Sustainable Biocatalytic Synthesis of (*S*)-β-Nitro Alcohols using a Promiscuous (*R*)-Selective Hydroxynitrile Lyase

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I, **D. H. Sreenivasa Rao**, hereby declare that this thesis entitled ***"Sustainable Biocatalytic Synthesis of (S)-β-Nitro Alcohols using a Promiscuous (R)-Selective Hydroxynitrile Lyase"*** submitted by me under the guidance and supervision of **Dr. Santosh Kumar Padhi,** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date: 24/12/2021.

D. H. Sreenivasa Rao.

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CERTIFICATE

This is to certify that the thesis entitled ***"Sustainable Biocatalytic Synthesis of (S)-β-Nitro Alcohols using a Promiscuous (R)-Selective Hydroxynitrile Lyase"*** submitted by D. H. Sreenivasa Rao to University of Hyderabad, Hyderabad, for the award of the degree of Doctor of Philosophy is bonafide record of research work carried out by him under my supervision. The contents of this thesis, in full or parts, have not been submitted to any other University or Institution for the award of any degree or diploma.

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This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Parts of this thesis have been:

A. Published in the following publications:

1. **D. H. Sreenivasa Rao** and Santosh Kumar Padhi, title: Production of (*S*)-β-Nitro Alcohols by enantioselective C–C Bond cleavage with an (*R*)-selective hydroxynitrile lyase, ***ChemBioChem*, 2019**, 20, 371 – 378.

2. **D. H. Sreenivasa Rao**, Kummari Shivani, Santosh Kumar Padhi, title: Immobilized *Arabidopsis thaliana* hydroxynitrile lyase catalyzed retro-Henry reaction in the synthesis of (*S*)-β-nitro alcohols, ***Appl. Biochem. Biotechnol.*, 2021**, 193, 560–576.

3. **D. H. Sreenivasa Rao**, Ayon Chatterjee, and Santosh Kumar Padhi, Biocatalytic approaches for enantio and diastereoselective synthesis of chiral β-nitroalcohols, ***Org. Biomol. Chem.*, 2021**,19, 322-337.

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BC802	Research Ethics, Data Analysis and Biostatistics	4	Pass
BC803	Lab Seminar and Record	4	Pass

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Chapter 1

Introduction

1.1. Chiral β-nitroalcohols and their importance

A chiral β-nitroalcohol possesses two versatile, functional groups, a nitro and a hydroxyl, attached to vicinal carbon centers of which either one or both are asymmetric. They are essential precursors to many synthetically useful structural motifs and pharmaceuticals. The potential to transform both the nitro and hydroxyl group in a β-nitroalcohol structure could produce a diverse range of synthetic molecules with one or more different functional groups. The nitro group can be altered to amine, carbonyl, nitrile, nitrile oxide, oxime, hydroxylamine, etc.[1] Chiral vicinal amino alcohols that are key building blocks of a number of β-blockers used to cure cardiovascular diseases are synthesized by a simple reduction of the nitro group of corresponding chiral β-nitroalcohols.[2] The nitro group of a β-nitroalcohol is converted to a carbonyl using Nef reaction to produce α-hydroxy carbonyl.[3] It undergoes denitration where the nitro group is replaced by a hydrogen.[4] Similarly, the hydroxyl group can be subjected to elimination, Ritter reaction, oxidation, acetylation, etc. Simple elimination in case of a β-nitroalcohol produces nitro alkene.[5] In Ritter reaction, the hydroxyl group reacts with a nitrile under acidic conditions and converts it into corresponding N-(β-nitro)amide.[6] The hydroxyl group of a β-nitroalcohol can be derivatized, i.e., acetylated to produce acetylated nitroalcohol. A few examples of different drug molecules and natural products for whom (*S*)-β-nitroalcohols happened to be structural components are, chelonin A[7] (antimicrobial), and (*S*)-tembamide[8] (anti-HIV), (*S*)-toliprolol, (*S*)-moprolol[9], and (*S*)-propranolol[10], (*S*)-norphenylephrine[11] (β-adrenergic receptor blocking agents), (*S*)-sotalol[12] (antiarrhythmic agents), and (*S*)-miconazole[13] (antifungal) (**Figure 1.1**, upper). Similarly, among the other significant molecules (*R*)-tembamide[14] a natural product shows hypoglycemic activity, (*R*)-isoproterenol[15]

is a β_1-adrenergic receptor agonist, (*R*)-salmeterol[16] is a β_2-adrenoreceptor agonist, and (*R*)-denopamine[17] is a β_1-adrenoreceptor agonist (**Figure 1.1**, middle). Representative examples of natural products, biologically active molecules, and pharmaceuticals carrying β-nitroalcohol diastereomers as structural components are spisulosine[18], sphingosine[19], (*R*,*S*)-ephedrine, (*R*,*S*)-metaraminol[20], (*R*,*S*)-methoxamine[21], (*R*,*R*)-chloramphenicol[22], and AZD-5423[23] (shown with stereochemistry in **Figure 1.1**).

1.2. Biocatalytic approaches for synthesis of chiral β-nitroalcohols

As several chiral β-nitroalcohols are precursors to pharmaceuticals, there is a growing demand to develop clean and green catalysts for their synthesis. Biocatalysts (i) are nature's catalysts; thus, their use in the form of enzymes or whole cells in the reaction avoids the use of toxic/metal/hazardous catalysts, (ii) are more selective leading to high stereoselective products, (iii) work under mild reaction conditions, (iv) does not require protection of functional groups in the substrate, (v) in the process makes fewer byproducts, and (vi) are re-usable if immobilized. They could provide a sustainable solution to the asymmetric synthesis of β-nitroalcohols.

A systematic review on biocatalytic synthesis of β-nitroalcohols was done by Milner et al. in 2012 with literature available until 2011.[24] Different biocatalytic approaches to synthesize chiral β-nitroalcohol stereoisomers can be classified into the following six categories. They are (a) kinetic resolution of racemic β-nitroalcohols, mostly catalyzed by lipases, (b) dynamic kinetic resolution that uses multienzymes or a chemoenzymatic system, (c) Henry reaction by direct C-C bond formation between a carbonyl and nitroalkane substrate, mostly catalyzed by a hydroxynitrile lyase (HNL), (d) stereoselective cleavage of racemic β-nitroalcohols (retro-Henry reaction),

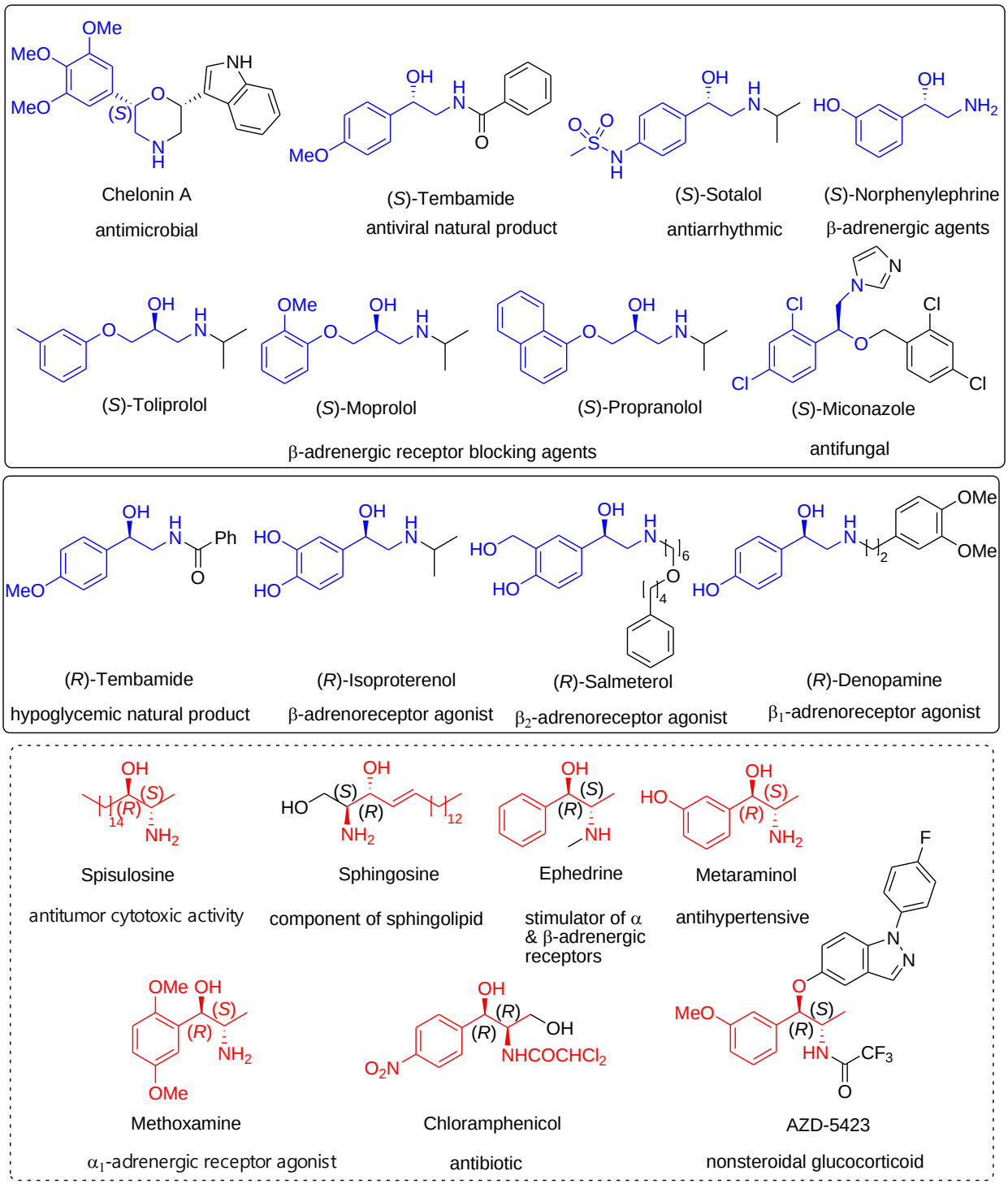


Figure 1.1: Examples of pharmaceuticals, biologically active molecules, and natural products derived from enantiopure (*S*)- (upper), (*R*)-β-nitroalcohols (middle), and chiral β-nitroalcohol diastereomers (lower dotted box).

(e) asymmetric reduction of α-nitroketones catalyzed by alcohol dehydrogenase (ADH) or corresponding whole cells, and (f) halohydrin dehalogenase (HheC) catalyzed enantioselective epoxide ring-opening (**Figure 1.2**).

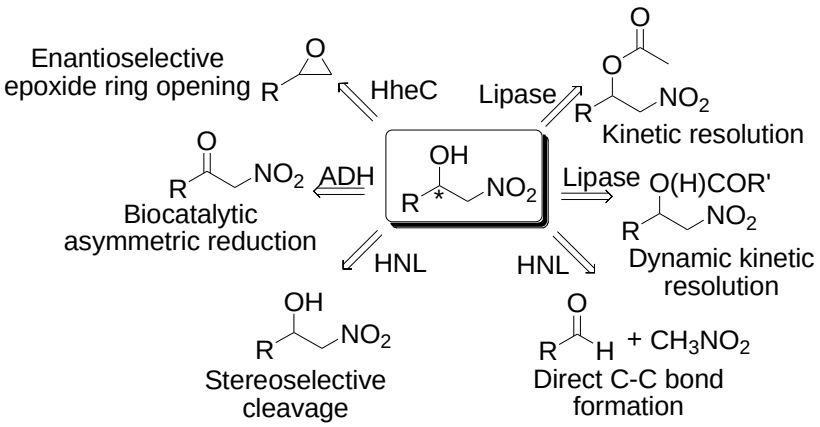


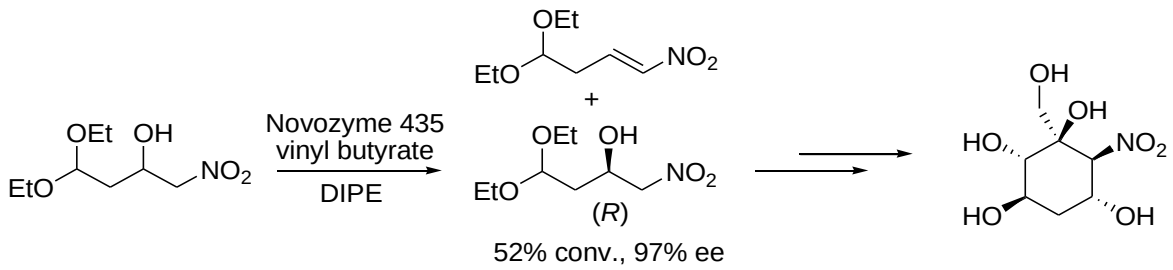
Figure 1.2: Different biocatalytic routes for the synthesis of chiral β-nitroalcohols.

1.2.1. Kinetic resolution (KR) and dynamic kinetic resolution (DKR)

KR is one of the early biocatalytic approaches that majorly used lipases and hydrolases in the enantioselective preparation of β-nitroalcohols. Among the different lipases tested in the KR, *Pseudomonas* sp.,[25,26] *Candida antarctica* lipase B (CALB),[27] *Pseudomonas fluorescens*,[28] and *Burkholderia cepacia* lipase (lipase PS, Amano IM)[29] were found to be effective against a number of aliphatic and aromatic β-nitroalcohols.

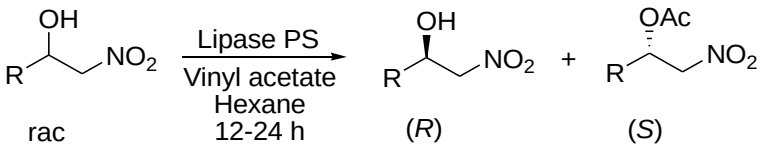
A chemo-enzymatic synthesis of aminocyclitols, known for their glycosidase inhibition properties, used lipase catalyzed KR of a β-nitroalcohol.[30] Among the three lipases, CAL-B (Novozyme 435), lipase PS and PC (Amano AK and PS) tested in the KR of 4,4-diethoxy-1-nitrobutan-2-ol efficient resolution was found by CAL-B in DIPE in the presence of vinyl butyrate as an acylating agent (**Scheme 1.1**).

Burkholderia cepacia lipase (lipase PS, Amano IM) catalyzed enantioselective transesterification



Scheme 1.1: CALB catalyzed KR of a racemic β-nitro alcohol with ketal functionality to enable chemo-enzymatic synthesis of aminocyclitol.

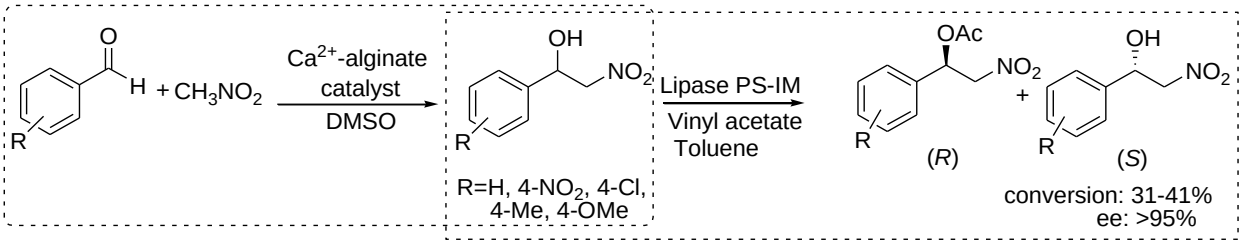
of eight racemic aromatic β-nitroalcohols has produced enantiopure (*R*)-2-nitroalcohols and the corresponding (*S*)-acetylated products (**Scheme 1.2**).[31] In 12 h, using 5:1 (w/w) of the enzyme to substrate ratio in hexane, the KR has produced 49% conversion, 98% *ee* of the (*R*)-2-nitro-1-phenylethanol (NPE), and >99% of the (*S*)-acetylated NPE and a *E* value was found to be >200. While *para* and *meta* substituted aromatic β-nitroalcohols were efficiently resolved *ortho* substituted ones (R=*o*-Me, *o*-Cl, **scheme 1.2**) gave poor performance. KR of 1-nitro-3-phenylpropan-2-ol gave only 72% *ee* of its (*R*)-alcohol, and 81% of the (*S*)-acetate.



