DOI: 10.1002/adsc.200700016

Hydroxynitrile Lyase in Organic Solvent-Free Systems to Overcome Thermodynamic Limitations

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Received: January 9, 2007; Revised: March 25, 2007

Dedicated to Prof. Dr. Maria-Regina Kula on the occasion of her 70th birthday.

Abstract: The overcoming of thermodynamic limitations in the synthesis of optically active ketone cyanohydrins by using organic solvent-free systems has been investigated. Therefore, substrates with known unfavorable results within hydroxynitrile lyase-catalyzed reactions were selected for the determination of limitations and bottlenecks in ketone cyanohydrin synthesis. The highly (*S*)-selective hydroxynitrile lyase from *Manihot esculenta* (*Me*HNL) has been chosen for the conversion of acetophenone and the corresponding derivatives, which are substrates that exhibit only low grades of conversion also with several other hydroxynitrile lyases. With organic solvent-free systems under optimized reaction conditions conversions up to 78% with >99.0 *ee* (*S*) were obtained. Finally, 5 mL of (*S*)-acetophenone cyanohydrin with an enantiomeric excess of 98.5% *ee* (*S*) were synthesized.

Keywords: acetophenone cyanohydrin; enzyme catalysis; hydroxynitrile lyase; *Manihot esculenta*; thermodynamics

Introduction

Chiral cyanohydrins are versatile intermediates in the synthesis of α-hydroxy acids, β-amino alcohols, amino nitriles, α-hydroxy ketones and aziridines.[1–4] Among the known methods for the synthesis of enantiomerically pure cyanohydrins, the use of hydroxynitrile lyases for the synthesis of cyanohydrins is currently the most effective approach for this class of compounds.[5,6] These hydroxynitrile lyases have been intensively studied during the last decades with excellent results for a variety of unnatural substrates yielding (*R*)- and (*S*)-cyanohydrins in high purities and high enantiomeric excess.[7–10] An interesting member of this group is the hydroxynitrile lyase from *Manihot esculenta* (E.C. 4.1.2.37) (*Me*HNL), which exhibits high enantioselectivity for a broad spectrum of substrates of aldehydes and ketones.[11–13] Moreover, the availability of several mutants is also beneficial due to the conversion of substrates with very bulky substituents, for example, 3-phenoxybenzaldehyde (mutant *Me*HNL-W128A).[14]

Unfortunately, low grades of conversion are still observed for many substrates using both non-enzymatic and enzymatic techniques. In the field of enzymatic cyanohydrin synthesis several ketone substrates showed, for example, low conversions using several sources of hydroxynitrile lyases.[12,15,16] On the one hand, this behavior is often explained by low reactivities of ketone substrates. On the other hand, other ketone cyanohydrins are obtained with high yields and excellent enantiomeric excess, for example, phenylacetone cyanohydrin with a yield of 97% and an enantiomeric excess of 98% *ee* (*S*) using an immobilized hydroxynitrile lyase from *Manihot esculenta* (*Me*HNL).[17] This indicates that the limitations in enzymatic ketone cyanohydrin synthesis are much more complicated.

By contrast, several non-enzymatic pathways were developed to overcome these limitations, for example, catalytic asymmetric cyanosilylation using titanium isopropoxide and trimethylsilyl cyanide as a cyanide donor.[18–20] However, the requirement of anhydrous conditions, including high pressure reactions conditions, and an additional hydrolysis step represents a significant drawback, which is directly related to high costs and low atomic efficiencies. Therefore, the enzy-

matic cyanohydrin synthesis is still the best pathway to obtain enantiopure cyanohydrins.

