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FULL PAPER

Preparation of (*S*)-β-nitro alcohols by a (*R*)-selective HNL via enantioselective C-C bond cleavage

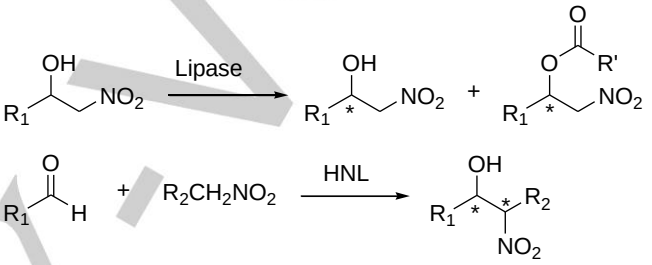
D.H. Sreenivasa Rao, [a] and Santosh Kumar Padhi*[a]

Abstract: Hydroxynitrile lyase (HNL) catalysed stereoselective synthesis of β-nitro alcohols is considered as an efficient biocatalytic approach. However, there exist only one (*S*)-selective HNL i.e. *Hevea brasiliensis* (*Hb*HNL) to synthesize (*S*)-β-nitro alcohols from corresponding aldehydes. Further, *Hb*HNL synthesis is limited by low specific activity and moderate yield. We prepared a number of (*S*)-β-nitro alcohols using a (*R*)-selective HNL from *Arabidopsis thaliana* (*At*HNL). Optimization of the reaction conditions of *At*HNL catalyzed stereoselective C-C bond cleavage of racemic 2-ntro-1-phenylethanol (NPE) produced (*S*)-NPE up to 99% ee and 47% conversion. This is the fastest HNL catalyzed route known so far to synthesize a series of (*S*)-β-nitro alcohols. This approach widens the application of *At*HNL not only to synthesize (*R*)- but also (*S*)-β-nitro alcohols starting with appropriate substrate. Without discovering a new enzyme, rather using a retro-Henry approach, it synthesized a number of (*S*)-β-nitro alcohols by taking the advantage of the substrate selectivity of *At*HNL.

Introduction

Enantiopure β-nitro alcohols are versatile synthetic intermediates. They can be transformed into various fine chemicals and chiral intermediates e.g. dehydration to conjugated nitro alkenes, reduction to vicinal amino alcohols, denitration to alcohols, oxidation to nitro carbonyl compounds and Nef reaction to α-hydroxy carbonyl compounds etc.[1–3] Nitroaldol reduced products i.e. β-amino alcohols are structural intermediates of many pharmaceuticals, such as (−)-arbutamine,[4] ritonavir,[5] (*R*)-salmeterol,[6] pindolol,[7] propranolol[8] and epinephrine[9] and fungicides.[10] Further they are important chiral building blocks in the synthesis of several bioactive molecules e.g. codonopsinine,[11] spisulosine,[12] taxotere[13] and nummularine F.[14]

Synthesis of optically pure β-nitro alcohols are reported using chemical and biocatalytic methods. Biocatalytic synthesis has advantages over chemical catalyst i.e. high regio- and stereoselectivity, mild reaction conditions, biodegradable catalyst etc. Two major biocatalytic approaches used to synthesize them are (i) kinetic resolution of racemic β-nitro alcohols, and (ii) stereoselective C-C bond formation between a carbonyl centre and nitroalkane (Scheme 1).



Scheme 1. (top) Lipase catalyzed kinetic resolution of a racemic β-nitroalcohol. R_1 is an alkyl group and R' is part of the acylating agent. (bottom) HNL catalyzed synthesis of chiral β-nitroalcohols. R_1 is an alkyl group and R_2 is usually H or another alkyl group.

Kinetic resolution of racemic β-nitro alcohols has been reported with several lipases. Kitayama et al reported stereoselective transesterification of four aliphatic β-nitroalcohols using lipase from *Pseudomonas* sp. (Amano AK) in organic solvents but found highest 78% ee with two (*S*) β-nitroalcohols.[15] Sorgedragor et al screened different lipases in the kinetic resolution of 1-nitro-2-pentanol and found highest *E* value with Novozym 435 when succinic anhydride was used for acylation in presence of TBME.[16] They found the (*S*)-enantiomers as succinic esters in 43 to 97% ee in 24 h. However this process requires one more step to deprotect the succinyl group to prepare (*S*)-β-nitroalcohols. CALB catalyzed kinetic resolution of 4,4-diethoxy-1-nitrobutan-2-ol using vinyl butyrate as acyl donor in DIPE produced its (*R*)-enantiomer in 92% ee and 50% conversion in 7 days.[17] Milner et al carried out the kinetic resolution of 2-nitrocyclohexanol to prepare its all four diastereomers.[18] This method used hydrolase-catalyzed transesterification of 2-nitrocyclohexanol and hydrolase-catalyzed hydrolysis of 2-nitrocyclohexyl acetate but in 24 h.[18] Xu et al performed a two step biocatalytic reaction that involved D-aminoacylase-catalyzed synthesis of racemic β-nitroalcohols and its kinetic resolution by immobilized lipase PS.[19] They prepared seven (*S*)-β-nitroalcohols in 84 to 97% ee and 46 to 53% conversion in 12 h. Li et al demonstrated enantioselective transesterification of racemic β-nitroalcohols using *Burkholderia cepacia* lipase.[20] They obtained the acyl esters of seven (*S*)-β-nitroalcohols in 81 to 99% ee in 12 h.

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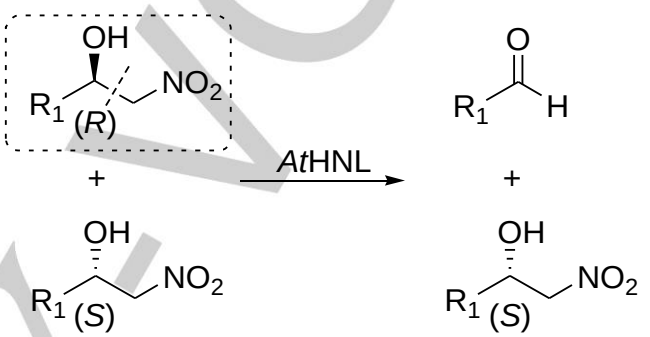
Kuhbeck et al prepared racemic β-nitroalcohols using Ca^{2+}-alginate hydrogel beads in DMSO and subjected to immobilized lipase PS catalyzed kinetic resolution to prepare five (*S*)-β-nitroalcohols in 31 to 41% yield and >95% ee.[21] This analysis clearly indicates that lipase catalyzed kinetic resolution of racemic β-nitroalcohols suffers with either long reaction time i.e. 12 to 24 h (in some cases up to 7 days)[17–21] or an additional step to deprotect the acyl derivative product. Ramström and coworkers have demonstrated dynamic kinetic resolution approach in one-pot synthesis of enantioenriched β-nitro alcohol derivatives using triethyl amine and *Pseudomonas cepacia* (PS-C I).[22] However, this method synthesized (*R*)-products in 2 to 4 days reaction time and in the form of acyl derivatives.