R= Ph, 4-ClPh, 4-MePh	98 to >99% ee	98 to >99% ee	*E*= >200
R= 3-ClPh, 3-MePh	>99% ee	91 to 98% ee	*E*= 61-116

Scheme 1.2: Lipase PS catalyzed KR of aromatic β-nitroalcohols

Kühbeck et al. reported a two-step chemoenzymatic reaction in the production of (*S*)-β-nitroalcohols.[32] The first step involved a Ca^{2+} –alginate beads catalyzed racemic β-nitroalcohol synthesis in DMSO and second, immobilized lipase PS-catalyzed kinetic resolution of aromatic racemic β-nitroalcohols in toluene (**Scheme 1.3**). A combination of these two steps in one pot was not possible due to the incompatibility of the solvents used in the two different reactions. The lipase PS catalyzed KR has produced (*S*)-β-nitroalcohols and (*R*)-β-nitroalcohol esters. Five aromatic β-nitroalcohols studied in the KR have provided their corresponding enantiopure (*S*)-β-nitroalcohols in 31-41% yield and >95% *ee*.

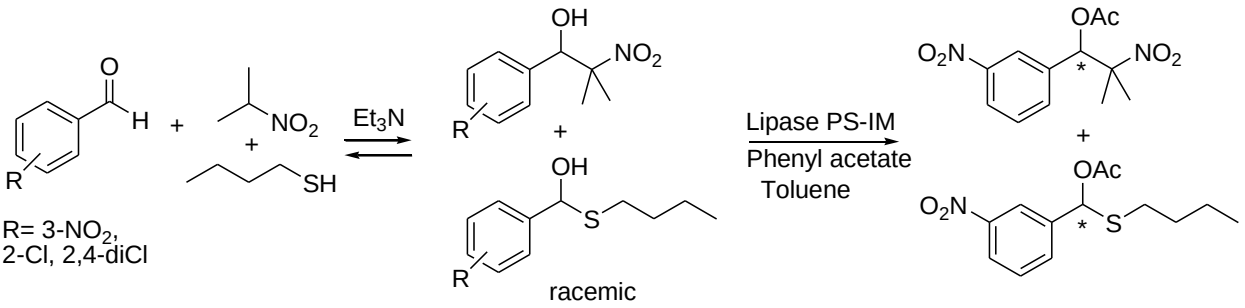


Scheme 1.3: Two-step biocatalysis, racemic β-nitroalcohol synthesis by calcium alginate catalyst followed by lipase PS catalyzed KR.

The limited (maximum of 50%) yield of chiral β-nitroalcohols by KR has been overcome by DKR.

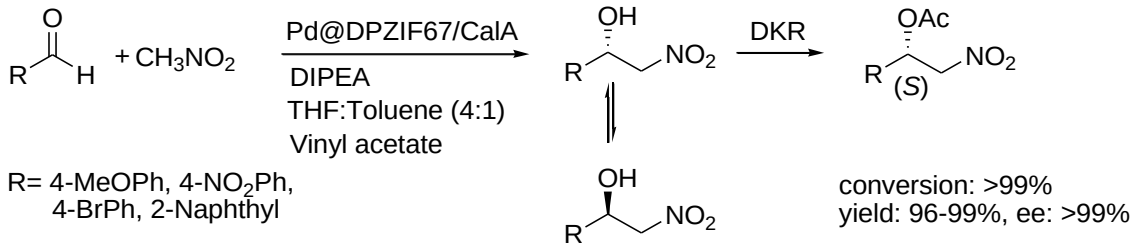
Ramstrom and coworkers have used a lipase based dynamic combinatorial resolution (DCR) approach that involved (i) triethylamine catalyzed 2-nitropropane addition to different aromatic aldehydes in the preparation of racemic β-nitroalcohols followed by (ii) *Pseudomonas cepacia* lipase catalyzed resolution using *para*-chlorophenyl acetate as an acylating agent.[33] The same group have latter developed a DKR that used a one-pot nitroaldol reaction with lipase catalyzed KR at mild conditions.[34] Similar to the DCR, a double parallel dynamic resolution has been reported to prepare enantiopure β-nitroalcohols.[35] In this approach, two parallel dynamic systems were prepared by the addition of 2-nitropropane and 1-butanethiol to three different aromatic aldehydes in the presence of the base trimethylamine. This has produced two simultaneous dynamic equilibrium systems between the aldehydes and (a) racemic β-nitroalcohols, and (b) corresponding racemic hemithioacetals. *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) lipase (PS-IM) and *Pseudozyma antarctica* (formerly *Candida antarctica*) lipase B) were investigated in the stereoselective resolution of the double parallel dynamic system (**Scheme 1.4**). CALB did not give any product, while PS-IM gave a higher ratio of acetylated hemithioacetal in 10 to 11 days of reaction time. The lipase PS-IM showed a high preference for the hemiacetal compared to nitroaldol. Among the three aldehydes, i.e., 3-nitrobenzaldehyde, 2-chlorobenzaldehyde, and 2,4-dichlorobenzaldehyde used in this study,

the lipase could produce the nitroaldol product of only 3-nitrobenzaldehyde, probably due to the less preference of the enzyme towards the *ortho* substituted substrates.



Scheme 1.4: *Burkholderia cepacia* catalyzed double parallel dynamic resolution in the enantioselective synthesis of β-nitroalcohol esters

A recent multi-step chemo-biocatalytic DKR process involves conversion of aldehydes into (*S*)-β-nitroalcohol esters.[36] The cascade was a combination of three reactions, nitroaldol, KR, and racemization (**Scheme 1.5**). Multimodal catalytic nanoreactors (MCNRs) were designed and synthesized to achieve this cascade reaction in a one-pot system. The MCNR, Pd@DPZIF67/CalA had an assembly of three catalysts, i.e., (a) a mesoporous metal organic framework (MOF), (b) *Candida antarctica* lipase A (CalA), and (c) metal nanocrystals to carry out racemic β-nitroalcohol synthesis, KR, and racemization respectively. Optimization of the cascade starting with *p*-anisaldehyde and nitromethane showed >97% yield and >99% *ee* of the corresponding (*S*)-acetyl nitroalcohol in THF: toluene (4:1) at room temperature with vinyl acetate as the acyl donor and in the presence of 0.1 mol % of *N*,*N*-diisopropylethylamine in 20 h (**Scheme 1.5**). Four different aromatic aldehydes tested in the cascade have shown very high conversion and % *ee* of their corresponding (*S*)-β-nitroalcohol acetyl derivatives (>99% conversion and >99% *ee*). The Pd@DPZIF67/CalA was tested for recyclability, and the catalyst showed >89% residual activity and gave 86% yield and >80% *ee* of the product after five successive cycles of reuse. However, in the consecutive cycles, the reaction time increased from 20 to 35 h.



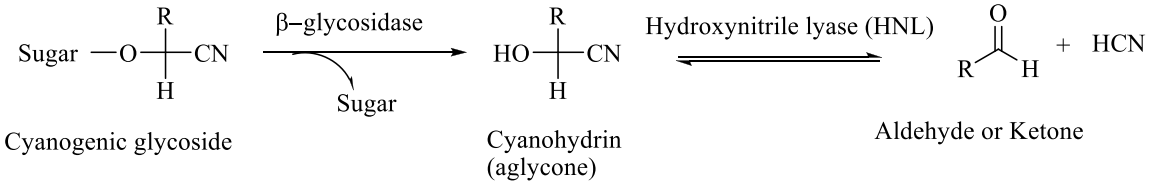
Scheme 1.5: One-pot DKR based chemo-enzymatic cascade using *Candida antarctica* lipase A in the synthesis of (*S*)-β-nitroalcohol esters

1.2.2. Direct C-C bond formation

Stereoselective direct C-C bond formation between electrophilic carbonyl carbon and nucleophilic nitroalkane is one of the important transformation for the synthesis of chiral β-nitroalcohols. This is a nitroaldol reaction, also known as Henry reaction. It is catalyzed by a few hydroxynitrile lyases (HNLs).

1.2.2.1. Hydroxynitrile lyase

Hydroxynitrile lyases (HNL), belong to E.C. 4.1.2.X, X = 10, 11, 46 and 47. Most of the known HNLs are from higher plants, athough they are also found in some arthropods, bacteria, ferns, lichens and fungi. In nature HNLs catalyze the cleavage of cynohydrins that are resulted due to the hydrolysis of cyanogenic glycosides by a glycosidase (**Scheme 1.6**).[37] This process of decomposition of cyanohydrin to a carbonyl compound and HCN is known as cyanogenesis, which plants use to protect them from herbivores and pathogens.[37]



Scheme 1.6: Catabolism of cyanogen glycoside to carbonyl compound and HCN

Based on the stereoselectivity in the synthesis of cyanohydrins, HNLs can be categorized as (*R*)-, and (*S*)-selective HNLs. Prominent examples of (*R*)-selective HNLs are *Arabidopsis thaliana* HNL (*At*HNL), *Prunus amygdalus* HNL (*Pa*HNL), *Linum usitatissimum* HNL (*Lu*HNL), *Chamberlinius hualienensis* HNL (*Chua*HNL), *Parafontaria tonominea*

(*Pton*HNL), *Prunus serotine* (*Ps*HNL),[38] *Prunus amygdalus turcomanica* (*Pat*HNL),[39] *Prunus mume* (*Pm*HNL),[40-41] *Amygdalus pedunculata* Pall (*AP*HNL),[42] *Passiflora edulis* (*Pe*HNL),[43-45] *Prunus communis* (*Pc*HNL),[46] HNL from wild apricot i.e. *Prunus armeniaca* L. (*Pars*HNL),[47-49] *Prunus serotina* var. capulli), peach (*Prunus persica*) HNL, *Acidobacterium capsulatum* ATCC 51196 (*Ac*HNL) and *Granulicella tundricula* (*Gt*HNL),[50] etc. and (*S*)-selective HNLs are *Baliospermum montanum* HNL (*Bm*HNL),[51] *Hevea brasiliensis* HNL (*Hb*HNL), *Manihot esculenta* HNL (*Me*HNL) and *Sorghum bicolor* HNL (*Sb*HNL), etc.

Similarly, based on the presence of co-factor they may be classified into (a). HNLs with FAD: *Pa*HNL and other *Prunus* sp. HNLs, and (b) HNLs without FAD: *At*HNL, *Phlebodium aureum* HNL (*Fa*HNL), *Lu*HNL, *Passiflora edulis* HNL (*Pe*HNL) and *Xylella fastidiosa* HNL (*Xf*HNL), *Sorghum bicolor* HNL (*Sb*HNL), *Ximenia americana* HNL (*Xa*HNL), *Hb*HNL, *Me*HNL and *Bm*HNL.

Apart from the cleavage of cyanohydrins in higher plants as described earlier, HNLs also catalyse the reverse reaction i.e., the synthesis of optically pure cyanohydrins, an important class of organic molecules having a lot of industrial significance. The asymmetric biocatalytic synthesis of chiral cyanohydrins using HNLs is a very cost effective, easy and eco-friendly process.[37,52-56] HNLs catalyze the asymmetric synthesis of chiral cyanohydrins having hydroxyl and cyano groups attached to the same carbon atom using HCN and aldehydes as substrates,[57-58] and the enantiopure cyanohydrins formed are a valuable chiral intermediates used in pharmaceuticals, agrochemicals, fine chemicals and various other chemically useful products.[37,56,59]

1.2.2.2. *Arabidopsis thaliana* HNL (*At*HNL)

Andexer et al., in 2007 reported a HNL from the non-cyanogenic plant *Arabidopsis thaliana* (*At*HNL) (mouse-ear cress) as the first (*R*)-selective HNL (EC:4.1.2.10) that belongs to α/β-hydrolase superfamily.[60] It is structurally related to the (*S*)-selective HNLs of α/β-hydrolase

superfamily such as *Hevea brasiliensis* (*Hb*HNL), *Manihot esculenta* (*Me*HNL) and *Bm*HNL. *At*HNL shows 45% identity and 67% similarity in sequence with the (*S*)-selective *Hb*HNL.[61] Despite of such high sequence similarity *At*HNL shows opposite stereoselectivity in cyanohydrin synthesis. In 2012, crystal structure of *At*HNL was solved, which confirmed its dimeric form (PDB ID: 3dqz).[62] Further it revealed that *At*HNL's active site consists of a catalytic triad i.e., Ser-His-Asp, which is conserved in other α/β-hydrolase fold HNLs. While Thr11 and Lys236 are known to play crucial catalytic role in *Hb*HNL for cyanogenesis, a similar role in *At*HNL is believed to be played by Asn12 side chain, main chain NH of Phe82, and Ala13.[62] The inverse enantioselectivity of *At*HNL compared to other α/β-hydrolase fold HNLs (especially *Hb*HNL) was investigated and elaborated by Kazalauskas and his coworkers.[63] *At*HNL has shown high enantioselectivity towards a wide range of substrates, aliphatic and aromatic aldehydes and catalysed the synthesis of corresponding (*R*)-cyanohydrins.[60,64-65] Its promiscuous catalytic activity in the stereoselective synthesis of β-nitroalcohols is elaborated below.