To describe limitations in enzymatic ketone cyanohydrin synthesis we have chosen acetophenone and the corresponding derivatives as substrates, which showed low grades of conversion and only moderate enantioselectivities with several hydroxynitrile lyases.[12,16,21–23] Enantiopure acetophenone cyanohydrin is a very useful building block in the atrolactic acid synthesis.[24,25]

Results and Discussion

In biocatalytic reactions several limitations, like substrate or product inhibition, may occur, which directly lead to a limitation in productivity. On the one hand, one way to overcome the problem of inhibition is the right choice of reactor type, where a substrate inhibition can be overcome, for example, by a fed-batch process or by performing the reaction in a continuously operated stirred-tank reactor (CSTR). In case of a product inhibition an *in situ* product removal would be the best choice. Usually aqueous/organic two-phase systems and XAD-resins (for adsorption of the product) are used for this approach.[26] Additionally, thermodynamic limitations can by overcome be the removal of the product.

On the other hand, the hazardous properties of hydrogen cyanide allow for large-scale applications usually only simple batch reactions. Only small-scale applications, for example, small continuously operated membrane reactors, have proven their practicability under the safety circumstances of hydrogen cyanide.[27] Additionally, a continuous removal of the product (cyanohydrin) is not known so far in enzymatic cyanohydrin synthesis.

Finally, the undesired chemically side reaction (non-enzymatic formation of racemic cyanohydrins) can be reduced by decreasing the pH value within the aqueous phase[27,44] or by decreasing the reaction temperature.[28] The mass transfer limitation is also a powerful tool to enhance the enantiomeric excess.[29,30]

In order to understand the underlying limitations within hydroxynitrile lyase-catalyzed ketone cyanohydrin synthesis, we investigated several different reaction systems to overcome the limitations (Figure 1).

Figure 1. (*S*)-Acetophenone cyanohydrin synthesis using the hydroxynitrile lyase from *Manihot esculenta* (*Me*HNL).

Homogeneous Aqueous-Ethanol System

At the beginning, a one-phase-system with ethanol as co-solvent (to increase the solubility of acetophenone) was chosen for the determination of possible substrate and product inhibitions. However, first results showed clearly that an inhibition of the enzyme is not the limiting effect. On the contrary, the unfavorable position of the thermodynamic equilibrium prohibits high grades of conversions, which can be explained by a higher stability of the substrate acetophenone compared to the product acetophenone cyanohydrin. Within the homogenous aqueous-ethanol system, the initial substrate concentration is directly correlated with the thermodynamic equilibrium. An acetophenone concentration of only 10 mmol/L yields only a very low grade of conversion, while an initial acetophenone concentration of 500 mmol/L allows the highest grade of conversion (Figure 2). This increase of the thermodynamic equilibrium by an increase of the substrate concentration can be explained by the principle of Le Châtelier. In this case, 2 molecules (the substrates: acetophenone and hydrogen cyanide) are transformed into 1 molecule (the product:

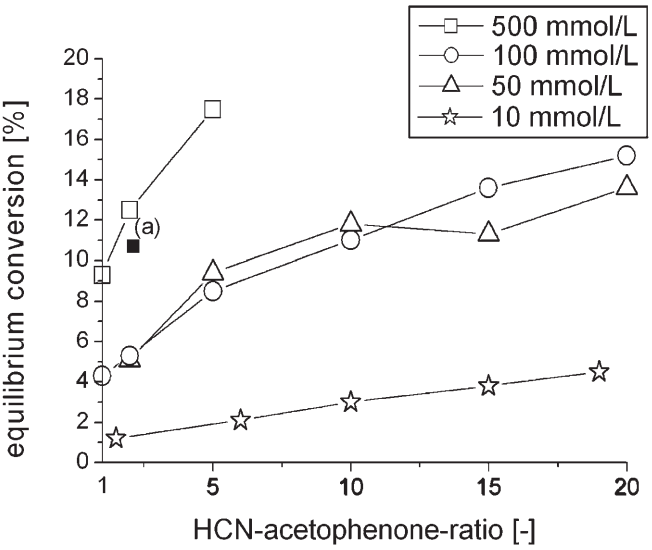

Figure 2. Acetophenone cyanohydrin synthesis in one-phase-system consisting of a mixture of ethanol and citrate buffer pH 4.0: (-☆-) 10 mmol/L acetophenone: 100% (v/v) buffer, (-△-) 50 mmol/L acetophenone: 25% (v/v) ethanol, 75% (v/v) buffer, (-○-) 100 mmol/L acetophenone: 35% (v/v) ethanol, 65% (v/v) buffer, (-□-) 500 mmol/L acetophenone 50% (v/v) ethanol, 50% (v/v) buffer. *Reaction conditions:* 5 °C, 50–500 mmol/L citrate buffer pH 4.0/ethanol, 400 rpm. Equilibrium constant: K = 0.0006 L/mmol. Due to the determination of the thermodynamic equilibrium no enzyme was required (reaction yielding the racemate). [a] [Bühler et al. 2003][12] – 200 mmol/L acetophenone, 400 mmol/L hydrogen cyanide (ratio 1:2) in diisopropyl ether with immobilized *Me*HNL on nitrocellulose (pure organic *one-phase* system).

Table 1. Enzyme stability and enzyme activity with the addition of ethanol at 5 °C.[a]

Addition of ethanol [(v/v) %]	Enzyme stability, half life period [h]	Relative enzyme activity [%]
0	>500	100.0
5	>500	18.1 ± 3.8
10	>500	17.3 ± 1.2
15	n.d.	16.1 ± 2.5
25	40	8.0 ± 3.5
50	<1	9.6 ± 1.9

[a] *Reaction conditions:* 5 °C, 50-500 mmol/L citrate buffer pH 4.0, 400 rpm; n.d. - not determined

acetophenone cyanohydrin), which is a decrease of the overall concentration of the reactants. Therefore, the reaction system responds with an increase of the equilibrium conversion when the acetophenone concentration is increased. This behavior was also found with the (better soluble) substrate benzaldehyde.[31]

Interestingly, Bühler et al. reported similar results by using pure organic one-phase systems, whereas the enzyme was immobilized on nitrocellulose (Figure 2).[12] By using an HCN to acetophenone ratio of only 2 and an initial acetophenone concentration of 200 mmol/L, 13 % conversion was obtained, which correlates with our findings. Some authors discussed a difference of the equilibrium constants between enantioselective and racemic cyanohydrin formation.[28]

On the other hand, the high concentration of ethanol has a negative effect on the enzyme stability and activity (Table 1). With increasing amount of ethanol the enzyme stability decreases dramatically from a half life period of over 500 h to only 40 h at 25 % (v/v) and less than 1 hour at 50 % (v/v) ethanol. Unfortunately, due to the extremely low enzyme stability at an ethanol content of 50 % (v/v), the enantioselective catalysis is completely suppressed and only the non-enzymatic addition of hydrogen cyanide occurs, yielding the racemate.

Two-Phase System

Two-phase systems are well established and investigated within hydroxynitrile lyase-catalyzed reactions using a variety of substrates and hydroxynitrile lyases from several sources.[29,32–34] Unfortunately, only low grades of conversion were obtained for acetophenone cyanohydrin synthesis by using a two-phase system consisting of diisopropyl ether as the organic phase and citrate buffer as the aqueous phase. Even an increase of the substrate concentration leads only to a slight increase of the equilibrium conversion (Table 2).

Table 2. Acetophenone cyanohydrin synthesis in a two-phase system.[a]

Overall acetophenone concentration within the two-phase system (DIPE buffer)	Equilibrium conversion [%]
10 mmol/L acetophenone	2.0
100 mmol/L acetophenone	2.5

[a] *Reaction conditions:* substrate to HCN ratio 1:2; diisopropyl ether to buffer ratio 1:1; citrate buffer pH 4.0; 10 °C, 400 rpm, 20 U mL^{-1} *Me*HNL

This very low equilibrium conversion is associated with the results from the homogeneous aqueous-ethanol system. Due to the high partition coefficient of acetophenone, the acetophenone concentration within the aqueous phase is very low (both experiments <5 mmol/L). Hence, these low substrate concentration result in low grades of conversion for the two-phase system.

Organic Solvent-Free System

In contrast to the one- and two-phase systems an organic solvent-free system consists only of the substrate, which represents in this special case also the organic phase, and the aqueous phase, containing the hydroxynitrile lyase and the buffer salts.