HNL catalyzed stereoselective synthesis of β-nitro alcohols is considered to be an efficient biocatalytic approach because it is a single step transformation that uses easily available aldehydes as substrates and has the potential to achieve 100% yield. So far there exist three (*R*)-selective HNLs i.e. *Arabidopsis thaliana* (*At*HNL),[23] *Granulicella tundricola* (*Gt*HNL),[24] and *Acidobacterium capsulatum* (*Ac*HNL)[24], while only one (*S*)-selective HNL i.e. *Hevea brasiliensis* (*Hb*HNL)[25,26] to synthesize corresponding stereoselective β-nitro alcohols. *Hb*HNL is the first HNL to be reported for nitroaldolase activity.[25] However *Hb*HNL catalyzed synthesis of chiral Henry products is limited by low specific activity (Table S2). Further the yield and ee for (*S*)-2-ntro-1-phenylethanol was reported to be moderate i.e. 63% and 92% ee respectively.[25] When ee of the products was increased by lowering the pH of the reaction, corresponding yield was decreased.[26] Therefore both lipase and HNL catalyzed methods for the synthesis of (*S*)-β-nitro alcohols are limited with not only long reaction time but also moderate yield/conversion to products. Devamani et al reported nitroaldol activity in the ancestral enzyme HNL1 which showed (*S*)-selectivity in the synthesis of 2-ntro-1-phenylethanol.[27] Yu et al described acyl-peptide releasing enzyme from *Sulfolobus tokodaii* (ST0779) catalyzed Henry reaction. However the absolute configuration of the Henry products have not been mentioned.[28]

Compared to the synthesis, HNLs show a higher rate in the cleavage of cyanohydrins.[29] A similar effect has been observed with β-nitro alcohols.[30] Kinetic studies of *Hb*HNL catalyzed Henry reaction reported k_{cat} of 0.013 s^{-1} for the synthesis (*S*)-NPE while 0.16 s^{-1} for the cleavage of racemic NPE into benzaldehyde and nitromethane.[30] Therefore exploiting the cleavage reaction rather than the synthesis, to prepare enantiopure NPE appears to be beneficial. Yuryev et al showed *Hb*HNL catalyzed retro Henry reaction in the preparation of (*R*)-NPE from its racemic counterpart.[31] Their study was limited to finding a process where they could minimize benzaldehyde inhibition, thus they reported the use of HCN that converted benzaldehyde to mandelonitrile. Further, they have not investigated nor showed any scope of their method to synthesize various (*R*)-β-nitro alcohols. However, it suggests that the cleavage reaction could be advantageous to synthesize enantioenriched β-nitro alcohols of opposite stereopreference of the HNL. We used a simlar approach but a different HNL to synthesize (*S*)-β-nitro alcohols.

Here we report for the first time *At*HNL catalysed stereoselective C-C bond cleavage of racemic β-nitro alcohols in the preparation of a number of (*S*)-β-nitro alcohols i.e. opposite stereo preference of *At*HNL (Scheme 2). We exploited this cleavage reaction and optimized its reaction conditions that produced (*S*)-β-nitro alcohols with ee up to 99% and 47% conversion in 3 h.



Scheme 2. *At*HNL catalyzed stereoselective cleavage of racemic β-nitro alcohols.

This is the first biocatalytic approach to synthesize (*S*)-Henry products using a (*R*)-selective HNL. This route has advantages over the existing biocatalytic routes for synthesis of chiral β-nitro alcohols. We believe this retro-Henry reaction approach is the fastest HNL catalyzed approach to synthesize a series of (*S*)-β-nitro alcohols known so far. It uses HNL for a C-C bond cleavage reaction which is closer toward the natural reaction of a HNL than a C-C bond formation. This approach widens the application of *At*HNL not only to synthesize (*R*)- but also to prepare (*S*)-β-nitro alcohols starting with appropriate substrate. It gives opportunity to synthesize a number of (*S*)-β-nitro alcohols by exploiting the substrate selectivity of *At*HNL without discovering a new enzyme but using a different approach.

Results and Discussion

Prior to exploring stereoselective C-C bond cleavage by *At*HNL as a method to prepare enantioenriched β-nitro alcohols, the potential of this method was evaluated by determining the kinetic parameters using racemic NPE as substrate. Due to unavailability of (*R*)-NPE commercially, racemic substrate was used in the kinetic experiments. NPE concentrations ranging from 0.2 to 4 mM was used in this cleavage reaction along with purified *At*HNL. Formation of benzaldehyde due to stereoselective cleavage of NPE was monitored at 280 nm. Michaelis-Menten plot (Fig 1)

showed K_m: 0.012 mM, k_{cat}: 30.8 min^{-1}, k_{cat}/K_m: 2571 min^{-1} mM^{-1} and V_{max}: 1.1 U/mg. *At*HNL catalysed synthesis of NPE appeared to be even slower for which kinetic parameters were not measured. Purified *At*HNL was also used to determine kinetic parameters using mandelonitrile (MN) as substrate. This was performed to measure *At*HNL's biocatalytic potential and also to compare its catalytic efficiency between NPE cleavage vs MN. The kinetic parameters of MN cleavage are found to be, K_m: 2 mM, k_{cat}: 4424 min^{-1}, k_{cat}/K_m: 2212 min^{-1} mM^{-1} and V_{max}: 158 U/mg (Fig S 1).