1.2.2.3. HNLs and other enzyme catalyzed synthesis of opticallypure β-nitroalcohols

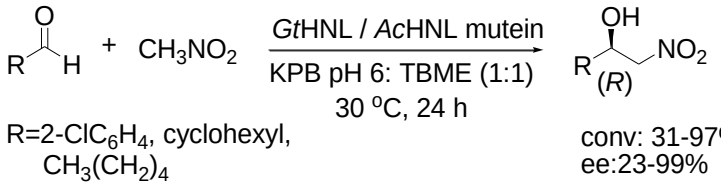
HNLs are frequently used for stereoselective synthesis of cyanohydrins.[57,66-68] A few of them in their native or engineered form catalyze the synthesis of chiral β-nitroalcohols (Henry reaction). This is a promiscuous reaction for the HNLs. HNL catalyzed direct C-C bond formation between a carbonyl and nitroalkane is an important approach for the synthesis of chiral β-nitroalcohols because it has the potential to provide high yield and enantioselective product. Purkarthofer et al. reported the first biocatalytic enantioselective nitroaldol synthesis using *Hevea brasiliensis* HNL (*Hb*HNL), an (*S*)-selective HNL.[69] Further study on the *Hb*HNL catalyzed nitroaldol was performed by Griengl and coworkers.[70] Exploting the (*R*)-selectivity of *Arabidopsis thaliana* HNL (*At*HNL), Fuhshuku and Asano have synthesized a series of (*R*)-

β-nitroalcohols.[71] Both the *Hb*HNL and *At*HNL nitroaldol synthesis were elaborated by Milner et al.[24]

Subsequently two metal dependent cupin fold containing bacterial HNLs *Ac*HNL (*Acidobacterium capsulatum* ATCC 51196) and *Gt*HNL (*Granulicella tundricula*) were reported in the stereoselective synthesis of (*R*)-NPE.[50] While wild type *Ac*HNL showed moderate enantioselectivity (79% *ee*) and poor yield (37%), wild type *Gt*HNL did not catalyze the reaction at all, despite of its 77% sequence identity with *Ac*HNL and several identical active site residues, i.e., A40, V42, and Q110.[72] The *Gt*HNL and *Ac*HNL muteins (A40H, A40R, and A40H-V42T-Q110H) created earlier to improve the activity of cyanohydrin synthesis were tested for the synthesis of (*R*)-NPE. The *Ac*HNL muteins have shown 66-74% conversion and 93-97% *ee* of (*R*)-NPE in 24 h reaction time. In 2 to 4 h, up to >99% *ee* of the product was achieved, but the % conversion was affected, i.e., 11-72%. Long reaction time (24 h) has increased the yield but decreased % *ee* of the product. Among the three *Ac*HNL muteins, A40H showed the highest conversion of 74%, with 97% *ee* of the (*R*)-NPE. Similar to *Ac*HNL, all three *Gt*HNL muteins gave the highest conversion in 24 h biotransformation, compared to the 2 or 4 h reaction. The *Gt*HNL muteins produced 15-75% conversion and 94-98% *ee* of the (*R*)-NPE in 24 h. In a study to find out the importance of metal ion on *Gt*HNL, the bound Mn was replaced with Ni, Co, Fe, and Zn. *Gt*HNL-A40H-V42T-Q110H with Fe and Co showed higher % conversion to (*R*)-NPE than the Mn counterpart in 4 h, while the % *ee* remained high (>97%).

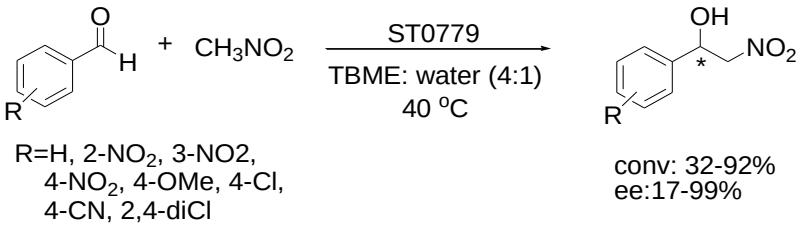
Two *Gt*HNL (A40R, and A40H/A42T/Q110H) and three *Ac*HNL muteins (A40H, A40R, and A40H/A42T/Q110H) were tested toward the enantioselective addition of nitromethane to 2-chlorobenzaldehyde, cyclohexanecarboxaldehyde and hexanal (**Scheme 1.7**). For 2-chloro benzaldehyde, *Ac*HNL-A40H and A40R gave 89-95% conversion with 80-83% *ee* of the corresponding (*R*)-β-nitroalcohol, which was comparatively better than the results by other *Ac*HNL and *Gt*HNL variants. In the case of cyclohexanecarboxaldehyde the triple variants of

both *Ac*HNL and *Gt*HNL showed 80-86% conversion and 96.9-98% *ee* of the product. Similarly, for hexanal, both the triple variants gave 93.7-95% conversion and >99% *ee* of the corresponding (*R*)-β-nitroalcohol.



Scheme 1.7: Enantioselective nitroaldol catalyzed by *Gt*HNL and *Ac*HNL muteins.

Yu et al. studied the catalytic ability of several hydrolases (cloned and purified enzymes) and two commercial lipases for promiscuous nitroaldol reaction.[73] Among the seven biocatalysts evaluated, an acyl peptide releasing enzyme from *Sulfolobus tokodaii* (ST0799) gave the highest yield (92%) and 94% *ee* of the nitroaldol product in the condensation of 4-nitro benzaldehyde and nitromethane (**Scheme 1.8**). Several important biocatalytic parameters of the ST0799 catalyzed nitroadol condensation, i.e., solvent system, temperature, % of water content, reaction time, and the ratio of the two substrates, were optimized to achieve maximum yield and enantioselectivity of product. The optimized parameters include MTBE as the best organic solvent, at 40 °C, 15% (v/v) water content, 36 h reaction time, and 10:1 ratio of nitromethane to 4-nitrobenzaldehyde. The ST0799 has provided 89-92% yield with 93-99% *ee* of β-nitroalcohols in case of the benzaldehyde derivatives carrying electron withdrawing groups, i.e., 2-NO_2, 3-NO_2, 4-NO_2, and –4-CN in 18-24 h reaction time. Other substrates tested showed only 32-45% yield, with 17-94% *ee* in 60-90 h of long reaction time (**Scheme 1.8**). Although ST0799 has successfully synthesized a number of chiral β-nitroalcohols, the absolute configuration of the products was not clearly mentioned. From the HPLC profile of the products, it could be interpreted that enantiopreference of the catalyst varies with the electronic effects of the substituents on the benzaldehyde ring.



Scheme 1.8: Enantioselective nitroaldol catalyzed by acyl peptide releasing enzyme from *Sulfolobus tokodaii* (ST0799)

Yu et al. have used porcine pancreas lipase (PPL) among the biocatalysts screened for the stereoselective nitroaldol synthesis and further investigated it based on the reasonable activity and enantioselectivity of the product obtained with it.[73] PPL was also tested against the eight aromatic aldehydes in the nitroaldol reaction that are used with ST0799. Unlike ST0799, PPL showed low yield and enantioselectivity for all substrates except 4-cyanobenzaldehyde (71% yield, 93% *ee*).

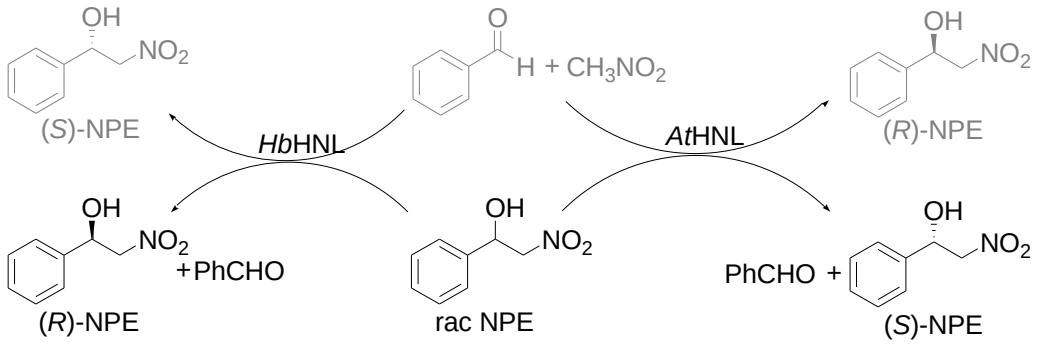
Devamani et al. have explored the catalytic promiscuity of several ancestral enzymes.[74] They hypothesized that ancestral enzymes are more promiscuous than their modern successors. To investigate their hypothesis, they reconstructed ancestral enzymes by predicting the sequences of four of them at different branch points during the esterase evolution to HNL. They have tested the ancestral enzymes and the modern HNLs, i.e., *Hb*HNL, *Me*HNL (*Manihot esculenta*), and a modern esterase *At*EST (*Arabidopsis thaliana*) for different promiscuous reactions, including retro-Henry reaction and Henry reaction. The *At*EST described here as an esterase by Devamani et al.[74] is the same enzyme that shows HNL activity, reported by Andexer et al. earlier.[60] Among the fourteen enzymes examined, only six showed promiscuous Henry reaction in the synthesis of chiral NPE (**Table 1.1**). Except for *Me*HNL, all others have shown high conversion and enantioselectivity in the NPE synthesis. HNL1-NJ, and HNL1 have shown higher % conversion (up to 98) and *ee* (up to 96) than *Hb*HNL in the (*S*)-NPE synthesis. Only *At*EST showed (*R*)-selectivity, while all other HNLs showed (*S*)-selectivity in the Henry reaction.

Table 1.1: Modern HNLs and their ancestral enzymes in the stereoselective Henry reaction in the synthesis of chiral NPE

Entry	Enzyme	Conv., % *ee*	major enantiomer
1	*Hb*HNL	63, 92	*S*
2	*Me*HNL	76, 5	*S*
3	HNL1-ML	85, 83	*S*
4	HNL1-NJ	98, 95	*S*
5	HNL1	91, 96	*S*
6	*At*EST	96, 88	*R*

1.2.3. Stereoselective cleavage of racemic β-nitroalcohols (retro-Henry reaction)

Two HNLs are reported to catalyze reversible Henry reaction, i.e., stereoselective cleavage of one enantiomer of a racemic β-nitroalcohol based on the stereopreference of the HNL. This results in the production of an aldehyde as the cleavage product, and the complementary enantiomer of the racemic β-nitroalcohol that remains unreacted. This method is also known as the asymmetric retro-Henry reaction. (**Scheme 1.9**).



Scheme 1.9: Retro-Henry reaction by *Hb*HNL and *At*HNL produces (*R*)- and (*S*)-NPE respectively, while Henry reaction by them is shown in grey.

Thus, along with this approach, a HNL can be used to synthesize both (*R*)- and (*S*)-β-nitroalcohols. Eventually, the substrate scope of the HNL is extended to synthesize enantiocomplementary β-nitroalcohols. For example, the substrate selectivity of *At*HNL can be used to synthesize (*S*)-β-nitroalcohols, while *Hb*HNL can be used to synthesize (*R*)-β-

nitroalcohols in the retro-Henry approach. Importantly, a HNL shows higher specific activity for C-C bond cleavage than a C-C bond formation. In the case of cleavage and synthesis of β-nitroalcohols by *At*HNL and *Hb*HNL, specific activity of both the enzymes are found to be more for retro-Henry reaction than for synthesis of Henry products.[71,75] However, the retro-Henry approach has multiple limitations. They are (i) maximum of 50% yield, (ii) one of the products, i.e., the aldehyde is often an inhibitor of the HNL, which can also denature the enzyme,[76] (iii) the starting substrate is not commercially available, its preparation requires an additional reaction, and (iv) loss of 50% of the product. The first biocatalytic retro-Henry reaction using *Hb*HNL[76] was elaborated elsewhere.[24] Although it was tested with the only substrate NPE, this methodology can be used to synthesize other (*R*)-β-nitroalcohols.

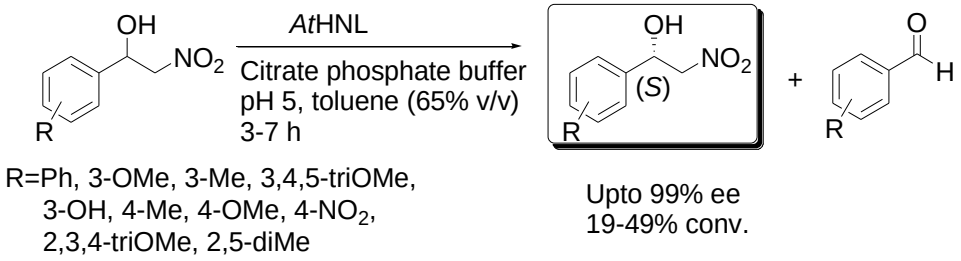
Langermann et al. have engineered *Hb*HNL to enhance its substrate preference for cyanohydrin synthesis but simultaneously tested the variants for retro-Henry reaction.[77] Several variants have shown higher specific activity in the corresponding spectrophotometric assay. The best variant with the triple substitution L121Y-F125T-L146M, showed a specific activity of 0.71 Umg^{-1} toward the cleavage of racemic NPE, which is ~5.5 fold higher than the wild type (0.13 Umg^{-1}) and k_{cat} 3.3 times higher than the wild type. The *Hb*HNL variants, however, were neither tested for stereoselective nitroaldol synthesis nor in the retro-Henry approach in the preparation of chiral β-nitroalcohols. Their biocatalytic potential and substrate selectivity remain to be explored, especially in the synthesis of chiral β-nitroalcohols.

Devamani et al. have also investigated the ancestral enzymes for promiscuous retro-Henry reaction.[74] As described in section 2.3, the same set of six enzymes showed retro-Henry activity in the cleavage of NPE (**Table 1.2**). Three ancestral HNLs, HNL1-ML, HNL1-NJ, and HNL1 were effective towards retro-Henry reaction, while two of them HNL1-NJ, and HNL1 had a higher cleavage rate of NPE than *Hb*HNL. The % *ee* of the cleavage products was not high for all the six enzymes. Except for *At*EST, other enzymes were (*S*)-selective.

Table 1.2: HNLs including their ancestral enzymes in the stereoselective cleavage of NPE

Entry	Enzyme	Rate of cleavage (min^{-1})	% *ee* (preferred enantiomer)
1	*Hb*HNL	7.2±0.3	49, *S*
2	*Me*HNL	0.3	1.1, *S*
3	HNL1-ML	3.4±0.4	5.6, *S*
4	HNL1-NJ	9.6±0.6	32, *S*
5	HNL1	14±1	32, *S*
6	*At*EST	4.6±3	13, *R*

We have synthesized ten aromatic (*S*)-β-nitroalcohols using *At*HNL catalyzed retro-Henry reaction with optimized biocatalytic reaction conditions (**Scheme 1.10**).[78] At least half a dozen aromatic (*S*)-β-nitroalcohols were prepared in up to 99% *ee* and 47% conversion and *E* value up to 84. Among the different racemic aromatic β-nitroalcohols tested, reasonable % *ee* was obtained with substrates having substituents at either *meta*, or *para*, or both positions of the aromatic ring. However, it was difficult to equate the % *ee* of *At*HNL catalyzed retro-Henry reaction with the effect of the nature of substituents in the aromatic rings or their positions because four more substrates with a single or multiple substituents in the phenyl ring showed very poor optical yield in the retro-Henry approach. Substrates with *ortho* substituents did not result in the production of a good % *ee* of products. Nevertheless, a preparative scale synthesis of (*S*)-NPE was reported that supports the synthetic feasibility of this approach.

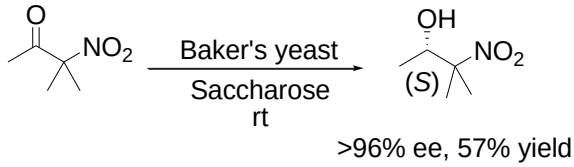


Scheme 1.10: *At*HNL catalyzed synthesis of (*S*)-β-nitroalcohols by retro-Henry approach

Recently we have shown celite immobilized *At*HNL catalyzed retro-Henry reaction and produced a dozen of (*S*)-β-nitroalcohols under optimized conditions.[79] Racemic NPE and its two analogous, with *meta* methoxy and *meta* methyl substituents in the aromatic ring, showed excellent enantioselectivity (97-98.5% *ee*), and 45-48% conversion in the retro-Henry reaction. Three other NPE analogous with *para* fluoro, *meta* chloro, and *para* methyl substituents in the aromatic ring produced corresponding (*S*)-β-nitroalcohols in 68–75% *ee* and 44–54% conversion. Four more substrates having single or multiple substituents in the *ortho*, *meta*, and *para* positions of the phenyl ring showed poor optical yield. The *para* allyloxy-NPE and racemic β-nitroalcohol of cinnamaldehyde showed poor % *ee* of the corresponding product in the celite-*At*HNL catalyzed retro-Henry reaction.

