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Screening hydroxynitrile lyases for (*R*)-pantolactone synthesis

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Abstract

The application of (*R*)-hydroxynitrile lyases ((*R*)-HNLs) enables a simple chemo-enzymatic approach towards (*R*)-pantolactone synthesis. For the first time, several new recombinant almond (*R*)-HNL isoenzymes were compared with native HNLs from different *Prunus* species with respect to cyanohydrin formation from hydroxypivalaldehyde providing the chiral key precursor in HNL based (*R*)-pantolactone synthesis. Recombinant *Pa*HNL5 (*R*-selective hydroxynitrile lyase, isoenzyme 5, from *Prunus amygdalus*) surpasses all other tested natural and recombinant HNL variants. At low pH even very low amounts of crude enzyme catalysed stereoselective hydroxypivalaldehyde cyanohydrin formation in water based reaction systems.

Keywords: Cyanohydrin; Hydroxynitrile lyase; HNL; *Prunus* sp.; Hydroxypivalaldehyde; (*R*)-Pantolactone

1. Introduction

Already 1940, Stiller et al. [1] reported on the first successful synthesis of pure pantothenic acid via the chiral key intermediate (*R*)-pantolactone. Today, manufacturing (*R*)-pantolactone is still an object of interest for the chemical industry because of its importance in calcium (*R*)-pantothenate and (*R*)-pantothenol synthesis. Both compounds represent vitamin B_5 precursors and find wide application in food, cosmetics and pharmaceuticals [2].

Most existing techniques to synthesise (*R*)-pantolactone represent long and complex routes including the formation of racemic pantolactone and subsequent enantioselective hydrolysis [3,4] and esterification [5], respectively, or the oxidation to ketolactone which is followed by stereoselective reduction back to (*R*)-pantolactone [6-15]. Upadhya et al. [16] reported a 4-step chemical synthesis of (*R*)-pantolactone using Sharpless asymmetric dihydroxylation as the key step for the introduction of chirality and obtained (*R*)-pantolactone with 60% yield and 92% ee. However, the synthesis of (*R*)-pantolactone can be simplified by asymmetric hydrocyanation of hydroxypivalaldehyde (**1a**) [17]. Thus, (*R*)-pantolactone was directly obtained in a 3-step synthesis via its chiral cyanohydrin using (*R*)-selective hydroxynitrile lyase (see Scheme 1).

In recent years, several efforts were made to optimise the stereoselective, enzymatic addition of HCN to **1a** [17,18]. Native (*R*)-HNL from almond extracts (*Pa*HNL) was employed as catalyst. Isolated and purified enzyme was used either in buffered aqueous solution, in biphasic systems (water/organic solvent) [18] or supported by solid carriers, in micro aqueous organic phase [17]. Effenberger et al. [17] reported on the highest enantiomeric excess so far (ee: 89%, yield: 84%), employing large amounts of highly purified *Pa*HNL immobilised on Avicel in diisopropyl ether (DIPE). Though *Pa*HNL was in principle able to perform enantioselective addition of HCN to **1a**, the necessary amount of enzyme was still very high and no industrial implementation is known so far. At the same time, the enantiomeric purity is still low. Native *Pa*HNL (i.e. a mixture of several isoenzymes) and also several other HNLs have been tested so far for the asymmetric addition of HCN to different sterically hindered aliphatic aldehydes [2,17-22]. Recombinant HNL from *Linum usitatissimum* (*Lu*HNL) was described to prefer aliphatic substrates. However, applying *Lu*HNL, immobilised on nitrocellulose and resuspended in DIPE [19], resulted in worse results, i.e. 73% ee of (*R*)-hydroxypivalaldehyde cyanohydrin (**2**) and low yield (47%), than conversions employing almond

Abbreviations: DIPE, diisopropyl ether; HNL, hydroxynitrile lyase; *Lu*HNL, *Linum usitatissimum* HNL; *Pa*HNL5, (*R*)-selective hydroxynitrile lyase, isoenzyme 5, from *Prunus amygdalus*; TBME, *tert*-butyl methyl ether.