By using this unusual reaction system the equilibrium conversion can be enhanced significantly. At a fixed acetophenone to buffer ratio of 1:20 (equivalent to 0.41 mol/L acetophenone) and an increasing acetophenone to HCN ratio up to 1:20, the thermodynamic equilibrium conversion has been raised up to 46 % (Table 3). Unfortunately, the enzyme stability decreases simultaneously with increasing hydrogen cyanide and acetophenone concentrations from >500 h in pure buffer (Table 1) *via* 29 h in the organic solvent free system with acetophenone and finally to 7 h with 2 mol/L hydrogen cyanide (also organic solvent-free system with an acetophenone to HCN ratio of 1:5). This prevents high grades of enantioselective conversion. The same behavior was also found for the closely related hydroxynitrile lyase from *Hevea brasiliensis*.[35–37]

Nevertheless, for an acetophenone to HCN ratio of 1:5 an enzyme stability of 7 h still enables 22 % conversion with excellent 97 % *ee* (*S*). Higher hydrogen cyanide concentrations permanently deactivate the enzyme and thus prevent higher grades of conversion, which is a general problem in hydroxynitrile lyase-catalyzed cyanohydrin synthesis.[37,38]

Furthermore, the acetophenone to buffer ratio has also a significant influence on the equilibrium conversion. By adjusting the acetophenone to buffer ratio

Table 3. Variation of acetophenone to HCN ratio.[a]

Acetophenone to HCN ratio	Enzyme stability (half life time) [h]	Equilibrium conversion [%]	pH 4.8		pH 4.0	
			Conversion [%]	Enantiomeric excess [%]	Conversion [%]	Enantiomeric excess [%]
1:0	29	0	0	0	0	0
1:2	8	10	10	95	n.d.	-
1:5	7	22	22	96	22	97
1:10	<0.5	31	6	6	22	97
1:15	<0.5	37	5	0	0	0
1:20	n.d.	46	5	0	0	0

[a] *Reaction conditions:* reaction time: 6 h; acetophenone to buffer ratio 1:20 (overall acetophenone concentration equivalent to 0.41 mol/L), citrate buffer pH 4.0 or pH 4.8, 5 °C, 400 rpm; 450 U mL^{-1} for pH 4.0; 150 U mL^{-1} for pH 4.5; n.d. = not determined.

Table 4. Variation of acetophenone to buffer ratio.[a]

Acetophenone to buffer ratio	Overall acetophenone concentration [mol/L]	pH 4.8; 150 U mL^{-1} *Me*HNL		pH 4.0; 450 U mL^{-1} *Me*HNL	
		Conversion [%]	Enantiomeric excess [%]	Conversion [%]	Enantiomeric excess [%]
1:100	0.085	n.d.	-	9	86
1:33	0.25	11	95	11	94
1:20	0.41	22	96	22	97
1:10	0.78	36	91	15	98
1:6.6	1.13	31	75	n.d.	n. d.

[a] *Reaction conditions:* reaction time: 6 h; acetophenone to HCN ratio 1:5; citrate buffer pH 4.0 or pH 4.8, 5 °C, 400 rpm; n.d. = not determined.

from 1:100 up to 1:10 the equilibrium conversion has been increased from 9% up to 36% with a slight decline of the enantioselectivity (Table 4).

Noteworthy, the hydrogen cyanide concentration increases simultaneously with the increasing acetophenone concentration at a constant acetophenone to HCN ratio of 1:5. This leads again to a loss of enzyme stability and prevents higher grades of conversion at even lower acetophenone to buffer ratios (1:6.6).

Reactions with Acetophenone Derivatives

Finally, the position and kind of substituents at the aromatic ring also influence the maximal conversion and enantiomeric excess within hydroxynitrile lyase-catalyzed reactions.[39] Therefore, several acetophenone derivatives were tested under the optimized conditions, but only substrates being that were liquid at 5 °C were applied.