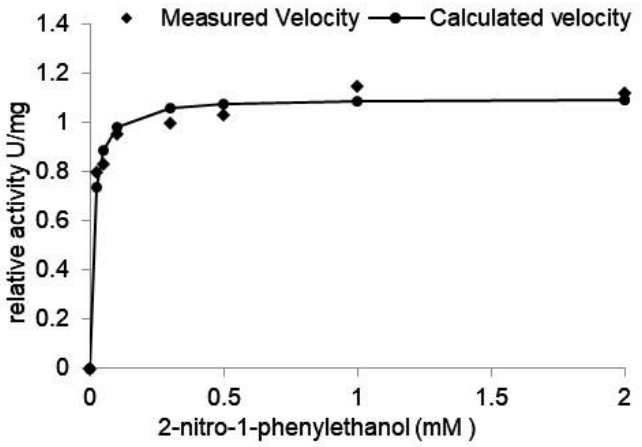


Figure 1. Michaelis Menten plot for the cleavage of rac NPE by *At*HNL

Comparison of V_{max} and k_{cat} for the above two reactions shows that, MN cleavage is faster than NPE cleavage. Similar observation has been made in case of *Hb*HNL.[32] Nitroaldol cleavage is usually a slower reaction compared to cyanohydrin cleavage by HNLs. Among the four HNLs i.e. *At*HNL, *Hb*HNL, *Ac*HNL and *Gt*HNL known to catalyse enantioselective synthesis of nitroaldols[23–26], kinetic studies for synthesis or cleavage of nitroaldol have not been reported for *Ac*HNL and *Gt*HNL. Between *At*HNL and *Hb*HNL, the rate and catalytic efficiency of retro-Henry reaction are high for *At*HNL. k_{cat} of the cleavage reaction by *At*HNL was found to be three fold higher than *Hb*HNL i.e. 30 min^{-1} vs 0.16 s^{-1}, although the stereo preference of both the enzymes differ.[30] Catalytic efficiency of NPE cleavage by *Hb*HNL was reported 3.8 $min^{-1}mM^{-1}$ only vs 2571 $min^{-1}mM^{-1}$ by *At*HNL (this study). Not only wild type, even if the nitroaldol activity by engineered *Hb*HNL is compared, maximum specific activity of the best mutant i.e. L121Y-F125T-L146M, for cleavage of racemic 2-nitro-1-phenylethanol is 0.71 U/mg.[32] As the synthesis reaction is slower than the cleavage one, therefore preparation of (*S*)-β-nitro alcohols by engineered *Hb*HNL would have lower specific activity than 0.71 U/mg. In contrary *At*HNL has showed 1.1 U/mg for the cleavage of racemic 2-nitro-1-phenylethanol. This process produces (*S*)-2-nitro-1-phenylethanol and hence *At*HNL catalyzed stereoselective cleavage appears to be catalytically efficient method for the production of (*S*)-β-nitro alcohols than *Hb*HNL catalyzed synthesis. This higher efficiency has motivated us to explore *At*HNL catalyzed stereoselective cleavage as a method to prepare (*S*)-β-nitro alcohols.

Different biocatalytic parameters for the stereoselective cleavage were optimized using racemic NPE as substrate to obtain highest % conversion and enantiomeric excess (ee) of its (*S*)-enantiomer. *At*HNL catalyzed enantioselective cleavage of racemic NPE at different pH was determined to know the effect of different pH of the buffer in this biotransformation. As below pH 5.0, wild type *At*HNL is reported to be less stable[33], we have selected the pH range from 5.0 to 6.0. This experiment showed not much difference in the % ee (96.5 to 97.1) and production (48.1 to 46.1) of (*S*)-NPE at five different pHs between 5.0 and 6.0 (Fig 2). The optimum pH was taken as 5.0 where 96.54% ee and 48.1% conversion of (*S*)-NPE was observed. *At*HNL catalyzed synthesis of (*R*)-cyanohydrin has been reported at pH 5.0[34] and (*R*)-β-nitroalcohol at pH 7.[23]

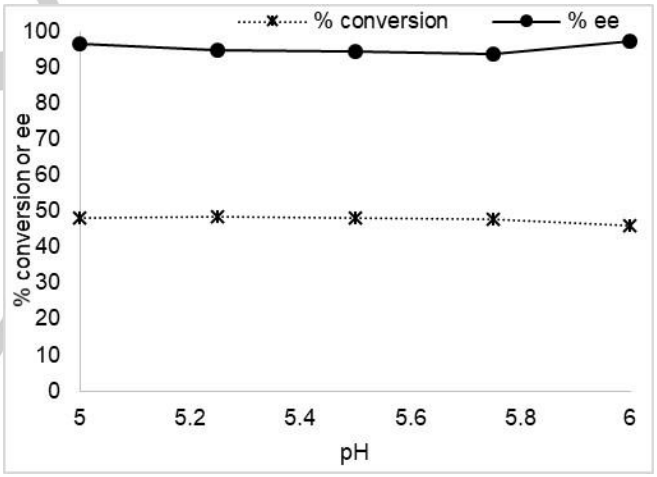


Figure 2. Effect of pH on enantioselective cleavage of rac NPE

*At*HNL catalyzed stereoselective cleavage of racemic NPE was performed at different substrate concentrations. The concentration of NPE was varied from 0.81 to 6.50 mM in the biotransformation (Fig 3). At higher substrate concentration, decrease in enantiopurity of product was observed. This decrease in ee could be due to product inhibition. Increase in the formation of benzaldehyde at higher NPE concentration may be a possible reason for this. A similar argument is done by Yuryev et al in case of retro-Henry reaction catalyzed by *Hb*HNL.[31] We have selected 3.25 mM as the optimum substrate concentration where 93.79% ee and 38.94% conversion of (*S*)-NPE was found.

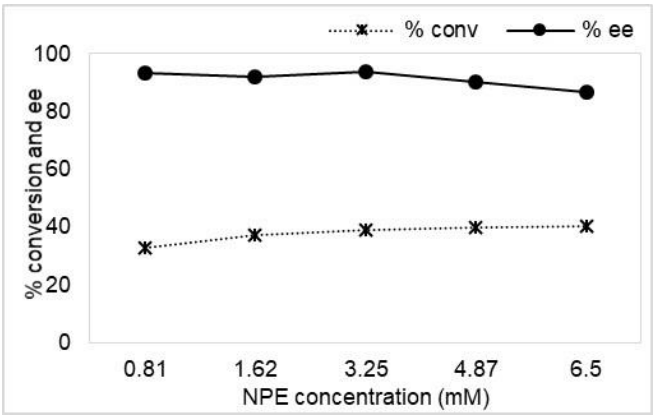


Figure 3. Effect of substrate concentration on enantioselective cleavage of rac NPE.