1.2.4. Biocatalytic asymmetric reduction

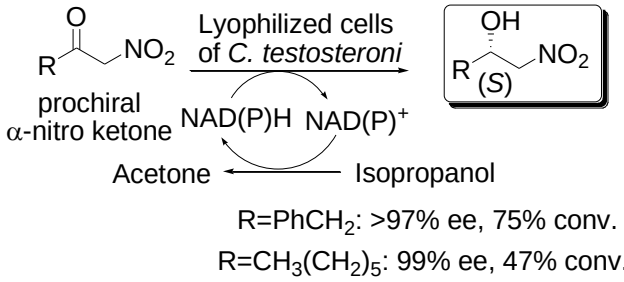
Asymmetric reduction of prochiral α-nitro ketones by whole cells and purified alcohol dehydrogenases produces chiral β-nitroalcohols. The first example is the asymmetric reduction of 3-methyl-3-nitro-2-butanone by baker's yeast in the presence of saccharose at room temperature that produced the (*S*)-enantiomer of 3-methyl-3-nitro-2-butanol in >96% *ee*, and 57% yield (**Scheme 1.11**).[80]



Scheme 1.11: Baker's yeast catalyzed asymmetric reduction of an α-nitro ketone

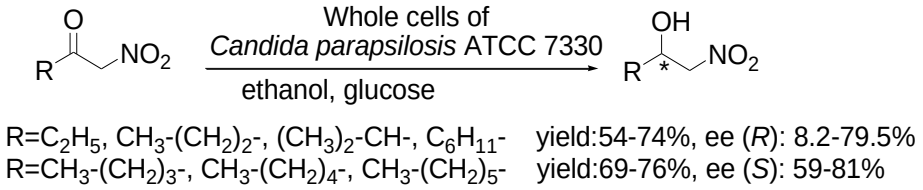
Asymmetric reduction of two α-nitro ketones by lyophilized cells of the bacterium *Comamonas testosteroni* DSM 1455 has produced their corresponding (*S*)-β-nitroalcohols in the presence of 20% (v/v) of 2-propanol as a hydrogen donor (**Scheme 1.12**).[81] Here the alcohol dehydrogenase has shown anti-Prelog preference. Both the substrates have shown very high stereoselectivity; for example, the β-nitroalcohol of aliphatic α-nitro ketone was obtained in

>99% *ee* and 47% conversion by GC while the one corresponds to an aromatic ketone in >97% *ee* and 75% conversion by HPLC. However, the reduction of the aliphatic substrate took 48 h compared to 24 h for the aromatic one.



Scheme 1.12: Alcohol dehydrogenase catalyzed asymmetric reduction of α-nitro ketones

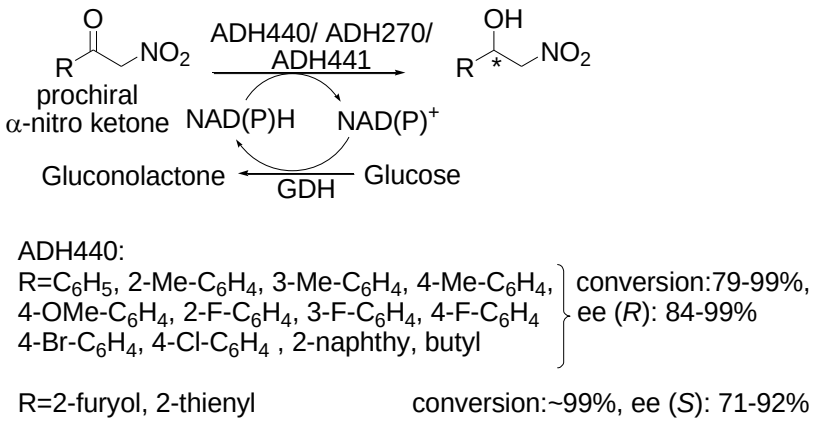
Venkataraman and Chadha showed an asymmetric reduction of aliphatic α-nitro ketones by whole cells of *Candida parapsilosis* ATCC 7330 using ethanol as co-solvent and glucose as co-substrate, in the synthesis of aliphatic β-nitroalcohols (**Scheme 1.13**).[82,83] The biocatalyst was very selective for keto reduction than the nitro group; however, it showed diverse enantiopreference toward the seven aliphatic α-nitro ketones reduced by it. Four substrates with short alkyl chains attached to the keto carbon were reduced to their corresponding (*R*)-β-nitroalcohols in 8.2 to 79.5% *ee* and 54 to 74% yield, while three others with long carbon chain produced their (*S*)-enantiomers in 59 to 81% *ee* and 69 to 76% yield.



Scheme 1.13: *Candida parapsilosis* ATCC7330 catalyzed asymmetric reduction of aliphatic α-nitro ketones

Screening of eighteen commercially available alcohol dehydrogenases for asymmetric reduction of α-nitro acetophenone gave suceesss results with eight of them.[84] The best three of the eight were used in the asymmetric reduction of fifteen α-nitroketones along with glucose dehydrogenase from *Bacillus megaterium* for NAD(P)H regeneration. Among the three

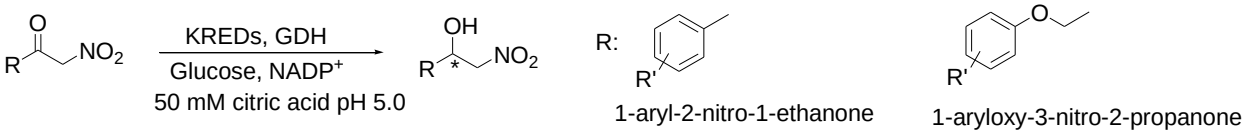
dehydrogenases, ADH440 has mostly produced (*R*)-β-nitroalcohols in 79-99% conversion and 92-99% *ee*, while ADH270 and ADH441 have produced (*S*)-β-nitroalcohols from their corresponding α-nitroketones. In the case of the 2-furyl and 2-thienyl α-nitro ketones, ADH440 produced their corresponding (*S*)-β-nitroalcohols, while ADH270, and 441 produced the (*R*)-β-nitroalcohols. This opposite stereoselectivity is due to the change in the priority of the substituents attached to the chiral center because of the presence of the heteroaromatic ring. Inverse enantioselectivity was observed in the asymmetric reduction of *ortho* fluro derivative (R= *o*-F-C_6H_4-), and 1-nitro-butan-2-one (R=Et, **Scheme 1.14**) by ADH441, and 270 respectively, where the corresponding (*R*)-β-nitroalcohols were formed.



Scheme 1.14: Alcohol dehydrogenase catalyzed reduction of prochiral α-nitroketones

Ketoreductases (KREDs) were used in the asymmetric reduction of class I (1-aryl-2-nitro-1-ethanone) and class II α-nitro ketones (1-aryloxy-3-nitro-2-propanone) (**Scheme 1.15**).[85] Evaluation of 13 KREDs in the reduction of α-nitro acetophenone uncovered (*R*)-selectivity with RasADH from *Ralstonia* species, while YGL039w showed (*S*)-selectivity. YGL039w showed (*S*)-selectivity for all tested substrates except for 1-naphthyl, benzyl, and phenethyl derivatives. It preferred *para* substituents than *ortho*, and *meta*, and electron donating groups than electron withdrawing groups in the aromatic ring. RasADH showed excellent enantioselectivity for most of the class I substrates (up to >99% *ee*). It preferred *ortho* and *para* substituted 1-aryl-2-nitro-1-ethanones (except *p*-*t*Bu).

Screening of KREDs against a class II α-nitro ketone model substrate, 1-benzyloxy-3-nitro-2-propanone, again identified YGL039w and RasADH as the stereocomplementary dehydrogenases that produced 96 and 58% *ee* of (*R*)- and (*S*)-products respectively. Further screening of KREDs against the same substrate has identified SyADH (*Sphingobium yanoikuyae* ADH) with 96% *ee* of the (*S*)-product. Ten class II α-nitro ketones were reduced using YGL039w and SyADH.



the % ee and conv of products by YGL039w are in blue, RasADH are in red, and SyADH are in green

For the products of 1-aryl-2-nitro-1-ethanones:

R':	H	2-Cl	2-Me	2-OMe	3-Cl
	>99%, 99% ee (*S*)	63%, 90% ee (*ND*)	31%, 74% ee (*S*)	50%, 31% ee (*S*)	73%, 29% ee (*S*)
	>99%, 98% ee (*R*)	>99%, >99% ee (*ND*)	81%, >99% ee (*R*)	79%, 84% ee (*R*)	72%, 96% ee (*R*)
R':	3-Me	3-OMe	3-CF_3	4-Cl	4-Me
	95%, 93% ee (*S*)	73%, 92% ee (*S*)	87%, 92% ee (*S*)	>99%, 98% ee (*S*)	96%, 96% ee (*S*)
	94%, 91% ee (*R*)	59%, 79% ee (*R*)	>99%, 85% ee (*R*)	98%, 98% ee (*R*)	>99%, 86% ee (*R*)
R':	4-OMe	4-CF_3	4-F	4-Br	4-$NHCOCH_3$
	96%,>99% ee (*S*)	43%, 72% ee (*S*)	93%, 68% ee (*S*)	76%, 96% ee (*S*)	55%, 96% ee (*S*)
	>99%, 99% ee (*R*)	85%, 98% ee (*R*)	85%, 96% ee (*R*)	86%, 98% ee (*R*)	99%, 89% ee (*R*)
R':	4-NO_2	4-(*tert*-butyl)	2,4-*di*Cl	3,4-*di*Cl	
	62%, 77% ee (*S*)	35%, 66% ee (*S*)	80%, 32% ee (*S*)	45%, 82% ee (*S*)	
	88%, 98% ee (*R*)	31%, >99% ee (*R*)	>99%, 99% ee (*R*)	55%, 92% ee (*R*)	
R:	1-Naphthyl	2-Naphthyl	Benzyl	Phenethyl	
	18%, 37% ee (*R*)	74%, 99% ee (*S*)	82%, 83% ee (*R*)	48%, 6% ee (*R*)	
	37%, 92% ee (*R*)	86%, 96% ee (*R*)	65%, 17% ee (*S*)	64%, 90% ee (*R*)	

For the products of 1-aryloxy-3-nitro-2-propanones:

R':	H	2-Me	2-OMe	3-Me	4-Me
	>99%, 96% ee (*R*)	90%, 94% ee (*R*)	64%, 98% ee (*R*)	83%, 85% ee (*R*)	75%, 99% ee (*R*)
	75%, 96% ee (*S*)	80%, 57% ee (*S*)	>99%, 97% ee (*S*)	>99%, 77% ee (*S*)	95%, 86% ee (*S*)
R':	4-OMe	4-F	4-Cl	4-NO_2	R = 1-Naphthyl
	>99%, 98% ee (*R*)	83%, 95% ee (*R*)	83%, 92% ee (*R*)	93%, 55% ee (*R*)	49%, 98% ee (*R*)
	>99%, 97% ee (*S*)	88%, 79% ee (*S*)	77%, 94% ee (*S*)	74%, >99% ee (*S*)	21%, 93% ee (*S*)

Scheme 1.15: KREDs catalyzed reduction of α-nitroketones (1-aryl-2-nitro-1-ethanone, and 1-aryloxy-3-nitro-2-propanone)

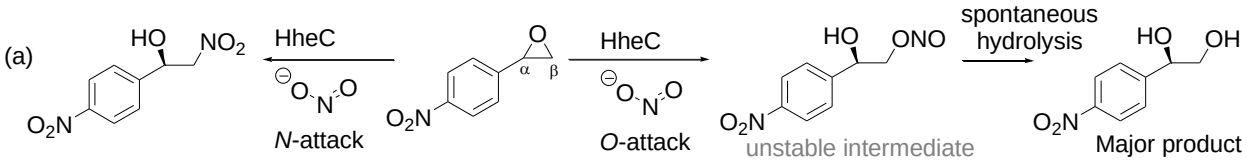
As the phenyl ring was not directly connected to the carbonyl group in these substrates, hence steric hindrance and electronic effects were less important for class II α-nitro ketones than the class I. Most of the substrates were converted to their corresponding products with good to

excellent conversion (up to >99%) and excellent *ee* (up to >99%), except the naphthyl derivative one.

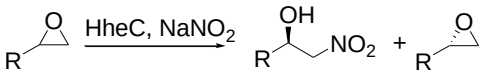
1.2.5. Enantioselective epoxide ring opening

Halohydrin dehalogenases catalyze the dehalogenation of vicinal halohydrins to produce the corresponding epoxides.They also catalyze the reverse reaction, i.e., ring opening of epoxide by a nucleophile such as nitrite, cyanide, or azide to produce corresponding β-substituted alcohols. A halohydrin dehalogenase (HheC) from the bacterium *Agrobacterium radiobacter* AD1 catalyzes both the ring closure of halohydrin to epoxide and enantioselective nucleophilic ring opening of the epoxide.[86] HheC accepts nitrite as a nucleophile in the ring opening reaction and shows β-regioselectivity, i.e., nucleophile attacks at the terminal carbon of the oxirane ring. However, in the oxygen vs. nitrogen selectivity in the nitrite attack, the enzyme produces a vicinal diol due to oxygen attack, as the major product (**Scheme 1.16a**). In the case of *p*-nitrostyrene oxide ring opening corresponding nirite ester, an unstable intermediate was formed by oxygen attack, which subsequently hydrolyzed spontaneously to its 1,2-diol. Simultaneously, β-regioselective nitrite attack happens through the nitrogen of the nitrite to produce the minor enantiopure β-nitroalcohol (**Scheme 1.16a**). The ratio of 1,2-diol and β-nitroalcohol was 4:1. This is a kinetic resolution, where the (*R*)-epoxide undergoes ring opening to produce the corresponding β-nitroalcohol, while the (*S*)-epoxide remains unreactive. Six aromatic epoxides were tested for the nitrite-mediated ring opening by wild type HheC, and its W249F variant. All of them showed β-regioselectivity, and except 2-((4-nitrophenoxy)methyl)oxirane, the other five showed (*R*)-selectivity in the halohydrin dehalogenase catalyzed kinetic resolution. The reverse enantioselectivity in the case of 2-((4-nitrophenoxy)methyl)oxirane is not due to the opposite enantio preference by the enzyme, rather due to change in the priority of the substituents around the chiral center. The

enantioselectivity, *E* of HheC wild type, and its W249F variant in the KR based enantioselective ring opening of all the substrates are shown in **Scheme 1.16b**. The wild type enzyme showed good to excellent enantioselectivity for the first three substrates. The mutein has shown higher enantioselectivity in the KR compared to the WT enzyme in the case of all the substrates, except 2-((4-nitrophenoxy)methyl)oxirane.



(b)



	4-NO_2-C_6H_4	3-Cl-C_6H_4	4-NO_2-C_6H_4-OCH_2	C_6H_5	4-Cl-C_6H_4	4-Me-C_6H_4
WT	*E*: 200	55	45	5	12	5
W249F	*E*: >200	100	28	40	90	193

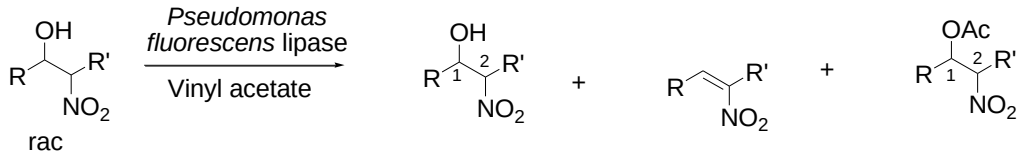
Scheme 1.16: Halohydrin dehalogenase catalyzed regio and enantioselective ring opening of epoxide by nitrite in the synthesis of chiral β-nitroalcohols, along with *E* values.