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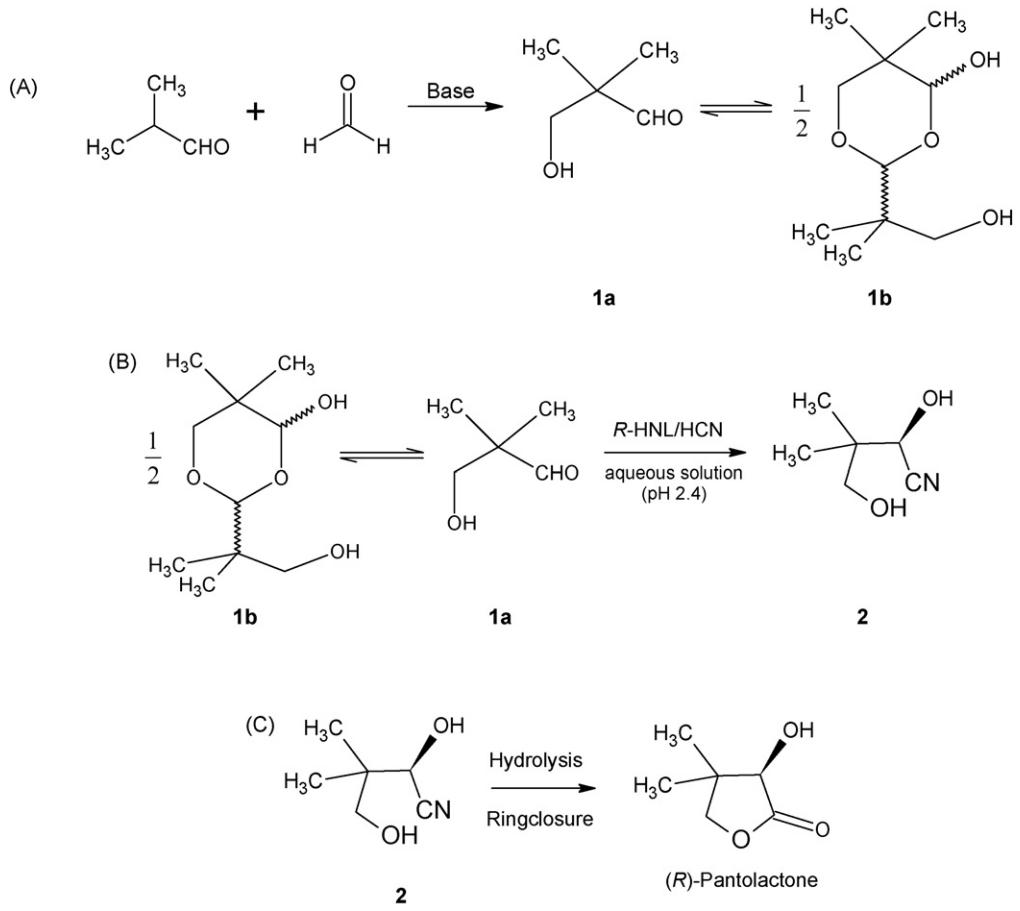

Scheme 1. Three-step synthesis of (*R*)-pantolactone.

HNL. In contrast, with another branched aliphatic aldehyde, i.e. pivalaldehyde (**3**), most HNLs showed excellent stereoselectivity. In this case, apple [20] or Japanese apricot HNL [21,22] led to the (*R*)-enantiomer in 90 and 96% ee, respectively. More reactive substrates like hydroxypivalaldehyde (**1a**) resulted in significantly decreased ee due to the competitive, non-selective chemical background reaction occurring side by side with the enzymatic cyanation [21]. Ognyanov et al. [18] expected the higher hydrophilicity of **1a** to be responsible for decreased enantiomeric purity compared to **3** when applying a biphasic reaction system (buffer/ether) and that water-immiscible organic solvents help to suppress the chemical background reactions primarily with hydrophobic substrates, making water based conversions of the aliphatic substrate **1a** problematic. However, they also worked at relatively high pH (5.0) in order not to decrease the rate of the enzymatic reaction. This led to the racemisation of the product and non-selective chemical addition of HCN to the aldehyde. As cyanohydrins are stable at acidic pH but decompose to HCN and carbonyls at neutral or basic pH-values [23], the enzymatic production of **2** has to be performed at low pH. Doing the biotransformation at low temperature favours high stereoselectivity but on the other hand enzymatic reaction rates become low. To sum up shortly, much work was done to optimise this biocatalytic reaction. Nevertheless, industrial production of (*R*)-pantolactone, employing *Pa*HNL seems to be out of reach. However, tailoring an HNL enzyme by enzyme engineering could help. Therefore, we met the challenge to find the most effective catalyst in order to establish a good starting point for future improvements by molecular engineering.