On the one hand, substrates with electronegative substituents like fluoro, chloro and nitro groups showed the best results, for example, yields up to 71% could be obtained for the substrate 2′-fluoroacetophenone (Table 5). Additionally, the position of the substituents also displays a significant influence on the maximum conversion. For instance, 4′-fluoroacetophenone was converted to the corresponding cyanohydrin only in 14% yield, while 2′-fluoroacetophenone showed a conversion of 71% (as already stated above). This can be explained by an intramolecular hydrogen bond, which stabilizes the cyanohydrin and facilitates high grades of conversion (Figure 3).[40] In contrast, by applying a two-phase system (diisopropyl ether-citrate buffer) for 2′-fluoroacetophenone the conversion decreases to 8%, the effect was pointed out in the section 'Two-Phase System'. The intramolecular stabilization by a hydrogen bond occurs also for 2-nitroacetophenone cyanohydrin. In contrast to this, 2′-methoxyacetophenone cyanohydrin and 2′-hydroxyacetophenone result still only in low grades of conversion. Potentially, the positive mesomeric effect of the oxygen atom is still too strong and thus prevents higher equilibrium conversions.

Additionally, the conversions of 2′-chloroacetophenone and 2′-bromoacetophenone result in lower yields and enantiomeric purities, which can be explained by steric effects due to the size of the substituent.

On the other hand, electropositive substituents like methyl and amino groups resulted in extremely low conversions of less than 1%. 2′,3′,4′,5′,6′-Fluoroacetophenone affords also a very low conversion, while the reasons for this behavior are not completely known till now.

Table 5. Acetophenone derivative cyanohydrin formation.[a]

Acetophenone (AP) derivative	Time [h]	Conversion [%]	*ee* (*S*) [%]
AP	6	22	96
4′-F-AP	1.5	14	>99
3′-F-AP	**3**	**48**	**>99**
2′-F-AP	**3**	**71**	**>99**
2′,3′,4′,5′,6′-F-AP	6	<1	-
4′-Cl-AP	6	18	97
3′-Cl-AP	**6**	**23**	**97**
2′-Cl-AP	4.5	6	80
4′-Br-AP	solid		
3′-Br-AP	6	10	>99
2′-Br-AP	6	9	68
4′-I-AP	solid		
2′-I-AP	6	<1	-
4′-Me-AP	6	<1	-
3′-Me-AP	6	<1	-
2′-Me-AP	6	<1	-
4′-MeO-AP	solid		
3′-MeO-AP	6	<1	-
2′-MeO-AP	6	<1	-
4′-NO_2-AP	solid		
3′-NO_2-AP	solid		
2′-NO_2-AP	**1.5**	**40**	**>99**
4′-NH_2-AP	solid		
3′-NH_2-AP	solid		
2′-NH_2-AP	6	<1	-
4′-OH-AP	solid		
3′-OH-AP	solid		
2′-OH-AP	6	<1	-

[a] *Reaction conditions:* reaction time: 1.5–6 h; 0.4 mmol acetophenone derivative: 2 mmol hydrogen cyanide; 1 mL citrate buffer pH 4.0, 5 °C, 450 U mL^{-1}, 400 rpm; solid = not determined due to the absence of an organic layer.

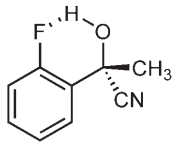

Figure 3. Possible intramolecular hydrogen bond of (*S*)-2′-fluoroacetophenone.

Conclusions

We investigated the use of organic solvent-free systems for their practicability to overcome limitations and bottlenecks within acetophenone cyanohydrin synthesis. We pointed out that organic solvent-free systems enable good conversions also for substrates which otherwise showed unsatisfactory results using conventional methods, like aqueous-organic two-phase systems.

The organic solvent free system has several advantages.

Firstly, the substrate concentration within the aqueous phase correlates directly with the enzyme activity. Especially at substrate concentrations below the K_M value (Michaelis–Menten constant) the enzyme activity will be reduced dramatically. In the case of an aqueous organic two phase-system, for example, with diisopropyl ether as the organic solvent and a buffer as the aqueous layer, the partition coefficient of the substrate generates only a low substrate concentration within the aqueous phase, which leads directly to a low enzyme activity. The kinetics within the two-phase system were intensely studied during the last years.[33,36] On the other hand, the organic solvent-free system affords always a maximum enzyme activity, because the substrate concentration is only limited by the solubility of the substrate in the aqueous phase.