In another study HheC catalyzed nitrite mediated ring opening of an aliphatic epoxide 1,2-epoxybutane showed only 26% conversion, and 96% *ee* of (*R*)-1-nitro-butan-2-ol with *E*=28, and 97% β-regioselectivity (terminal position).[87]

1.3. Biocatalytic approaches for diastereoselective synthesis of chiral β-nitroalcohols

Biocatalytic approaches to synthesize β-nitroalcohol diastereomers include lipase catalyzed KR and HNL catalyzed asymmetric Henry reaction. In the *Pseudomonas flourescens* lipase catalyzed KR, Borah et al. have used several β-nitroalcohols that included three aromatic substrates each having two chiral centers (**Scheme 1.17**).[28] The KR has produced the (*R*)-β-nitroalcohols of the 4-Cl-Ph and 4-COOMe-Ph derivatives, while the chirality of the second chiral carbon with $–NO_2$ was not specified in the case of both of these substrates. KR of the third substrate 2-hydroxy-3-nitro-3-phenyl-1-benzyl-oxypropane has resulted in the synthesis

of the (*R*,*S*)-diastereomer in 85% *ee*, 50% yield, and the (*S*,*R*)-diastereomer of the 2-acetoxy-3-nitro-3-phenyl-1-benzyl-oxypropane in 82% *ee*, 45% yield.

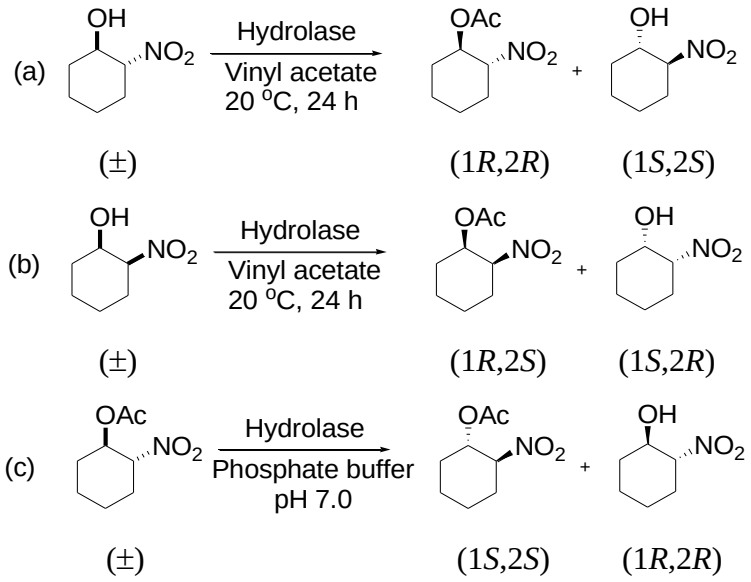


R= 4-Cl-C_6H_4	R'=Me	66% conv., 71% ee (1*R*)	27%	not found
R= 4-COOMe-C_6H_4	R'=Me	68% conv., 82% ee (1*R*)	30%	not found
R= $C_6H_5CH_2$-OCH_2	R'=Ph	50% conv., 85% ee (1*R*,2*S*)	not found	45% conv., 82% ee (1*S*,2*R*)

Scheme 1.17: *Pseudomonas flourescens* lipase catalyzed KR in the synthesis of β-nitroalcohol diastereomers.

Milner et al. investigated KR of racemic 2-nitrocyclohexanol and 2-nitrocyclohexyl acetate via transesterification and hydrolysis respectively in the production of diastereomers of 2-nitrocyclohexanol.[88] Eighteen different hydrolases were screened in the stereoselective transacetylation of racemic *trans*-2 nitrocyclohexanol using vinyl acetate as an acylating agent and solvent (**Scheme 1.18a**). Two lipases *Pseudomonas flourescens* and CALB showed optimum activity in the KR with 49-50% conversion and >98% *ee* of each of the stereoisomers. Preparative scale transacetylation by *Pseudomonas flourescens* has produced (1*R*,2*R*)-*trans*-2-nitrocyclohexyl acetate in 99% *ee*, 49% yield, and (1*S*,2*S*)-*trans*-2-nitrocyclohexanol in 99% *ee*, 48% yield. Similarly, screening of ten different hydrolases for the transesterification of racemic *cis*-2-nitrocyclohexanol was done in the presence of vinyl acetate (**Scheme 1.18b**). Four enzymes, *Candida cylindracea* C1, *Pseudomonas stutzeri*, *Alcaligenes* spp. and *Pseudomonas flourescens* showed *E* >400, with a conversion range of 47-81% and up to >98% *ee* of the product. The *cis*-2-nitrocyclohexanol on preparative scale transesterification produced (1*R*,2*S*)-*cis*-2-nitrocyclohexyl acetate and (1*S*,2*R*)-*cis*-2-nitrocyclohexanol in 99% *ee*, however, the *cis*-acetate was decomposed during silica gel based separation. Similarly, among the several hydrolases studied for the hydrolysis of racemic *trans*-2-nitrocyclohexyl acetate, *Candida cylindracea* C1, *Alcaligenes* spp., *Pseudomonas cepacia* P1, *Pseudomonas stutzeri*, and *Pseudomonas cepacia* P2 have shown high enantioselectivity (**Scheme 1.18c**). The

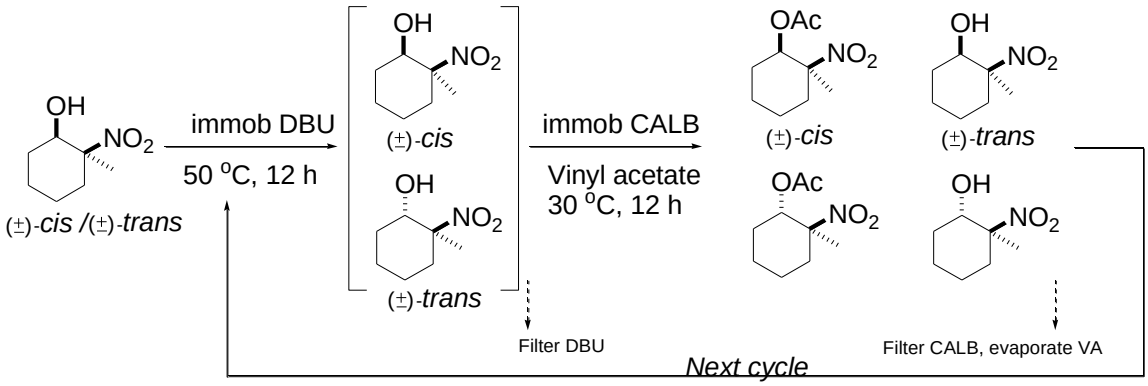
hydrolysis based KR by these enzymes has produced (1*S*,2*S*)-*trans*-2-nitrocyclohexyl acetate in 88-99% *ee*, 47-53% conversion, and (1*R*,2*R*)-*trans*-2-nitrocyclohexanol in 91-99% *ee*.



Scheme 1.18: Screening of racemic 2-nitrocyclohexanol and 2-nitrocyclohexyl acetate via transesterification and hydrolysis in the synthesis of β-nitroalcohol diastereomers.

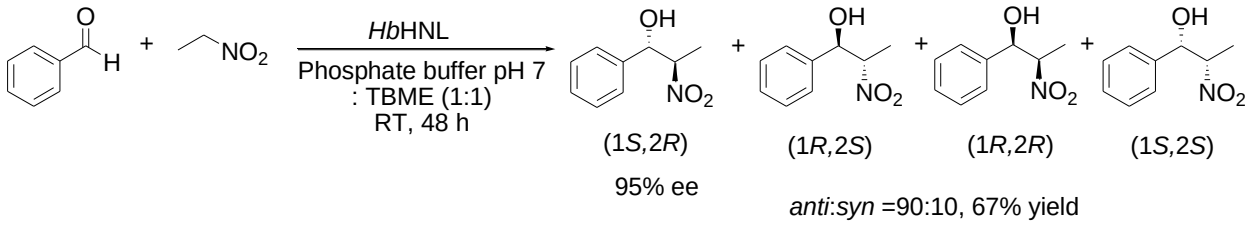
Recently, a chemo-enzymatic DKR was reported for diastereoselective synthesis of β-nitro alcohols.[89] The DKR of 2-methyl-2-nitrocyclohexanol was exemplified using a one-pot two-step process that combines the intramolecular nitroaldol (Henry) reaction and lipase catalyzed resolution. In the first step, dynamic interconversion between *cis* and *trans* 2-methyl-2-nitrocyclohexanol occurs via ring opening and closing of the corresponding aldehyde, i.e., 6-nitroheptanal in the presence of the base 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU). The next step is the immobilised CALB catalysed transesterification of both *cis* and *trans* 2-methyl-2-nitrocyclohexanol in the presence of vinyl acetate as an acylating agent and solvent. The one-pot process, however, suffered with (i) competing chemical acylation, and (ii) lipase inhibition by the base. A two-pot process, where the two steps carried out separately, the catalysts removed and added to the corresponding individual reactions in the next cycle, has produced the (1*R*,2*R*)-*trans*-2-methyl-2-nitrocyclohexyl acetate in >98% *ee* and 57% conversion. Further attempt to optimize the reaction conditions for a one-pot process has led to exploring the use of immobilized DBU, toluene, and 50 °C for the interconversion process. In the optimized one-

pot DKR, interconversion and the resolution were carried out separately for three consecutive cycles with reuse of the catalysts (**Scheme 1.19**). While the DBU was filtered at the end of interconversion and in the resolution, the CALB was filtered, and vinyl acetate was evaporated, before the reaction mixture was re-exposed to the next cycle. Under optimized conditions, the DKR has produced the (1*R*,2*S*)-*cis*-2-methyl-2-nitrocyclohexyl acetate and (1*R*,2*R*)-*trans*-2-methyl-2-nitrocyclohexyl acetate in 97 and 96% *ee* with 18 and 53% conversion respectively (49.3% de, *trans*), while corresponding alcohols had 21.4% de (*trans*).



Scheme 1.19: One-pot DKR with repeated interconversion and lipase based KR

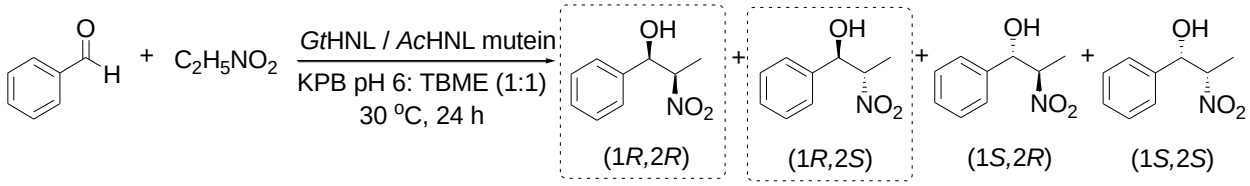
*Hb*HNL catalyzed C–C bond formation between benzaldehyde and nitroethane has produced a mixture of diastereomers of 2-nitro-1-phenylpropanol (NPP) in 67% yield (**Scheme 1.20**).[69] *Hb*HNL has shown high diastereoselectivity and preferred *anti* stereoisomers over the *syn* (*anti*:*syn* =90:10, 80% de, *anti*). The major *anti* stereoisomer obtained was (1*S*,2*R*)-2-nitro-1-phenylpropanol in 95% *ee*. No substrate scope was studied in this *Hb*HNL catalyzed diastereoselective synthesis approach.



Scheme 1.20: *Hb*HNL catalyzed diastereoselective Henry reaction

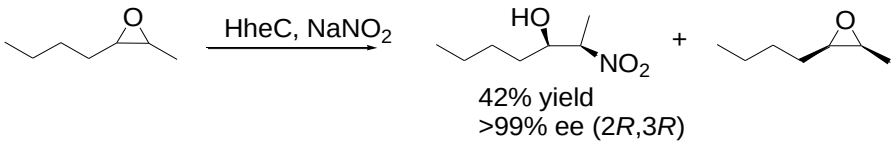
The *Gt*HNL (A40R, and A40H/A42T/Q110H) and *Ac*HNL muteins (A40H, A40R, and A40H/A42T/Q110H) were tested for diastereoselective Henry reaction (**Scheme 1.21**).[50]

Nitroethane addition into benzaldehyde in the presence of these variants resulted mainly in the production of *anti* product, i.e., (1*R*, 2*S*)- (NPP), while a few gave (1*R*, 2*R*)-NPP. *Ac*HNL wild type produced *anti*/*syn* in 2:1 ratio, with ~85% *ee* of (1*R*, 2*S*)-NPP, however, the total conversion was only 20% in 2 h. In 24 h, the total conversion increased to 77%, but the *anti*/*syn* ratio was decreased to 53:47. The *Ac*HNL-A40H has shown maximum *anti* selectivity (54% de, *anti*). The *anti*/*syn* ratio was found to be 77:23 with >85% *ee* of the (1*R*, 2*S*)-NPP, but the conversion was only 16% in 2 h. The % conversion to NPP was increased to 66% in 24 h, with a decreased *anti*/*syn* ratio than the 2 h results. *Ac*HNL-A40R, *Gt*HNL-A40R, and *Gt*HNL-A40H/A42T/Q110H, have shown their preference for the *syn* product, i.e., (1*R*, 2*R*)-NPP.



Scheme 1.21: *Gt*HNL and *Ac*HNL muteins catalyzed diastereoselective Henry reaction

The halohydrin dehalogenase (HheC) catalyzed regio and enantioselective ring opening of epoxide by nitrite was tested with 2,3-epoxyheptane, which still showed high regioselectivity in the nitrite attack to the sterically less crowded position to produce (2*R*,3*R*)-2-nitroheptan-3-ol in >99% *ee* and 42% yield (**Scheme 1.22**).[87] This diastereoselective synthesis of β-nitroalcohol has, however, not been tested with more than one substrate.



Scheme 1.22: Halohydrin dehalogenase catalyzed ring opening by nitrite in the synthesis of β-nitroalcohol diastereomer.

1.4. Outline of the thesis

The current era is demanding for greener synthetic methods for industrially important fine chemicals and chiral intermediates. Exploring the diverse selectivity (chemo, regio, and stereo)

of the natural enzymes and the tailor made enzymes have provided greener routes for numerous asymmetric syntheses, including the chiral β-nitroalcohols. Having elaborated the significance of enantioenriched β-nitroalcohols and their applications, and discussed about the six different biocatalytic approaches for the asymmetric synthesis of chiral β-nitroalcohols, it was realized that the best biocatalytic approach would be the HNL catalyzed Henry reaction because of its own advantages and the substrates used are widely available and economical. The retro-Henry reaction appeared even more fascinating as it could be used to prepare enantioenriched β-nitroalcohols having absolute configuration opposite to that of the stereopreference of a HNL. Without discovering a new enzyme, rather using a retro-Henry approach, a HNL can be used to prepare β-nitroalcohols of its opposite stereopreference. Not only that, but also rate of enantioselective cleavage of a β-nitroalcohol is high as compared to the synthesis, which makes the retro-Henry reaction an efficient biocatalytic approach.