Asano and coworkers reported the effect of different organic solvents in the *At*HNL catalyzed synthesis of (*R*)-β-nitro alcohols.[23] They observed highest % ee and yield in case of *n*-butylacetate among diethyl ether, diisopropyl ether (DIPE), *tert*-butyl methyl ether (TBME), ethyl acetate, hexane, cyclohexane, toluene and xylene. We have selected most of these organic solvents except ethyl acetate which is similar to *n*-butylacetate, to examine their effect on the *At*HNL catalyzed enantioselective cleavage of rac NPE. The best result was obtained in case of toluene that showed the highest enantiomeric excess i.e. 96.3% of (*S*)-NPE (Fig 4). Use of tetrahydrofuran (THF) did not contribute toward the enantioselectivity of product while hexane has resulted in poor conversion. DIPE (81%), toluene (96.3%) and *n*-butyl acetate (76.2%) has showed moderate to high % ee of product. Earlier reports shows the use of toluene in biocatalysis to prepare enantiopure β-nitroalcohols.[19,23] Lipases have been reported to show high activity when toluene is used as a solvent in the kinetic resolution of β-nitro alcohols and (*S*)-1-chloro-3-(4-(2-methoxyethyl)phenoxy) propan-2-ol.[19,35] Biocatalysis without any organic solvent resulted in 93.6% ee and 35% conversion of (*S*)-NPE. Thus, comparison of stereoselective cleavage of racemic NPE to its (*S*)-enantiomer by *At*HNL in different organic solvents, with the reaction without solvent (only buffer) showed marginal improvement in both % ee and conversion by organic solvent. This result is similar to our recent findings that *Bm*HNL catalysed biotransformation in organic solvent has marginally increased the ee of cyanohydrins [manuscript under revision], and thus we have selected toluene as the best organic solvent and further optimisations were planned. Previous two experiments were conducted using DPIE as organic solvent, however both Fig 1 and 2 showed higher % ee of (*S*)-NPE compared to Fig 3. This vary in enantiopurity of product could be because (a) enzymes of different batches were used and hence the purity may vary, (b) water content of the biotransformation that influences stereoselectivity of the enzyme varies in each set of experiment due to the vary in volume of enzyme used based on their concentration.

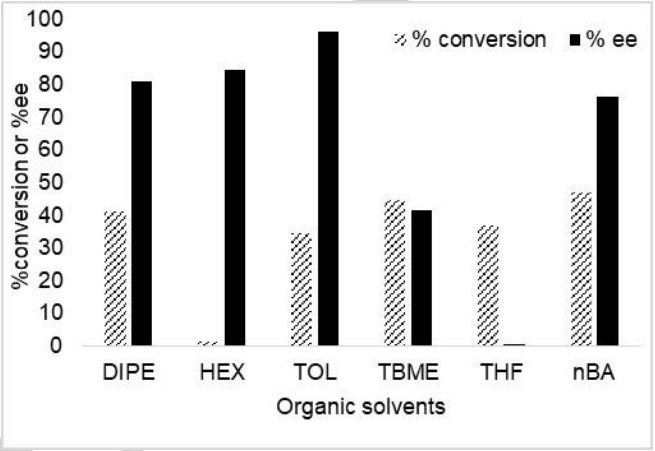


Figure 4. Effect of different organic solvents on the enantioselective cleavage of rac NPE. DIPE: diisopropyl ether, HEX: hexane, TOL: toluene, TBME: tert-butyl methyl ether, THF: tetrahydrofuran, nBA: n-butyl acetate.

The effect of amount of enzyme in the biocatalysis was studied by using 0.01 to 0.3 µmol (12.5 to 200 units) of pure *At*HNL. The ee of (*S*)-NPE was attained maximum at 0.15 mM of *At*HNL. Further increase in enzyme amount had showed negligible improve in % ee of product (Fig. 5). The optimum amount of enzyme was selected as 0.15 µmol where 99.73% ee and 53.94% conversion was observed.

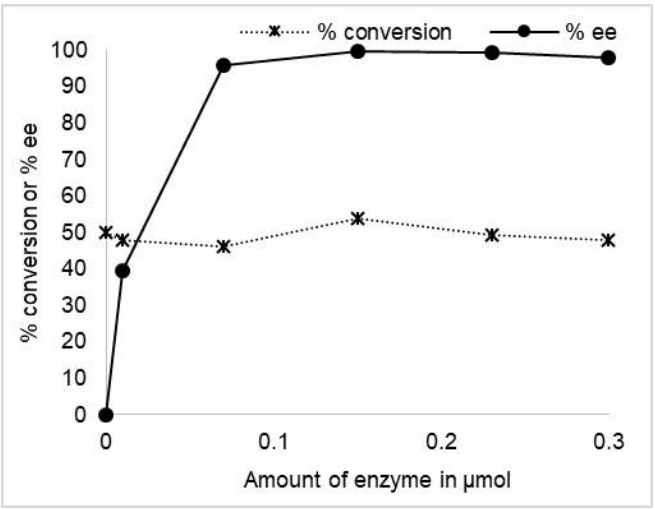


Figure 5. Effect of amount of enzyme on the enantioselective cleavage of rac NPE.

Although use of biphasic systems are known to enhance selectivity of enzymes but stability of enzyme in presence of organic solvents usually decreases. Excess organic solvent may denature the enzyme. Therefore it is necessary to find out the content of organic solvent that would be good enough to provide highest selectivity in a biocatalysis. We tried to find out the % volume of toluene in the enantioselective cleavage of racemic NPE. The % volume of toluene was varied from 0 to 65, of the overall reaction. There was not much difference in % ee of (*S*)-NPE i.e. 97.4 to 98% when 35 to 65% of toluene was used, whereas high conversion 45.77% was observed with 65%. Hence 65% of toluene was chosen as optimum solvent content to pursue further optimization experiments.

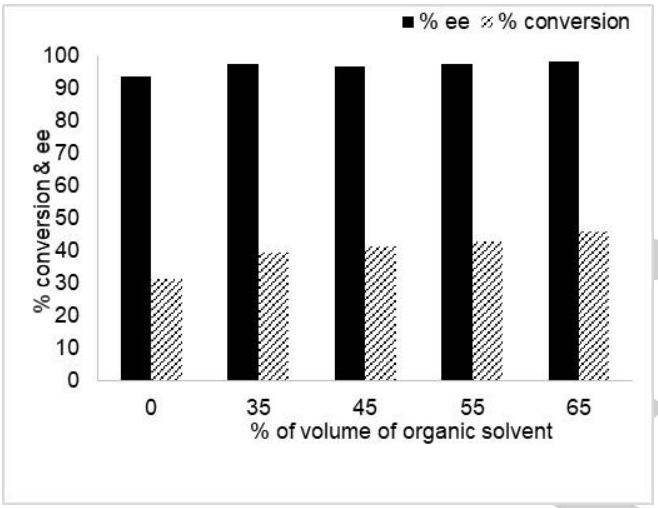


Figure 6. Effect of different ratio of toluene on the enantioselective cleavage of rac NPE.

