aldehydes towards nucleophilic attack of cyanides. The competing non-enzymatic addition of cyanide to the aldehyde therefore has a more pronounced effect on the overall selectivity. The observed values of rate, reaction time, conversion and enantioselectivity fall in the same range as with optimised bench scale two-phase experiments.

Much to our satisfaction, the lysate could be readily applied in the microreactor without causing any undesired clogging of the channels. It must be noted though, that in all microscale experiments an undefined plug flow was observed, rather than an initially anticipated laminar flow, which may be due to the presence of detergents and other surfactants in the crude cell lysate.

Since conservative collection and stabilisation times were employed the overall time to screen the reaction landscape of the HNL-catalysed cyanide addition to an aldehyde amounted to about 4 h, consuming 400 μL for both phases. Currently we are further optimising the screening procedure, which will lead to a significant reduction in both analysis time and use of reagents.

4. Conclusion

In conclusion, we have successfully demonstrated that a crude cell lysate containing a hydroxynitrile lyase can be used for the enantioselective synthesis of several cyanohydrins in a microchannel. These enzymatic reactions showed a high initial reaction rate and enantioselectivity, which in a batchwise process can only be achieved by vigorous stirring. Currently, additional substrates are under investigation and the results will be compared in terms of selectivity and reaction rate in a more quantitative manner with classical batchwise methods.

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