Till today a countable number of biocatalytic approaches exist for diastereoselective synthesis of β-nitroalcohols. The existing chemo-enzymatic DKR lacks the efficiency to obtain high conversion (maximum 57%) of diastereomers and suffers from the compatibility of the two steps involved in it.[89] This opens opportunities to explore new enzymatic routes to synthesize different diastereomers of β-nitroalcohols.

The retro-Henry reaction despite of its potential significane, had remained as an underdeveloped method to prepare enantiopure β-nitroalcohols. In order to prepare (*S*)-β-nitroalcohols by this approach, we have envisioned to use *Arabidopsis thaliana* (*At*HNL), a (*R*)-selective HNL, known to catalyze the promiscuous stereoselective nitromethane addition to aldehydes to prepare (*R*)-β-nitroalcohols.

We aimed not only to develop the *At*HNL catalyzed enantioselective C-C bond cleavage to prepare a diverse range of (*S*)-β-nitroalcohols, but also to prepare immobilized enzyme to

improve the enzymatic stability, and activity, and use it further in the retro-Henry reaction. We also intended to exploit engineered *At*HNL variants to enhance the substrate scope of the enzyme in the enantioselective preparation of (*S*)-β-nitroalcohols. To improve the existing methods of diastereoselective synthesis of β-nitroalcohol, we aimed to develop a one-pot dynamic kinetic resolution cum nitroaldol reaction in the synthesis of β-nitroalcohol diastereomers. Hence, the major objectives of the thesis are framed as follows.

Objectives of the present study

1. To develop a method for enantioselective cleavage of racemic β-nitroalcohols using purified *At*HNL to prepare (*S*)-β-nitroalcohols.
2. To prepare immobilized *At*HNL, optimize corresponding biocatalytic parameter and explore it in the preparation of (*S*)-β-nitroalcohols using retro-Henry approach.
3. To investigate the biocatalytic potential of *At*HNL variants in the enantioselective preparation of (*S*)-β-nitroalcohols.
4. To explore *At*HNL catalyzed dynamic kinetic resolution cum asymmetric nitroaldol reaction in the diastereocomplementary synthesis of (2*S*,3*R*)-β-aryl-α-hydroxynitroalkane.

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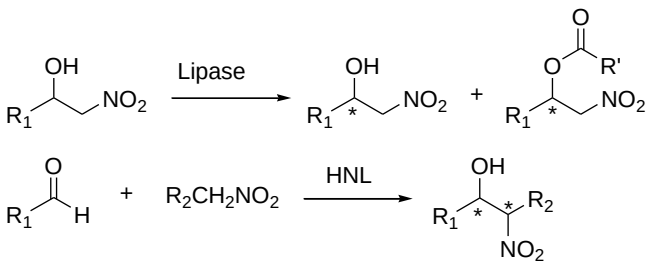
Chapter 2

*At*HNL catalysed enantioselective C-C bond cleavage in the preparation of (*S*)-β-nitroalcohols

2.1. Introduction

Enantiopure β-nitroalcohols 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] 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-nitroalcohols 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-nitroalcohols, and (ii) stereoselective C-C bond formation between a carbonyl center and nitroalkane (**Scheme 2.1**).



Scheme 2.1: (top) Lipase catalysed kinetic resolution of a racemic β-nitroalcohol. R_1 is an alkyl group and R' is part of the acylating agent. (bottom) HNL catalysed 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-nitroalcohols 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 *tert*-butyl methyl ether (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. *Candida antarctica* lipase B (CALB) catalysed kinetic resolution of 4,4-diethoxy-1-nitrobutan-2-ol using vinyl butyrate as acyl donor in diisopropyl ether (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-catalysed transesterification of 2-nitrocyclohexanol and hydrolase-catalysed hydrolysis of 2-nitrocyclohexyl acetate but in 24 h.[18] Xu et al performed a two-step biocatalytic reaction that involved D-aminoacylase-catalysed 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

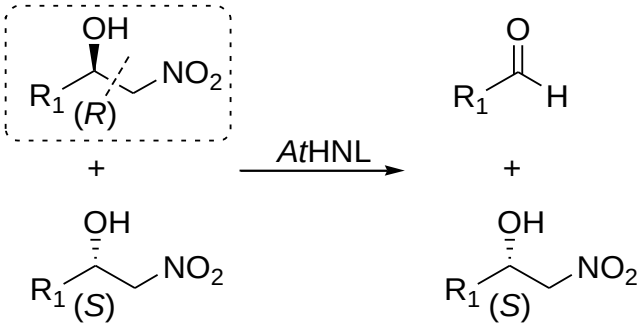
to 99% *ee* in 12 h. Kuhbeck et al prepared racemic β-nitroalcohols using Ca^{2+}-alginate hydrogel beads in DMSO and subjected to immobilized lipase PS catalysed kinetic resolution to prepare five (*S*)-β-nitroalcohols in 31 to 41% yield and >95% *ee*.[21] This analysis clearly indicates that lipase catalysed 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-nitroalcohol 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 catalysed stereoselective synthesis of β-nitroalcohols 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-nitroalcohols. *Hb*HNL is the first HNL to be reported for nitroaldolase activity.[25] However *Hb*HNL catalysed synthesis of chiral Henry products is limited by low specific activity (0.13 U/mg). Further the yield and *ee* for (*S*)-2-ntro-1-phenylethanol (NPE) 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 catalysed methods for the synthesis of (*S*)-β-nitroalcohols 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 NPE.[27] Yu et al described acyl-peptide releasing enzyme from *Sulfolobus tokodaii* (ST0779) catalysed Henry reaction. However, the absolute configuration of the Henry products has not been mentioned.[28]

Recently in our laboratory, we found the promiscuous nitroaldolase activity of *Baliospermum montanum* HNL (*Bm*HNL), which was exploited in an enzyme cascade reaction to convert primary alcohols into (*S*)-β-nitroalcohols.[29]

Compared to the synthesis, HNLs show a higher rate in the cleavage of cyanohydrins.[30] A similar effect has been observed with β-nitroalcohols.[31] Kinetic studies of *Hb*HNL catalysed 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.[31] Therefore exploiting the cleavage reaction rather than the synthesis, to prepare enantiopure NPE appears to be beneficial. Yuryev et al showed *Hb*HNL catalysed retro Henry reaction in the preparation of (*R*)-NPE from its racemic counterpart.[32] 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-nitroalcohols. However, it suggests that the cleavage reaction could be advantageous to synthesize enantioenriched β-nitroalcohols of opposite stereopreference of the HNL. We used a similar approach but a different HNL to synthesize (*S*)-β-nitroalcohols.

Here we report for the first time *At*HNL catalysed enantioselective C-C bond cleavage of racemic β-nitroalcohols in the preparation of a number of (*S*)-β-nitroalcohols, i.e., opposite enantiopreference of *At*HNL (**Scheme 2.2**). We exploited this cleavage reaction and optimised its reaction conditions that produced (*S*)-β-nitroalcohols with *ee* up to 99% and 47% conversion in only 3 h reaction time.



Scheme 2.2: *At*HNL catalysed enantioselective cleavage of racemic β-nitroalcohols

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-nitroalcohols. We believe this retro-Henry reaction approach is the fastest HNL catalysed approach to synthesize a series of (*S*)-β-nitroalcohols known so far. It uses HNL for a C-C bond cleavage reaction, which is closer toward the natural reaction of 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-nitroalcohols starting with appropriate substrate. It gives opportunity to synthesize a number of (*S*)-β-nitroalcohols by exploiting the substrate selectivity of *At*HNL without discovering a new enzyme but using a different approach.

2.2. Objectives

1) To optimise the biocatalytic parameters of the *At*HNL catalysed retro-Henry reaction and demonstrate it in the preparation of (*S*)-β-nitroalcohol.

2) To illustrate the *At*HNL catalysed retro-Henry reaction in the preparation of diverse (*S*)-β-nitroalcohols and also establish the process in a preparative scale.

2.3. Materials and methods

2.3.1. Chemicals and 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.

2.3.2. 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 analyzed 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.

2.3.3. 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.[30] 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.

2.3.4. Optimisation of biocatalysis parameters for enantioselective cleavage of racemic NPE

2.3.4.1. 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 µL 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]

2.3.4.2. 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 were done according to the method described in the above paragraph.

2.3.4.3. 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 were done according to the method described above.

2.3.4.4. 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 were done according to the method described above.

2.3.4.5. 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 were done according to the method described above.

2.3.4.6. Study of time course of the reaction under optimised 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 an 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.

2.3.5. 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.[33]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-nitroalcohols were characterized by ^{1}H and ^{13}C-NMR as per literature.

2.3.6. *At*HNL catalysed enantioselective cleavage of different racemic β-nitroalcohols 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-nitroalcohol, 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 ºC for 5 min. A 20 µL of the organic layer was analyzed in a HPLC (Agilent) using Chiralpak® IB chiral column using HPLC conditions described earlier.

2.3.7. Preparative scale preparation of (*S*)-NPE using *At*HNL catalysed enantioselective 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.

2.4. Results

2.4.1. NMR characterization of racemic β-nitroalcohols

Numbers after the product name corresponds to the serial number in **tables 2.1** and **2.2**

2-nitro-1-phenylethanol 1[23]

^{1}H NMR (400 MHz, $CDCl_3$): δ 2.01 (1H, s), 4.51 (1H, dd, *J* = 3.2, 13.2 Hz), 4.62 (1H, dd, *J* = 9.6, 13.2 Hz), 5.45 (1H, dd, *J* = 2.8, 9.6 Hz), 7.28-7.44 (5H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.9, 81.2, 125.9 (X2), 128.9, 128.9 (X2), 138.3.

1-(3-Methoxyphenyl)-2-nitroethanol 2[23]

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.45 (1H, brs), 3.80 (3H, s), 4.49 (1H, dd, *J* = 3, 13.5 Hz), 4.58 (1H, dd, *J* = 9.5, 13.0 Hz), 5.39 (1H, dd, *J* = 2.5, 9.5 Hz), 6.88 (1H, ddd, *J* = 0.5, 2.5, 8.0 Hz), 6.93-6.95 (2H, m), 7.31 (1H, dd, *J* = 1.0, 8.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.3, 70.8, 81.2, 111.5, 114.3, 118.1, 130.0, 139.9, 159.9.

1-(3-Methylphenyl)-2-nitroethanol 3[23]

^{1}H NMR (500 MHz, $CDCl_3$) δ 2.39 (3H, s), 3.13-3.19 (1H, m), 4.47-4.50 (1H, m), 4.56-4.60(1H, m), 5.40 (1H, d, *J* = 9.5 Hz), 7.17-7.21 (3H, m), 7.31 (1H, dd, *J* = 7.5, 4.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.3, 71.0, 81.2, 123.0, 126.6, 128.9, 129.6, 138.1, 138.8.

1-(3,4,5-Trimethoxyphenyl)-2-nitroethanol 4[34]

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.34 (1H, d, *J* = 2.4 Hz), 3.79 (3H, s), 3.83 (6H, s), 4.51 (1H, d, *J* = 3.2, 12.8 Hz), 4.62 (1H, dd, *J* = 9.6, 12.8 Hz), 5.38 (1H, d, *J* = 9.6 Hz), 6.57 (2H, d, 0.4 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.1, 60.8, 71.1, 81.3, 102.8, 134.1, 137.9, 153.5.

1-(3-Hydroxyphenyl)-2-nitroethanol 5[35]

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.82 (1H, d, *J* = 4 Hz), 4.55 (1H, dd, *J* = 3, 13.5 Hz), 4.59-4.63 (1H, m), 4.96 (1H, s), 5.43-5.44 (1H, m), 6.83-6.85 (1H, m), 6.93-6.98 (2H, m), 7.24 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 70.7, 80.8, 112.6, 116.1, 118.0, 130.1, 139.6, 156.0.

1-(4-Methylphenyl)-2-nitroethanol 6[23]

^{1}H NMR (500 MHz, $CDCl_3$) δ 2.38 (3H, s), 3.05 (1H, d, *J* = 3.5 Hz), 4.50 (1H, dd, *J* = 3.0, 13.0 Hz), 4.61 (1H, dd, *J* = 9.5, 13.0 Hz), 5.42 (1H, ddd, *J* = 3.0, 3.5, 9.5 Hz), 7.23 (2H, d, *J* = 8 Hz), 7.29 (2H, d, *J* = 8.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.1, 70.8, 81.2, 125.9 (X2), 129.7(X2), 135.2,138.9.

1-(4-Methoxylphenyl)-2-nitroethanol 7[23]

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.99-2.0 (1H, m), 3.77-3.78 (3H, m), 4.41-4.45 (1H, m), 4.52-4.58 (1H, m), 5.31-5.35 (1H, m), 6.86-6.90 (2H, m), 7.26-7.28 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.3, 70.6, 81.2, 114.3(X2), 127.3(X2), 130.5, 159.8.

1-(4-nitrophenyl)-2-nitroethanol 8[34]

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.51 (1H, d, *J* = 7, 14 Hz), 4.56-4.64 (2H, m), 5.63 (1H, dd, *J* = 5, 10 Hz), 7.65 (2H, d, *J* = 10 Hz), 8.29 (2H, d, *J* = 9 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 69.6, 80.1, 124.3 (2X), 126.6 (2X), 145.1, 148.1.

1-(2,3,4-Trimethoxyphenyl)-2-nitroethanol 9[36]

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.1 (1H, s), 3.87 (6H, d, *J* = 5.5 Hz), 3.9 (3H, s), 4.55-4.62 (2H, m), 5.56 (1H, d, *J* = 3.5 Hz), 6.70 (1H, d, *J* = 9 Hz), 7.11 (1H, d, *J* = 9 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.0, 60.7, 61.15, 67.4, 80.4, 107.3, 121.5, 123.4, 141.8, 150.6, 154.2.

1-(2,5-Dimethoxyphenyl)-2-nitroethanol 10[37]

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.25 (1H, d, *J* = 5.6 Hz), 3.79 (3H, s), 3.85 (3H, s), 4.58 (1H, dd, *J* = 9.6, 13.2 Hz), 4.68 (1H, dd, *J* = 2.8, 12.8 Hz), 5.62 (1H, dd, *J* =4.4, 9.2 Hz), 6.84-6.85 (2H, m), 7.04-7.05 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 29.6, 55.7, 67.7, 79.8, 111.5, 113.7, 114.2, 126.9, 150, 154.