This study for the first time compares several recombinant HNL isoenzymes from *Prunus amygdalus* (*Pa*HNLs) with native HNLs from different *Prunus* species regarding their potential for the stereoselective production of **2**, the chiral key precursor in HNL based (*R*)-pantolactone synthesis. While the effect of reaction parameters like temperature, amount of employed enzyme, addition of different organic cosolvents or even the use of different less reactive pivalaldehyde derivatives like *O*-protected hydroxy- or halogenated pivalaldehydes as starting compounds was known [2,17], this investigation especially targets at finding the most promising starting point for further enzyme engineering to develop an industrially feasible biocatalytic process. Thus, especially mild conditions and temperatures which are also acceptable for industrial scale up and which allow high enzymatic rates were applied. Due to these reasons and also since even high amounts of enzyme applied in organic solvents resulted in too low yields and ee for industrial application we started a search for the best HNL variant in the most preferred industrial solvent, i.e. water, although organic solvent based systems (i.e. not miscible with water) in which the undesirable

chemical addition is more or less suppressed were usually preferred for aldehydes that are poor substrates for HNLs [23]. Furthermore, to enable industrial implementation, additional reaction steps such as substrate derivatisation are not acceptable. Therefore, we focused on hydroxypivalaldehyde (**1a**), the most suitable starting compound for a large scale synthesis of (*R*)-pantolactone [17].

When performing the reaction in pure aqueous solution, the pH particularly influences stereoselectivity of the hydroxypivalaldehyde conversion. To deal with the high reactivity of this substrate in water, the effect of pH on the reaction was examined in detail for the first time.

2. Experimental

2.1. Materials

All solvents and chemicals except for **1a** and HCN were commercially available and purified if necessary.

2.1.1. Preparation of HCN

HCN was freshly prepared by adding a saturated NaCN solution dropwise to an aqueous solution of sulfuric acid (60%) which was heated at 80 °C. The formed HCN was dried over $CaCl_2$ and collected at −12 °C using a cooling trap. All reactions with HCN or cyanides were performed under a well-ventilated hood and for continuous warning, an electrochemical HCN sensor was installed.

2.1.2. Preparation of 3-hydroxy-2,2-dimethylpropanal (*1a*)

78.0 g of a 37% aqueous solution of formaldehyde (0.961 mol) were cooled at 0 °C and mixed with 61.5 g (0.853 mol) of isobutyraldehyde. Then, small portions of solid K_2CO_3 (122 g, 0.883 mol) were added to the stirred mixture at such a rate that the temperature of the reaction mixture did not exceed 20 °C. The reaction proceeded for another 1.5 h. Then, the mixture was brought to room temperature. The viscous liquid was extracted with ether, dried over Na_2SO_4, concentrated and yielded in 87 g of crude product (99% yield) as colourless oil, which slowly crystallised forming the dimer. The product was used without any further purification. NMR analysis proved a monomer/dimer mixture and the recorded data was consistent with the data reported by Upadhya et al. [16] and Toermaekangas et al. [24]. For monomerisation before application, the dimer was heated gently for 15 min at 80 °C.