*At*HNL catalyzed enantioselective cleavage of racemic NPE was monitored at different time intervals. An increase in % ee of (*S*)-NPE was observed with time (Fig 7). Although at 2 h, 97% ee of (*S*)-NPE was observed, at 3 h it reached 99.1% ee and 46.85% conversion. The increased % ee at longer reaction time is due to the cleavage of (*R*)-NPE to benzaldehyde and hence it increases the % ee of unreacted (*S*)-NPE. During this optimization study, we have quantified all the components of biocatalysis e.g. benzaldehyde, (*R*)-NPE, and (*S*)-NPE and found both % ee and conversion at various time points. A clear trend of increase in benzaldehyde up to ~ 50%, and decrease in concentration of (*R*)-NPE from 50 to 0% was observed in 3 h. (*S*)-NPE cleavage (represented as % conversion) was very slow and only 3% of its loss was noticed in 3 h. The conversion of total NPE was found to be 46.85% at 3 h. Comparison of this result with the (i) long reaction time as well as low specific activity of *Hb*HNL catalyzed synthesis of (*S*)-β-nitro alcohols[30,31], and (ii) low nitroaldol cleavage specific activity of *Hb*HNL variants (synthesis reaction should have even lower activity), clearly indicates that the current approach is the fastest HNL catalysed route known so far to synthesize (*S*)-β-nitro alcohols.

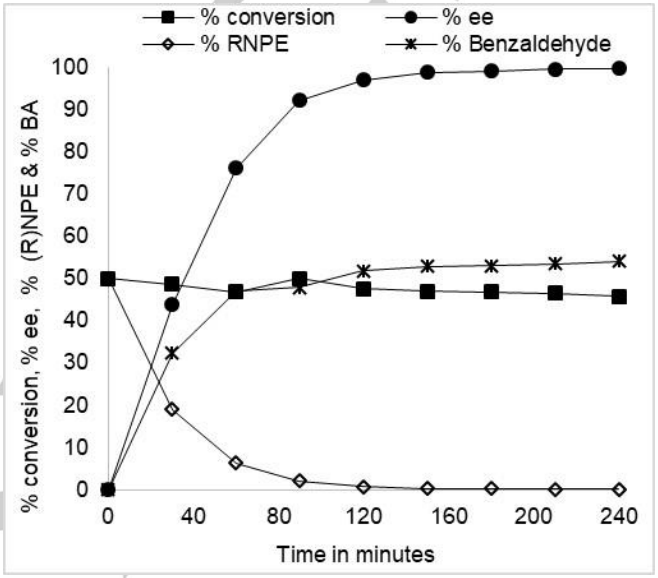
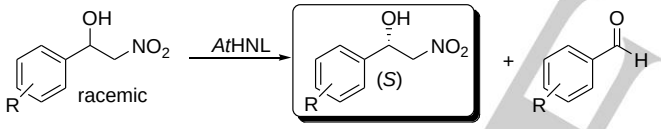


Figure 7. Time course of (*S*)-NPE preparation under optimized reaction conditions.

Using the optimized biocatalytic conditions substrates other than racemic NPE were used in the enantioselective cleavage by *At*HNL to prepare their corresponding (*S*)-β-nitro alcohols. Several racemic β-nitro alcohols having substituents at different positions of the aromatic ring were used to explore the catalytic potential of the enzyme as well as to measure the efficacy of the method. Along with NPE, 2-nitro-1-(3-methoxyphenyl)ethanol and 2-nitro-1-(3-methylphenyl)ethanol resulted in high ee (99%) with 35 to 47% conversion to their corresponding (*S*)-enantiomers. The high % ee and *E* value with these two substrates indicates *At*HNL's preference for *meta* substituted aromatic β-nitro alcohols. *At*HNL has been reported to show high % ee of product in the synthesis of similar *meta* substituted aromatic β-nitro alcohols from corresponding aldehydes.[23] However with 3,4,5-trimethoxy benzaldehyde and 3-hydroxybenzaldehyde, neither chiral cyanohydrin nor β-nitro alcohol synthesis using *At*HNL has been reported. For these two aldehydes, we report here for the first time *At*HNL catalyzed synthesis of (*S*)-2-nitro-1-(3,4,5-trimethoxyphenyl)ethanol, entry 4 of table 1 in 88% ee, 43% conversion and (*S*)-2-nitro-1-(3-hydroxyphenyl)ethanol, entry 5 of table 1 in 81% ee, 19% conversion. Among the *para* substituted aromatic β-nitro alcohols tested with *At*HNL, 2-nitro-1-(4-methylphenyl)ethanol was converted to its (*S*)-enantiomer in 85%

ee and 45% conversion. The 2-nitro-1-(4-methoxyphenyl)ethanol, however resulted in moderate (44%) ee of its (*S*)-enantiomer. In case of 2-nitro-1-(4-nitrophenyl)ethanol, very poor ee was observed i.e. 1%. The probable reason for this varied enantioselectivity for the *para* substituted aromatic substrates is not understood. *At*HNL has been reported to synthesize (*R*)-α-cyanohydrin of *p*-methoxy benzaldehyde in 68% ee but not with *p*-methylbenzaldehyde.[34] Further, *At*HNL has been reported to synthesize (*R*)-β-nitro alcohols of 4-methoxybenzaldehyde in 79% ee and 2% yield and 4-methylbenzaldehyde in 94% ee and 11% yield.[23] However *At*HNL has not tested in the synthesis of either (*R*)-α-cyanohydrin or (*R*)-β-nitro alcohol using 4-nitrobenzaldehyde as substrate. Effect of di- and tri-substituted aromatic substrates has not been studied earlier with *At*HNL either for enantioselective synthesis of α-cyanohydrins or for β-nitro alcohols. Our studies showed mixed results for three such compounds. While substrate **4** having substituents in three positions of the aromatic ring has resulted in 88% ee of its (*S*)-enantiomer, substrates **9** and **10** (table 1) having three and two methoxy substituents respectively have showed poor enantioselectivity. One of the probable difference between substrates **9** and **10** with **4** is, they are *ortho* substituted. However it is not clear whether *ortho* substitution is the reason for this poor enantioselectivity because earlier *At*HNL has been reported to synthesize (*R*)-α-cyanohydrin or (*R*)- β-nitro alcohol in high % ee with *ortho* substituted aromatic aldehydes.[23,34]



Scheme 3. *At*HNL catalyzed preparation of enantioenriched (*S*)-β-nitro alcohols from corresponding racemic β-nitro alcohols.

Table 1. Preparation of (*S*)-β-nitro alcohols by *At*HNL catalyzed stereoselective cleavage of corresponding racemic substrates.