^{1}H NMR and ^{13}C NMR spectra of racemic β-nitroalcohols

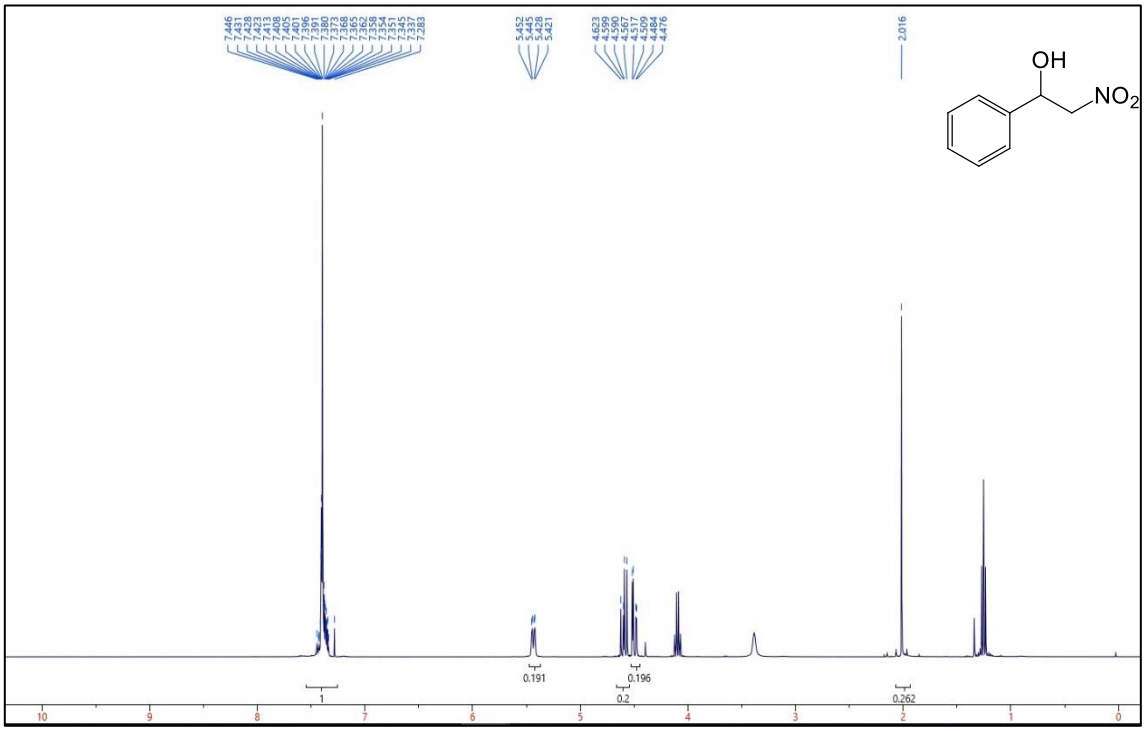


Figure 2.1.1: ^{1}H NMR spectrum of 2-nitro-1-phenylethanol

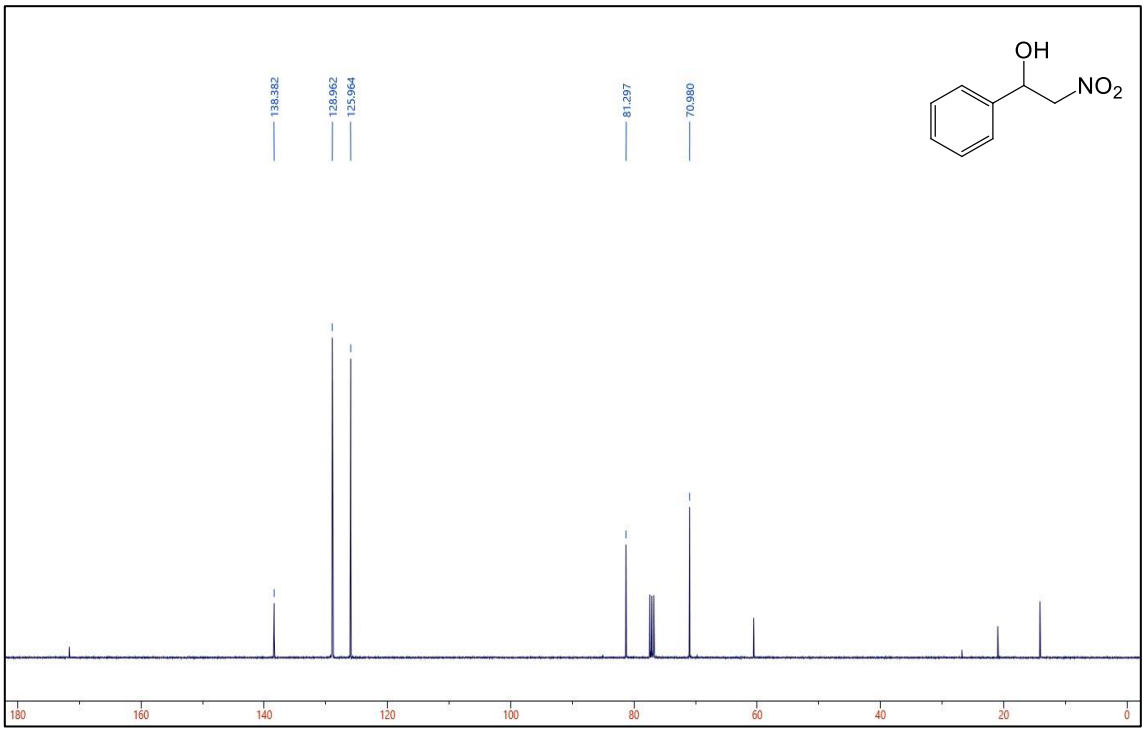


Figure 2.1.2: ^{13}C NMR spectrum of 2-nitro-1-phenylethanol

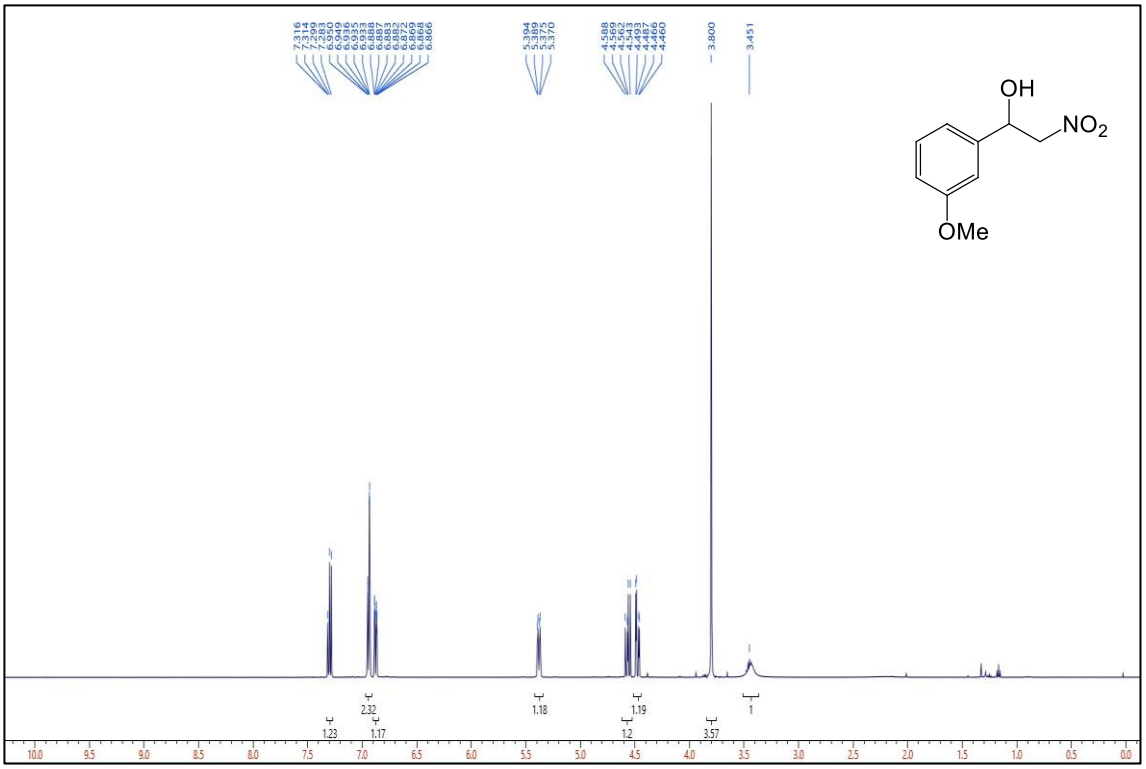


Figure 2.1.3: ^{1}H NMR spectrum of 1-(3-methoxyphenyl)-2-nitroethanol

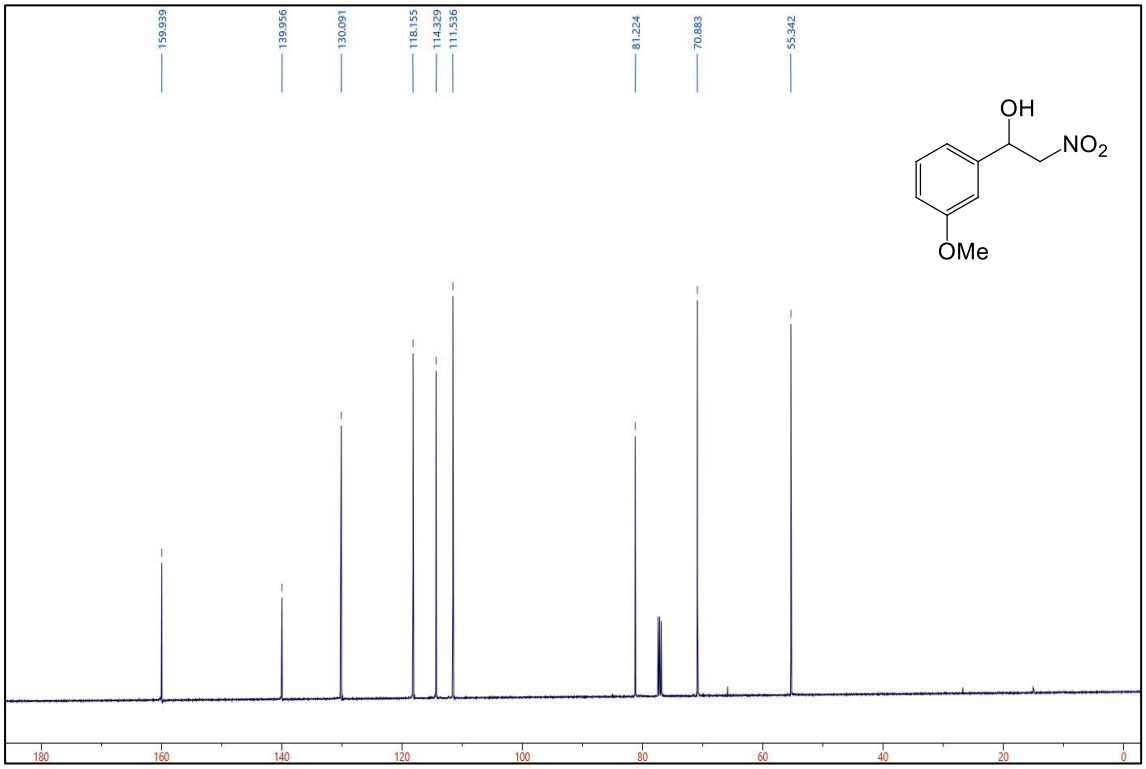


Figure 2.1.4: ^{13}C NMR spectrum of 1-(3-methoxyphenyl)-2-nitroethanol

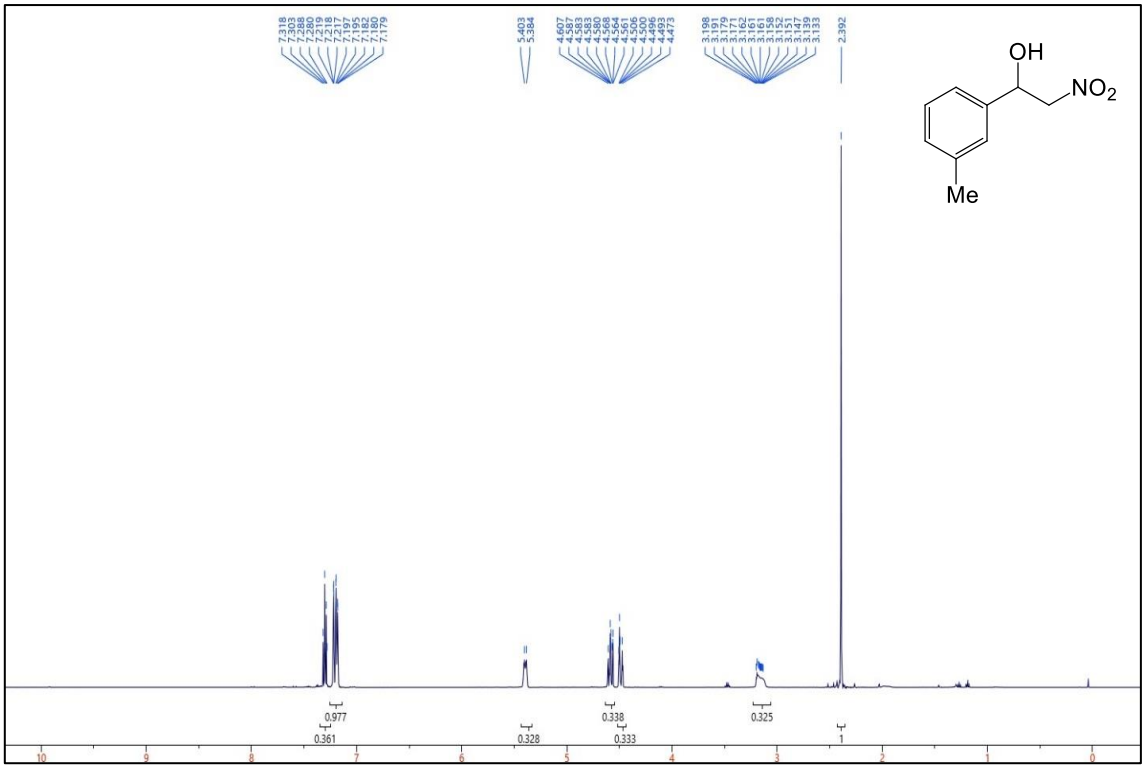


Figure 2.1.5: ^{1}H NMR spectrum of 1-(3-methylphenyl)-2-nitroethanol

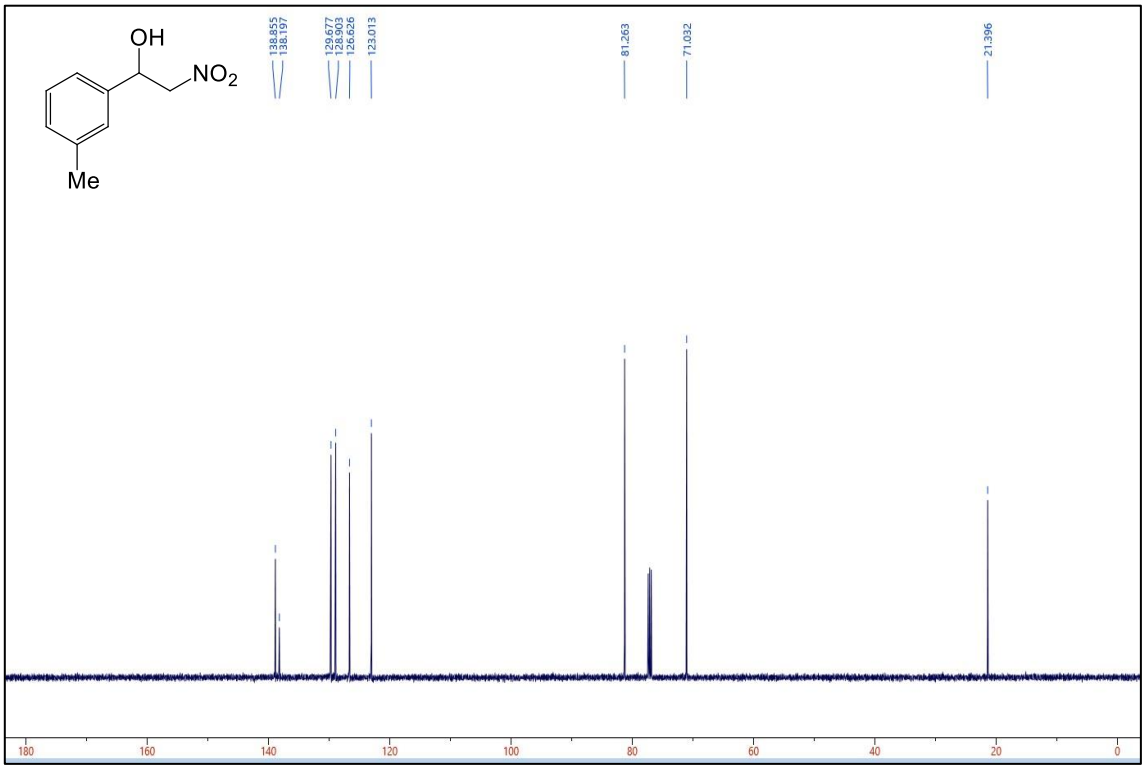