2.2. Isolation of native HNLs from Prunus species' flowers, leaves and seeds

Crude HNL enzyme extracts were prepared according to Kirschbaum et al. [25]. Frozen leaves, flowers or seeds of *Prunus* sp. were put in an appropriate dish and accurately powdered in liquid nitrogen using a pestle. Then, 1 g of the frozen powder was mixed with 10 mL of an 80 mM citrate buffer (pH 4.8) and stirred for 3 h at 4 °C. Insoluble cell debris were separated through centrifugation and the resulting enzyme preparations were tested for HNL activity analysing the cleavage of racemic mandelonitrile as described in Section 2.4.

2.3. Growth of Pichia pastoris for recombinant HNL production

The strains *P. pastoris* GS115 pHILD2-*Pa*HNL5α-L1Q [26] and *P. pastoris* GS115 pHILD2-*Pa*HNL*4* [27], respectively, were cultivated in 5 L bioreactors applying the following procedure: biomass was generated, first, during exponential and second, during linear growth. Then, recombinant *Pa*HNL5 was produced during an expression phase using methanol for induction.

The initial fermentation medium (73.5 mL ortho-phosphoric acid (85%), 3.15 g $CaSO_4{\cdot}2H_2O$, 50.05 g K_2SO_4, 42.7 g $MgSO_4{\cdot}7H_2O$, 11.55 g KOH, 140 g glycerol and deionised water to 3.5 L) was filled into the bioreactor and sterilised at 121 °C for 30 min. After cooling to 28 °C, the pH was adjusted with 25% ammonia (technical quality) to pH 5.0. 4.35 mL/L of the PTM1 trace element solution were added which consisted of 0.2 g/L biotin, 6.0 g/L $CuSO_4{\cdot}5H_2O$, 0.09 g/L KI, 3.0 g/L $MnSO_4{\cdot}H_2O$, 0.2 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.02 g/L H_3BO_3, 0.5 g/L $CoCl_2$, 42.2 g/L $ZnSO_4{\cdot}7H_2O$, 65 g/L $Fe(II)SO_4{\cdot}7H_2O$ and 5 mL/L H_2SO_4. Then, 400 mL of a preculture grown in YPD (prepared according to the '*Pichia* Expression Kit' provided by Invitrogen) were added for inoculation. The initial OD_{600} was about 1–2. For the cultivation the following parameters were chosen: temperature: 28 °C, aeration: 2.5–10 L air/min, agitation control: 500–1500 rpm, oxygen partial pressure: >30% of the saturation concentration and pH set point: 5.0. After a cultivation time of 15–20 h, the glycerol in the initial medium was consumed and glycerol feeding was initiated. The glycerol feeding medium consisted of 750 g glycerol, 12 mL/L of the PTM1 trace element solution and deionised water which was added to get a final volume of 1.5 L. After approximately 12 h and after reaching an OD_{600} of ∼180, the methanol feeding was started, first with a rate of 10–15 mL/h which was stepwise elevated to 45–60 mL/h during the first 12–15 h. The methanol feeding medium contained 0.9 L methanol and 12 mL/L of the PTM1 trace element solution. After about 72–80 h of methanol induction, the cells were separated through centrifugation and the enzyme preparation was obtained. The culture supernatant was further concentrated through ultrafiltration (Sartorius, Vivaflow 50, 30 kDa MWCO). During cultivation, HNL activity was continuously detected according to Section 2.4 and protein concentration was determined using the Biorad assay with *Pa*HNL from Sigma (M-6782 Lot 41H4016) as reference.

2.4. HNL enzyme activity assay

As a standard HNL assay, the cleavage of racemic mandelonitrile was measured photometrically. This was performed according to Weis et al. [28] by following the increase in absorption of benzaldehyde at 280 nm ($\varepsilon = 1.376$ L/mmol/cm). In general, 1 U corresponded to the amount of enzyme which

converted 1 μmol mandelonitrile to benzaldehyde and HCN per minute at pH 5.0 and room temperature.