Secondly, an increase of the substrate concentration within the aqueous phase results also in higher grades of conversion, as pointed out in the section 'Homogenous Aqueous-Ethanol System'. Therefore the organic solvent-free system is extremely advantageous, because the substrate concentration is only limited by the solubility of the substrate, resulting in very high grades of conversion.

Thirdly, the organic layer (in this case mainly the substrate) acts for the product (cyanohydrin) as an ordinary organic phase, resulting in a simple partition coefficient of the product. Therefore the product is selectively extracted into the organic phase, whereas the substrate concentration in the aqueous phase (where the enzymatic reaction takes place) is still maximal due to the limited water miscibility of acetophenone. This *in situ* product removal shifts the equilibrium to higher grades of conversion, depending also on the type and position of the substituents.

Which effect is more relevant to explain the observed higher conversion can only be confirmed with a thorough investigation. This would include exact determination of phase ratios, distribution coefficients and equilibrium constants, but this is complicated due to the volatility of hydrogen cyanide. That such studies are indeed possible has been shown recently for the enantioselective ketone reduction using alcohol dehydrogenases.[41,42]

The potential of the approach presented here was proven by synthesis of 5 mL of (*S*)-acetophenone cyanohydrin with an enantiomeric excess of 98.5% *ee* (*S*). This included also a distillation step without any decomposition or racemization of the product.[43]

In summary, the organic solvent-free system enables moderate to high conversions, even for substrates which were not obtained by conventional reaction systems, for example, the two-phase system using diisopropyl ether and buffer or immobilized enzyme in diisopropyl ether. Unfortunately, the enzyme stability is reduced by the very high concentrations of acetophenone and hydrogen cyanide, but the hydroxynitrile

lyase from *Manihot esculenta* still exhibited good enzyme stability and excellent enantioselectivity.

Experimental Section

*C*hemicals

2′-Methylacetophenone, 2′-fluoroacetophenone, 2′-iodoacetophenone, 2′-nitroacetophenone, 3′-chloroacetophenone and 3′-methylacetophenone were purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. 2′-Hydroxyacetophenone, 2′-bromoacetophenone, 3′-methoxyacetophenone, 3′-fluoroacetophenone, 3′-bromoacetophenone, 4′-fluoroacetophenone and 2′,3′,4′,5′,6′-pentafluoroacetophenone were obtained from Acros Organics, Geel, Belgium. 2′-Chloroacetophenone, 4′-chloroacetophenone, racemic mandelonitrile and acetophenone were products of Merck-Schuchardt, Hohenbrunn, Germany. 4′-Methylacetophenone, 2′-methoxyacetophenone and sodium cyanide were purchased from Fluka Chemie GmbH, Buchs, Switzerland.

Enzyme

The (*S*)-hydroxynitrile lyase from *Manihot esculenta* (recombinant in *E. coli*) was obtained from Julich Chiral Solutions GmbH (http://www.julich.com/), Jülich, Germany.

Enzyme Assay

The enzyme activity was determined by following the cleavage of *rac*-mandelonitrile into benzaldehyde and HCN at 25 °C. The formation of benzaldehyde was measured spectrometrically at 280 nm. The non-enzymatic cleavage reaction was monitored under identical conditions and subtracted. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the cleavage of 1 μmol mandelonitrile per minute under assay conditions.

Assay conditions: 700 μL citrate-phosphate buffer pH 5.0, 100 μL enzyme solution (dilution if required) and 200 μL mandelonitrile stock solution (60 mmol/L in citrate-phosphate pH 3.5) were mixed in a cuvette with 1 cm pathlength and the increase of absorbance at 280 nm was measured for 2 min.