S. No	R	Time (h)	% ee[b]	% conversion[a]	*E*[c]
1	H	3	99	47	84
2	3-OMe	6	99	41	30
3	3-Me	4	99	35	20
4	3,4,5-triOMe	6	88	43	19
5	3-OH	5	81	19	3
6	4-Me	7	85	45	25
7	4-OMe	6	44	41	6
8	4-NO_2	6	1	47	1
9	2,3,4-triOMe	6	3	49	5
10	2,5-diOMe	6	22	47	7

[a] % conversion or % c = $[\frac{S}{R+S+Ald*conversion\ factor}]$*100. *R*: % area of (*R*)-β-nitroalcohol, *S*: % area of (*S*)- β-nitroalcohol and Ald: % area of aldehyde in the biocatalytic product mixture; conversion factor: area of 1 mM racemic β-nitroalcohol /area of 1mM aldehyde. [b] % ee =$[\frac{(S-R)}{(S+R)}]$*100, [c] *E* was calculated by Sih's equation (see SI).

Preparative scale synthesis of (*S*)-NPE was carried out using purified *At*HNL as described in the experimental section. At the end of 3 h, the biocatalytic product mixture obtained was purified by column chromatography that produced (*S*)-NPE in 54% yield (with hexane as impurity) and 93% ee.

Conclusions

Biocatalytic application of (*R*)-selective *At*HNL was exploited in the synthesis of (*S*)-β-nitroalcohols. Retro Henry reaction by *At*HNL has successfully demonstrated as a new route to prepare (*S*)-β-nitroalcohols from their racemic counterparts. Measurement of kinetic parameters of the cleavage of rac NPE by *At*HNL has revealed K_m: 0.012 mM, k_{cat}: 30 min^{-1}, k_{cat}/K_m: 2571 min^{-1} mM^{-1} and V_{max}: 1.1 U/mg. This k_{cat} is found to be three fold higher and k_{cat}/K_m is more than 75 fold higher than the corresponding reaction by *Hb*HNL. Optimization of various biocatalytic reaction parameters of the stereoselective C-C bond cleavage by wild type *At*HNL using racemic NPE as the substrate was performed to find out optimal reaction conditions. Under optimized biocatalytic reaction conditions, this transformation resulted in 99% ee (*S*) and 47% conversion of the NPE, with *E* value of 84. Ten racemic β-nitro alcohols having substituents at different positions of the aromatic ring were used to prepare their corresponding (*S*)-β-nitro alcohols with varied enantioselectivity. This proves not only the broad substrate selectivity of *At*HNL but also the efficacy of the method. Preparative scale synthesis has produced (*S*)-NPE in 54% yield and 93% ee. We have demonstrated that this is the fastest HNL catalyzed route known so far to synthesize a series of (*S*)-β-nitro alcohols (Table S2). Along with this method, *At*HNL now can be used not only to synthesize (*R*)- but also (*S*)-β-nitro alcohols starting with appropriate substrate. This method of retro-

Henry reaction to prepare opposite enantioselective products can be extrapolated to other HNLs.

Experimental Section

Materials: The recombinant *At*HNL gene in pET28a plasmid was synthesized and purchased from Abgenex Pvt. Ltd, India. Culture media and ampicillin were purchased from HiMedia laboratory Pvt. Ltd, India. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from BR-BIOCHEM Pvt. Ltd, India. Aldehydes, nitromethane and mandelonitrile were purchased from Sigma Aldrich, AVRA, SRL and Alfa-Aesar. HPLC grade solvents were obtained from RANKEM, Molychem, FINAR, and SRL, India. Chemicals purchased were used without purification.

Expression and purification of *At*HNL: Expression and purification of *At*HNL was carried out using the method reported by Asano and co-workers.[23] Briefly, the recombinant *At*HNL gene in pET28a plasmid was transformed into *E. coli* BL21DE3 competent cells. Primary culture was prepared by inoculating a loop of transformed *E. coli* cells in 20 mL of LB broth containing 50 µg/mL of kanamycin grown for 12 hours in an incubator shaker at 37°C. Secondary culture was prepared by adding 1% (20 mL) of grown *E. coli* cells in 2 L of LB broth containing 50 µg/mL of kanamycin and incubated at 37°C until the OD reached ~0.5. The cells were then induced with 0.5 mM IPTG and incubated at 30°C for 6 h. Cells were harvested at 10000 rpm for 15 min at 4°C and the cell pellet was suspended in 20 mM potassium phosphate buffer (KPB), pH 7. All the purification steps were done at 4°C. The cell suspension was disrupted by sonication. Disrupted cells were centrifuged at 10000 rpm for 45 min. The supernatant and pellet were analysed by mandelonitrile cleavage assay to confirm HNL activity in soluble fraction. The supernatant was loaded into a Ni-NTA agarose column pre-equilibrated with twice its volume of binding buffer (20 mM imidazole, 300 mM sodium chloride, 20 mM KPB, pH 7). The column was subsequently washed with three supernatant volumes of wash buffer [50 mM imidazole, 300 mM sodium chloride, 20 mM KPB, pH 7), and finally eluted with one supernatant volumes of elution buffer (500 mM imidazole, 300 mM sodium chloride, 20 mM KPB, pH 7). The eluted protein solution was dialyzed in KPB buffer for 3 h, 3 times and later used for biocatalysis. (SDS-PAGE in Fig S2).

HNL assay and steady state kinetics: *At*HNL activity was measured by monitoring the continuous formation of benzaldehyde from racemic mandelonitrile at 280 nm in a spectrophotometer.[29] The reaction was performed in a 96 well microtiter plate. Each well of the plate consisted of 160 µl of 50 mM citrate phosphate buffer, pH 5, 20 µl of purified *At*HNL (1 mg/mL), and 20 µl of 67 mM mandelonitrile solution prepared in 5 mM citrate phosphate buffer, pH 3.15. The activity was calculated using molar extinction coefficient of benzaldehyde (1376 M^{-1} cm^{-1}). One unit of HNL activity is defined as the amount of enzyme which produced 1 µmol of benzaldehyde from mandelonitrile per minute. All measurements were performed in triplicates. Control experiment had all the components of reaction except the enzyme was replaced by its corresponding buffer. Steady state kinetic of *At*HNL was performed with 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 24 mM racemic mandelonitrile using the above cleavage assay, however the volume of assay buffer, enzyme (0.5 mg/mL) and substrate were taken as 140, 20 and 40 µl respectively. The reaction was monitored for 1 min. Absorbance of control resulted due to the spontaneous reaction was subtracted from the enzymatic reaction. Best fit of the data to Michaelis-Menten equation was done using Solver function of Microsoft excel. In case of steady state kinetics of NPE, 0.025, 0.05, 0.1, 0.3, 0.5, 1, 2, and 4 mM racemic NPE and 0.5 mg/mL enzyme concentration was used. The other reaction conditions and best fit of the data to Michaelis-Menten equation were same as mandelonitrile kinetic experiments.