2.5. Bioconversion of hydroxypivalaldehyde

Unless otherwise stated, typical reactions were performed as follows: 70–310 U of the corresponding HNL preparations were diluted in 5 mL of a 3 M citrate phosphate buffer (pH 2.4). Then, 120 mg (=1.2 mmol) of **1a** and 100 μL (=2.6 mmol) of anhydrous HCN were added. The reaction mixtures were stirred at 4 °C and 600 rpm applying the magnetic stirrer Variomag Poly 15. After scheduled time, 300 μL of the samples were taken, mixed with 400 μL *tert*-butyl methyl ether (TBME) and centrifuged. 200 μL of the organic phase were used for derivatisation with 100 μL acetic anhydride in presence of 100 μL pyridine and 800 μL dichloromethane. The samples were analysed by GC applying a Chirasil-DEX CB column (25 m × 0.32 mm, 0.25 μm film). Carrier gas: H_2; pressure: 1.0 bar; temperature program: 90 °C–2 min, 5 °C/min to 155 °C, 155 °C–1 min; FID detector: 250 °C. **1a**: 2.13 min; **1b**: 12.12 min, 12.44 min, 12.70 min, 13.05 min; **2**: 7.12 min; (*S*)-hydroxypivalaldehyde cyanohydrin: 7.63 min.

3. Results and discussion

In search of a promising starting point for engineering a valuable biocatalyst for **2** production, we first examined the recombinant HNLs *Pa*HNL1, overexpressed and secreted by *Schizosaccharomyces pombe* (Andryushkova et al., manuscript in preparation), *Pa*HNL4, produced by *P. pastoris* [27], and *Pa*HNL5 which was heterologously expressed in *P. pastoris* [26–28]. These recombinant isoenzymes can be supplied in large amounts and consistent composition. Furthermore, the cloned genes and their heterologous expression also provide a valuable basis for molecular engineering.

As previous studies indicated excellent stability of *Pa*HNL5 at acidic pH [26,29], we also investigated the pH effect on the conversion of **1a** to **2** (see Fig. 1). Best enantiomeric excess was obtained at pH 2.5. Elevating the pH-value of the reaction mixture led to increased conversion, whereas ee decreased significantly, especially at pH ≥ 4.0. This proved not only the above mentioned general law, valid for cyanohydrins [23], but also the high reactivity of **1a**. A possible cyclic structure of hydroxypivalaldehyde in its monomeric form includes a hydrogen bond between the hydroxy group hydrogen and the carbonyl oxygen leading to a more electrophilic carbonyl functionality (see Fig. 2). Thus, due to the fast, non-selective chemical background reaction, the enantiomeric excess of **2** was influenced negatively even at low pH-values. At pH 2.0, enzyme activity is reduced but still not totally lost. This resulted in decreased conversion but comparably high ee which slightly increased during the first 4 h. Further lowering the pH resulted in a sharp decline in ee and conversion, indicating the total loss of enzymatic activity. These results also demonstrate why all studies showing significant conversion with good stereoselectivity for the conversion of **1a** so far, described HNL applications in two phasic systems or organic solvents.