HCN Formation, General Procedure

The required amount of HCN was freshly distilled in a well ventilated hood. 4 grams of sodium cyanide were dissolved in 10 mL de-ionized water and 10 mL of 5 mol/L sulfuric acid were added dropwise within 2 min. Afterwards the combined solutions were heated up to 75 °C and formed HCN was trapped and stored at 5 °C. For the removal of traces of water traces a spatula tip of sodium sulfate was added. All waste solutions were collected and disposed.

An electrochemical HCN detector (Micro III G203, GfG-Gesellschaft für Gerätebau mbH, Dortmund, Germany) was placed in the hood for continuous monitoring.

Reaction Conditions for the Enzymatic Synthesis of Cyanohydrins

All reactions were carried out at 5 °C.

Homogenous Aqueous-Ethanol System

The required amount of ethanol (co-solvent) was added to 50–500 mM citrate buffer pH 4.0 in order to obtain the desired solubility of acetophenone in buffer. Afterwards acetophenone and hydrogen cyanide were added. Due to the determination of the thermodynamic equilibrium *no enzyme* was required.

Two-Phase System

The required amounts of acetophenone and hydrogen cyanide were added to the biphasic system, consisting of diisopropyl ether and 50–500 mM citrate buffer (pH 4.0, ratio 1:1). The enzymatic reaction was started by the addition of the enzyme solution (20 U mL^{-1}).

Organic Solvent-Free System, General Procedure

Acetophenone and hydrogen cyanide were added to 1 mL buffer, the reaction was initiated by the addition of the enzyme solution (450 U mL^{-1}).

Comparison of the Acetophenone Derivatives

0.4 mmol of the acetophenone derivative and 2 mmol of hydrogen cyanide were added to 1 mL 50–500 mM citrate buffer (pH 0.0 or pH 4.8). The enzymatic reaction was started by the addition of the required enzyme solution (150 or 450 U mL^{-1}).

Sample Taking

Homogeneous aqueous-ethanol system: A sample (100 μL) was extracted with 100 μL *n*-hexane. 50 μL of the organic phase (*n*-hexane) were added to a mixture of 500 μL dichloromethane, 50 μL trifluoroacetic anhydride and 50 μL pyridine for the acetylation procedure.

Two-phase system: 50 μL of the organic phase were added to a mixture of 500 μL dichloromethane, 50 μL trifluoroacetic anhydride and 50 μL pyridine for the acetylation procedure.

Organic solvent-free system: A sample (100 μL) of the suspension was added to 100 μL of diisopropyl ether for the extraction. 50 μL of the organic phase were added to a mixture of 500 μL dichloromethane, 50 μL trifluoroacetic anhydride and 50 μL pyridine for the acetylation procedure.

GC Analysis for Determination of Conversion and Enantiomeric Excess

The conversion of acetophenone (and the derivatives) to acetophenone cyanohydrin (derivatives) as well as their enantiomeric excess were determined by gas chromatographic analysis with a Chiraldex capillary gas chromatography column (G-PN – γ-Cyclodextrin, Propionyl) from astec using a CP3800 (Varian) with a flame ionization detector (FID). Carrier gas was helium at 2 mL min^{-1}. Temperature gradient: 80 °C for 0.5 min, rise with 10 °C min^{-1} to 130 °C and hold 130 °C for 15 min . The injector and detector temperatures were set to 250 °C.

Acknowledgements

This research was supported by the BMBF-Project -Biohydroform- (grant number: 0313402E). The authors also thank the other members of the BMBF-Project for fruitful discussions: Dr. Martina Pohl and Jan-Karl Guterl from the Institute for Molecular Enzymetechnology (IMET), University of Düsseldorf at the Research Centre Jülich; Dr. Harald Wajant from the University of Würzburg; Dr. Richard Wisdom, Dr. Andreas Meudt and Dr. Matthias Helms from Archimica GmbH, Frankfurt/Main and Hans-Georg Hennemann from Julich Chiral Solutions GmbH, Jülich. The authors also thank Julich Chiral Solutions GmbH, Jülich (http://www.julich.com), for the wild-type hydroxynitrile lyase from Manihot esculenta. Part of this work was also financially supported by Fonds der Chemischen Industrie.

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