Optimization of biocatalysis parameters for enantioselective cleavage of racemic NPE:

Effect of pH: The reaction mixture contained 94 units of purified *At*HNL in 20 mM KPB pH 7, 2 µmol of NPE, 0.37 ml (equal volume with respect to enzyme) of 50 mM citrate phosphate buffer of varied pH ranging from 5.0 to 6.0 and 0.4 ml (35% v/v) DIPE. In the control, enzyme was replaced by equal volume of 20 mM KPB. Reaction mixture was shaken at 1,000 rpm for 3 h at 30 °C in an incubator shaker. A 100 µl of aliquot from the organic layer was added to 700 µl of hexane:2-propanol = 9:1, centrifuged at 15,000×g, 4 °C for 5 min. A 20 uL of the organic layer was analyzed in a HPLC using Chiralpak® IB chiral column. HPLC conditions: *n*-hexane: 2-propanol = 90:10 (v/v); flow rate: 1 mL/min; absorbance: 210 nm. The retention times of benzaldehyde, (*R*)-NPE, and (*S*)-NPE are 4.6, 9.7, and 10.8 min respectively.[23]

Effect of substrate concentration: Purified *At*HNL, 94 units in 20 mM KPB pH 7, 0.4 ml (32.5% v/v) of 50 mM citrate phosphate buffer pH 5.0, 0.43 ml (35% v/v) of DIPE and racemic NPE ranging from 0.7 to 6.1 mM were taken in a 2 mL micro tube and shaken at 1000 rpm for 5 h at 30 °C in an incubator shaker. Aliquot extraction and analysis was done according to the method described in the above paragraph.

Effect of organic solvents: To a reaction mixture containing 94 units of purified *At*HNL in 20 mM KPB pH 7, and 4 µmol of NPE, 0.38 ml of 50 mM citrate phosphate buffer pH 5, 0.4 ml of organic solvent was added. Separate experiments carried out using different organic solvents e.g. DIPE, hexane, toluene, TBME, THF, and *n*-butyl acetate. Each reaction mixture was shaken at 1,000 rpm for 3 h at 30 °C in an incubator shaker. Aliquot extraction and analysis was done according to the method described above.

Effect of amount of enzyme: The reaction was performed in a 5 ml glass vial. Reaction mixture contained corresponding amount of purified *At*HNL in 20 mM KPB pH 7 ranging from 0.01 to 0.3 µmol (12.5 to 200 units), 3 µmol of NPE, 32.5% (v/v) of 50 mM citrate phosphate buffer pH 5, and 35% (v/v) of toluene. Reaction mixture was shaken at 1000 rpm for 4 h at 30 °C in an incubator shaker. Aliquot extraction and analysis was done according to the method described above.

Effect of organic solvent content: The reaction was performed in a 5 ml glass vial. Reaction mixture contained 133 units of purified *At*HNL in 20 mM KPB pH 7, 4 µmol of NPE, 0.62 ml of 50 mM citrate phosphate buffer pH 5, and corresponding volume of toluene ranging from 0-65% (v/v). Reaction mixture was shaken at 1000 rpm for 4 h at 30 °C in an incubator shaker. Aliquot extraction and analysis was done according to the method described above.

Study of time course of the reaction under optimized reaction conditions: A reaction mixture containing 133 units of purified *At*HNL in 20 mM KPB pH 7, 4 µmol of NPE, 0.59 ml (17.5% v/v) of 50 mM citrate

phosphate buffer pH 5, and 2.2 ml (65% v/v) of toluene was taken in a 5 ml glass vial. Reaction mixture was shaken at 1000 rpm for the corresponding time at 30 °C in incubator shaker. Different reactions were performed, each corresponding to different time interval ranging from 30 to 240 minutes. Reaction analysis was done as per methods described above.

Synthesis of racemic β-nitroalcohols: A mixture of nitroalkane (10 mmol), aldehyde (1 mmol) and $Ba(OH)_2$ (5 mol%) in H_2O (3 mL) was taken in a round bottom flask and stirred at room temperature for 30 to 60 minutes.[36] The reaction mixture was then extracted three times with ethyl acetate. The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The resulting residue was purified by silica gel column chromatography (eluents: hexanes-ethyl acetate). Purified racemic β-nitro alcohols were characterized by ^1H and ^{13}C-NMR as per literature.

***At*HNL catalysed stereoselective cleavage of different racemic β-nitro alcohols and their chiral analysis**: A reaction mixture of 133 units of purified *At*HNL in 20 mM KPB pH 7, 4 µmol of racemic β-nitro alcohol, 0.59 ml (17.5% v/v) of 50 mM citrate phosphate buffer pH 5, and 2.2 ml (65% v/v) of toluene was taken in a 5 ml glass vial. In the control, enzyme was replaced by equal volume of 20 mM KPB. Reaction mixture was shaken at 1000 rpm, 30 °C in an incubator shaker. The reaction was monitored at different time intervals. A 100 µl of aliquot from the organic layer was added to 700 µl of hexane:2-propanol = 9:1, centrifuged at 15,000×g, 4 °C for 5 min. A 20 uL of the organic layer was analyzed in a HPLC (Agilent) using Chiralpak® IB chiral column using HPLC conditions described earlier.

Preparative scale preparation of (S)-NPE using *At*HNL catalysed stereoselective cleavage process: A reaction mixture of 309 mg (5970 units) of purified *At*HNL in 20 mM KPB pH 7, 180 µmol of racemic NPE, 46.3 ml (17.5% v/v) of 50 mM citrate phosphate buffer pH 5, and 172 ml (65% v/v) of toluene was taken in a 500 ml Erlenmeyer flask. In the control, enzyme was replaced by equal volume of 20 mM KPB. Reaction mixture was shaken at 200 rpm, 30 °C in an incubator shaker for 3 h. The reaction mixture was extracted with diethyl ether, organic solvents were evaporated and crude product mixture was analyzed by HPLC as described earlier. Further product was purified by column chromatography, that resulted in 54% yield (with hexane impurity) and 93% ee of (*S*)-NPE.

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Keywords: *At*HNL • β-nitro alcohol • asymmetric synthesis • stereoselective C-C bond cleavage • biocatalysis.

[1] F. A. Luzzio, *Tetrahedron* **2001**, *57*, 915–945.

[2] S. E. Milner, T. S. Moody, A. R. Maguire, *European J. Org. Chem.* **2012**, 3059–3067.