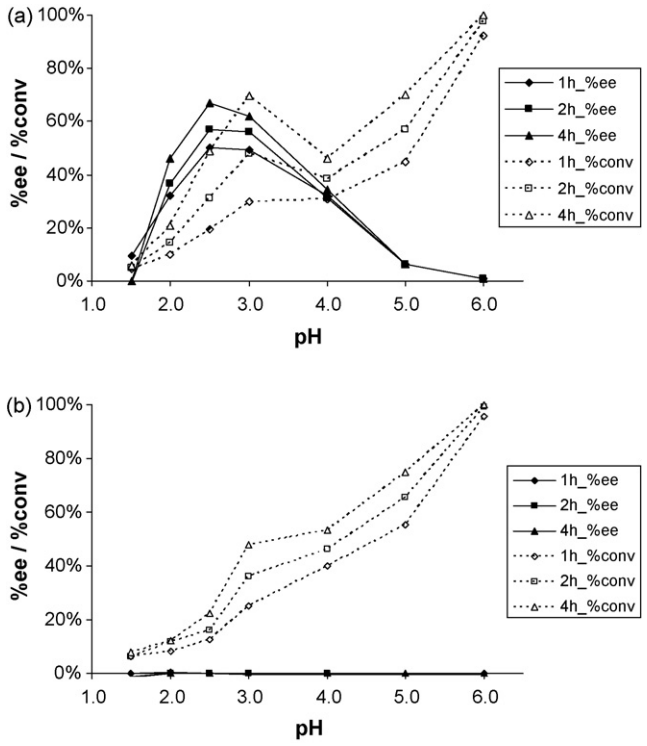

Fig. 1. pH effect on the conversion of **1a** into **2**: comparison of *Pa*HNL5 catalysed reaction (a) with non-selective chemical background reaction (b). Reaction conditions: (a) 1 mmol of **1a**, 2000 U of enzyme *Pa*HNL5 (~7.3 mg/mmol substrate) and 90 μL (=2.3 mmol) of anhydrous HCN were diluted in 5 mL of 1 M potassium phosphate buffer at pH 1.5–6.0. Reaction mixtures were stirred at 600 rpm and 10 °C. Samples were taken after 1, 2 and 4 h, respectively, and analysed by chiral GC.(b) like (a) but without enzyme. All reactions were performed at least in duplicate.

Assuming that nature might provide another, even better starting point for a laboratory evolution experiment, we investigated HNL activities of flower, leave and seed materials of several members of the rose family (Rosaceae). In particular, in members of the subfamilies Maloideae and Prunoideae, the cyanogenic glycosides prunasin and amygdalin can be found in seeds and other plant tissues. As cyanogenesis – the defence strategy of some plants against herbivores [30] – comprises the degradation of an α-hydroxynitrile into the carbonyl compound and HCN which can be catalysed by HNLs [31], the existence of cyanogenic glycosides indicates the possible coexistence of HNL enzymes. Moreover, Kiljunen and Kanerva [20] described HNL activity in apple seeds showing advantages for sterically hindered aldehydes. Interested in the performance of naturally occurring HNL isoenzymes for the synthesis of

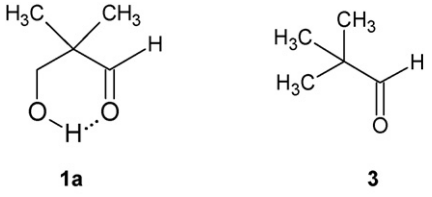

Fig. 2. Cyclic structure of hydroxypivalaldehyde (**1a**) in its monomeric form, and pivalaldehyde (**3**).

Table 1
Conversion of **1a** to the corresponding (*R*)-cyanohydrin (**2**) employing different HNL preparations

Entry	Sample name	Enzyme source/vegetative part	Scientific name	Used enzyme amount [U/mmol **1a**]	ee [%][a] (4 h)	ee [%][a] (20 h)
1	Blank	–	–	0	rac[b]	rac[b]
2[c]	*Pa*HNL5	Recombinant (*Pichia pastoris*)	–	2000	66.8	n.d.[d]
3	*Pa*HNL5	Recombinant (*Pichia pastoris*)	–	183	17.8	15.0
4	*Pa*HNL5	Recombinant (*Pichia pastoris*)	–	91.5	10.3	8.0
5	*Pa*HNL5	Recombinant (*Pichia pastoris*)	–	59.6	7.0	5.9
6	*Pa*HNL4	Recombinant (*Pichia pastoris*)	–	59.6	rac[b]	rac[b]
7	*Pa*HNL1	Recombinant (*Schizosaccharomyces pombe*)	–	59.6	rac[b]	rac[b]
8	*Pa*HNL	Sigma Aldrich (M6782)	*Prunus amygdalus*	183	rac[b]	rac[b]
9	Almond	Leaves	*Prunus amygdalus*	183	1.8	1.5
10	Apple	Seeds	*Malus domestica*	183	rac[b]	rac[b]
11	Apple	Seeds	*Malus domestica*	91.5	rac[b]	rac[b]
12	Service berry	Flowers	*Amelanchier ovalis*	91.5	rac[b]	rac[b]
13	Sweet cherry	Leaves	*Prunus avium*	91.5	rac[b]	rac[b]
14	Wild cherry	Leaves	*Prunus avium*	91.5	rac[b]	rac[b]

[a] Values are the average of 2–3 experiments.
[b] rac = racemic (enantiomeric excess <1.5%).
[c] Exact reaction conditions—see caption of Fig. 1.
[d] n.d. = not determined. We focused on comparing ee-values because conversions were only marginally higher than blank values when very low amounts of enzyme were employed. This was done to simulate feasible industrial conditions and also high throughput screening on small scale, as used in enzyme engineering.

the bulky product **2**, we chose flowers and leaves, respectively, of two traditional (and quite disease resistant) Styrian apple trees ('Kronprinz Rudolf' and 'Maschanska'), the service berry shrub, the sweet cherry tree, the wild cherry tree, leaves of the almond tree and a mixture of apple seeds from different varieties. Crude HNL enzyme extracts were prepared (see Section 2.2) and tested for HNL activity applying the standard HNL assay monitoring the cleavage of racemic mandelonitrile (see Section 2.4). Since the HNL content varies between protein extracts from different sources, we used the determined enzymatic activity to normalise the amount of employed enzyme. All enzyme preparations which showed HNL activity were concentrated applying Vivaspin 20 membrane tubes with a 10 kDa cutoff from Sartorius. The crude enzyme extracts were compared with recombinant HNLs. Furthermore, native *Pa*HNL from Sigma Aldrich was also tested for the conversion of **1a**. At the end, 183, 91.5 and 59.6 U/mmol of substrate **1a**, respectively, were applied for the asymmetric addition of HCN to **1a** in a buffered aqueous system at pH 2.4, taking into account the high impact of the non-selective chemical background reaction (already at pH > 2.5), enzyme stability and the pH optimum for the HNL mediated conversion of **1a**. Nevertheless, we employed very low amounts of enzyme to simulate feasible industrial conditions and also high throughput screening on small scale as employed in enzyme engineering in case a useful starting point enzyme can be identified. Individual samples were analysed by GC.

A glance at the ee-values (Table 1) indicates that none of the other employed enzymes could compete with recombinant *Pa*HNL5 under the applied conditions. Among the crude enzyme extracts, the extract from almond leaves performed slightly better than the rest, but all tested naturally occurring HNLs and the tested recombinant isoenzymes *Pa*HNL1 and *Pa*HNL4 showed decreased performance in the stereoselective conversion of **1a**, especially since high stability and activity at low pH are necessary for conversions in aqueous solution. In contrast to previous studies, we used relatively low amounts of HNL, since our main objective was to find the HNL with the highest fitness for **2** synthesis under conditions which are also acceptable for industrial scale up. Recently, we developed a new method for enzyme engineering employing the host *P. pastoris* [32] which in future will be employed to improve the features of *Pa*HNL5 for industrial synthesis of **2**.

4. Conclusion

This is the first report comparing the performance of recombinant and native HNL variants for stereoselective production of (*R*)-hydroxypivalaldehyde cyanohydrin. Recombinant *Pa*HNL5, produced in *P. pastoris*, provides the best starting point for engineering an improved biocatalyst for (*R*)-pantolactone synthesis. No other tested native or recombinant HNL showed similar performance. In addition, heterologous expression provides a predefined composition of the resulting enzyme preparation preventing variable isoenzyme composition as encountered in native enzyme extracts. In water based reactions, conversion at low pH using WT *Pa*HNL5 can result in **2** with similar ee as in biphasic or organic systems. An ee of more than 65% was obtained with an unpurified crude enzyme preparation. No enzyme immobilisation or complex biphasic reaction systems were needed. In addition, water based systems are easier to adapt for high throughput screening for enzyme engineering. Conversions employing very small amounts of enzyme combined with screening by GC analysis allow to discriminate between small differences in HNL activity for the synthesis of **2** and provide a basis for further enzyme developments by evolutionary engineering.

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