[3] L. Filippova, Y. Stenstr??m, T. V. Hansen, *Molecules* **2015**, *20*, 6224–6236.

[4] B. M. Trost, V. S. C. Yeh, H. Ito, N. Bremeyer, *Org. Lett.* **2002**, *4*, 2621–2623.

[5] A. Roy, L. A. Reddy, N. Dwivedi, J. Naram, R. Swapna, G. C. Malakondaiah, M. Ravikumar, D. Bhalerao, T. B. Pratap, P. P. Reddy, et al., *Tetrahedron Lett.* **2011**, *52*, 6968–6970.

[6] Z. L. Guo, Y. Q. Deng, S. Zhong, G. Lu, *Tetrahedron Asymmetry* **2011**, *22*, 1395–1399.

[7] M. S. Horikawa Sasai, Yoichi M.A. Yamada, Takeyuki Suzuki, *Tetrahedron* **1994**, *50*, 12313–12318.

[8] H. Sasai, N. Itoh, T. Suzuki, M. Shibasaki, *Tetrahedron Lett.* **1993**, *34*, 855–858.

[9] Y. Zhou, J. Dong, F. Zhang, Y. Gong, *J. Org. Chem.* **2011**, *76*, 588–600.

[10] L. A. Mikite Gyula, Jakucs Erzsebet, Kis-Tamas Attila, Darvas Ferenc, *Pestic. Sci.* **1982**, *13*, 557–562.

[11] D. Uraguchi, S. Nakamura, T. Ooi, *Angew. Chemie - Int. Ed.* **2010**, *49*, 7562–7565.

[12] K. Xu, G. Lai, Z. Zha, S. Pan, H. Chen, Z. Wang, *Chem. - A Eur. J.* **2012**, *18*, 12357–12362.

[13] I. Kudyba, J. Raczko, J. Jurczak, *J. Org. Chem.* **2004**, *69*, 2844–50.

[14] R. J. Heffner, J. Jiang, M. M. Joullié, *J. Am. Chem. Soc.* **1992**, *114*, 10181–10189.

[15] T. Kitayama, T. Rokutanzono, R. Nagao, Y. Kubo, M. Takatani, K. Nakamura, T. Okamoto, *J. Mol. Catal. - B Enzym.* **1999**, *7*, 291–297.

[16] M. J. Sorgedrager, R. Malpique, F. Van Rantwijk, R. A. Sheldon, *Tetrahedron Asymmetry* **2004**, *15*, 1295–1299.

[17] E. B. Lahssen, M. Ahbala, J. Bolte, M. Lemaire, *Tetrahedron: Asymmetry* **2006**, *17*, 2684–688.

[18] S. E. Milner, M. Brossat, T. S. Moody, C. J. Elcoate, S. E. Lawrence, A. R. Maguire, *Tetrahedron Asymmetry* **2010**, *21*, 1011–1016.

[19] F. Xu, J. Wang, B. Liu, Q. Wu, X. Lin, *Green Chem.* **2011**, *13*, 2359.

[20] N. Li, S. Bin Hu, G. Y. Feng, *Biotechnol. Lett.* **2012**, *34*, 153–158.

[21] D. Kühbeck, J. Mayr, M. Häring, M. Hofmann, F. Quignard, D. Díaz Díaz, *New J. Chem.* **2015**, *39*, 2306–2315.

[22] P. Vongvilai, R. Larsson, O. Ramström, *Adv. Synth. Catal.* **2008**, *350*, 448–452.

[23] K. I. Fuhshuku, Y. Asano, *J. Biotechnol.* **2011**, *153*, 153–159.

[24] M. Bekerle-Bogner, M. Gruber-Khadjawi, H. Wiltsche, R. Wiedner, H. Schwab, K. Steiner, *ChemCatChem* **2016**, *8*, 2214–2216.

[25] T. Purkarthofer, K. Gruber, M. Gruber-Khadjawi, K. Waich, W. Skranc, D. Mink, H. Griengl, *Angew. Chemie - Int. Ed.* **2006**, *45*, 3454–3456.

[26] M. Gruber-Khadjawi, T. Purkarthofer, W. Skranc, H. Griengl, *Adv. Synth. Catal.* **2007**, *349*, 1445–1450.

[27] T. Devamani, A. M. Rauwerdink, M. Lunzer, B. J. Jones, J. L. Mooney, M. A. O. Tan, Z. J. Zhang, J. H. Xu, A. M. Dean, R. J. Kazlauskas, *J. Am. Chem. Soc.* **2016**, *138*, 1046–1056.

[28] X. Yu, B. Pérez, Z. Zhang, R. Gao, Z. Guo, *Green Chem.* **2016**, *18*, 2753–2761.

[29] J. K. Guterl, J. N. Andexer, T. Sehl, J. von Langermann, I. Frindi-

Wosch, T. Rosenkranz, J. Fitter, K. Gruber, U. Kragl, T. Eggert, et al., *J. Biotechnol.* **2009**, *141*, 166–173.

[30] R. Yuryev, T. Purkarthofer, M. Gruber, H. Griengl, A. Liese, *Biocatal. Biotransformation* **2010**, *28*, 348–356.

[31] R. Yuryev, S. Briechle, M. Gruber-Khadjawi, H. Griengl, A. Liese, *ChemCatChem* **2010**, *2*, 981–986.

[32] J. Von Langermann, D. M. Nedrud, R. J. Kazlauskas, *ChemBioChem* **2014**, *15*, 1931–1938.

[33] D. Okrob, J. Metzner, W. Wiechert, K. Gruber, M. Pohl, *ChemBioChem* **2012**, *13*, 797–802.

[34] J. Andexer, J. Von Langermann, A. Mell, M. Bocola, U. Kragl, T. Eggert, M. Pohl, *Angew. Chemie - Int. Ed.* **2007**, *46*, 8679–8681.

[35] S. Soni, B. P. Dwivedee, V. K. Sharma, U. C. Banerjee, *RSC Adv.* **2017**, *7*, 36566–36574.

[36] M. Pandi, P. K. Chanani, S. Govindasamy, *"Applied Catal. A, Gen.* **2012**, *441–442*, 119–123.

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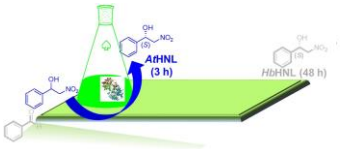

*D.H. Sreenivasa Rao [a] and Santosh Kumar Padhi**

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Preparation of (*S*)-β-nitro alcohols by a (*R*)-selective HNL via stereoselective C-C bond cleavage

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