Figure 2.1.6: ^{13}C NMR spectrum of 1-(3-methylphenyl)-2-nitroethanol

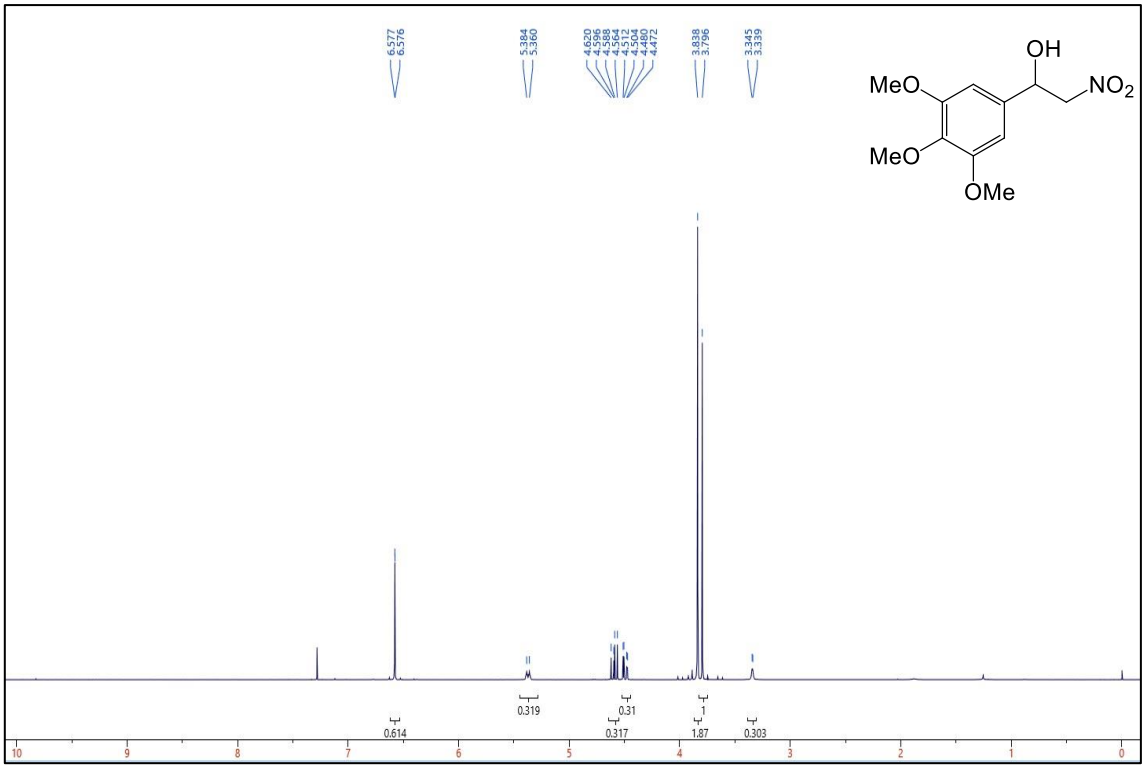


Figure 2.1.7: ^{1}H NMR spectrum of 1-(3,4,5-trimethoxyphenyl)-2-nitroethanol

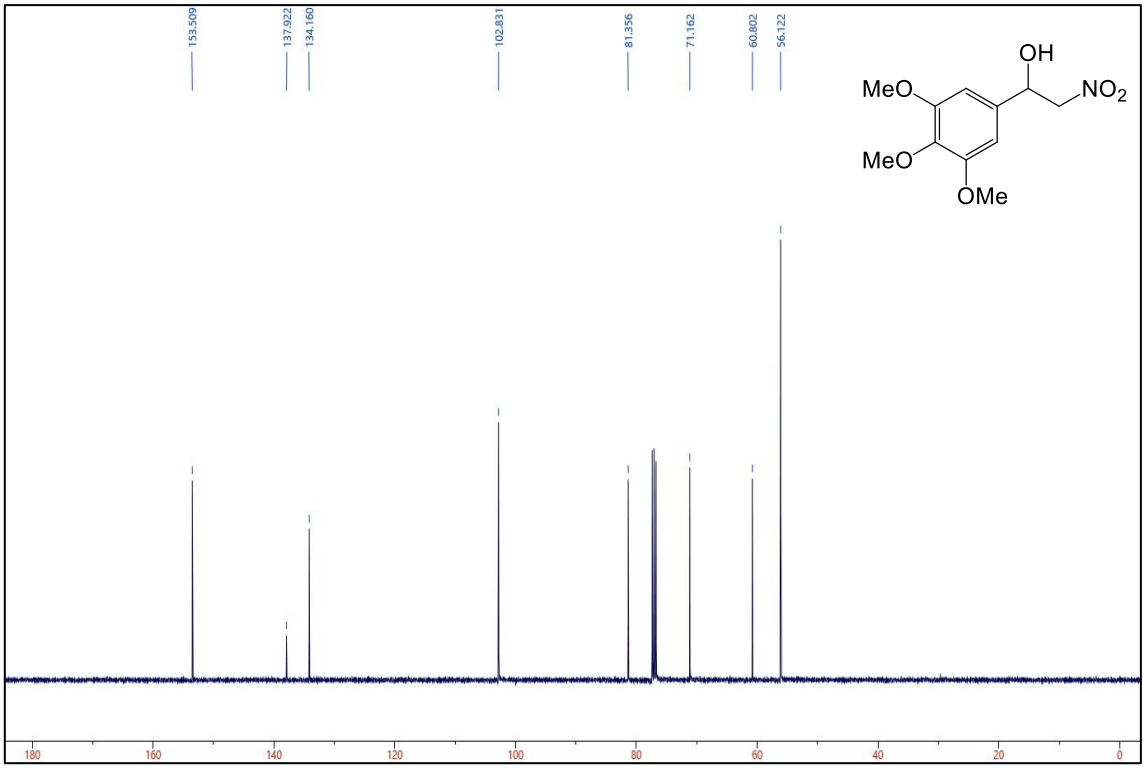


Figure 2.1.8: ^{13}C NMR spectrum of 1-(3,4,5-trimethoxyphenyl)-2-nitroethanol

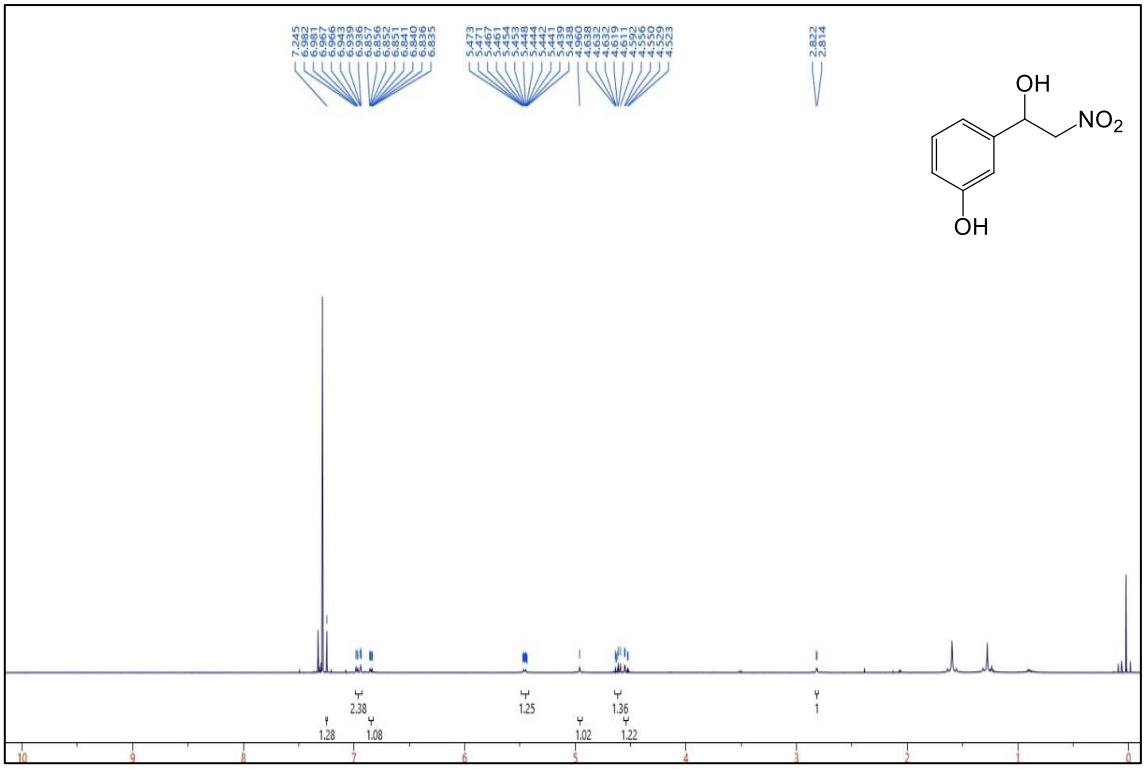


Figure 2.1.9: ^{1}H NMR spectrum of 1-(3-Hydroxyphenyl)-2-nitroethanol

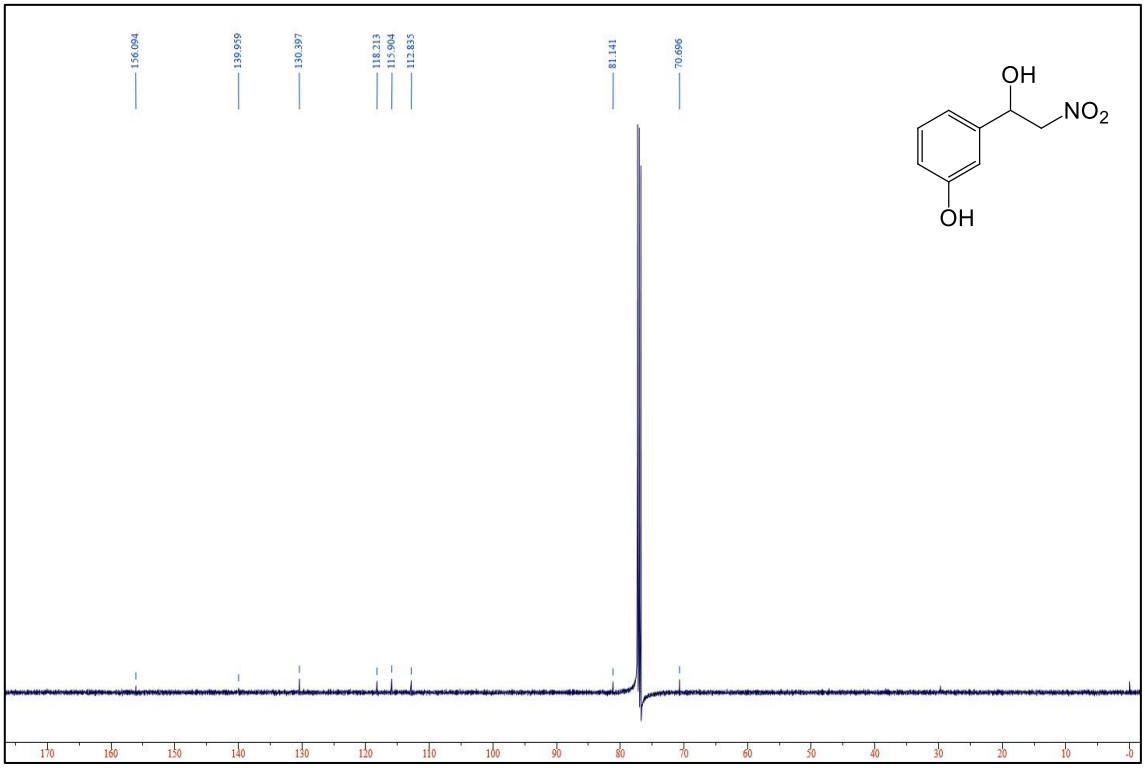


Figure 2.1.10: ^{13}C NMR spectrum of 1-(3-Hydroxyphenyl)-2-nitroethanol

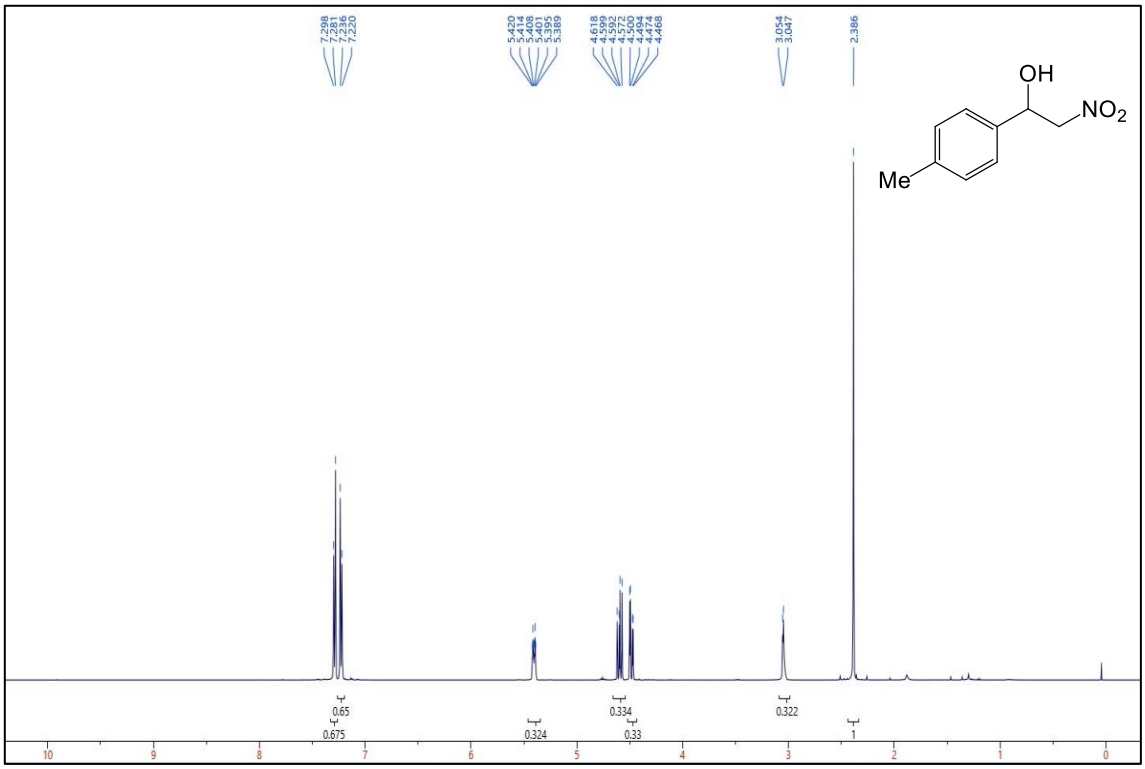


Figure 2.1.11: ^{1}H NMR spectrum of 1-(4-Methylphenyl)-2-nitroethanol

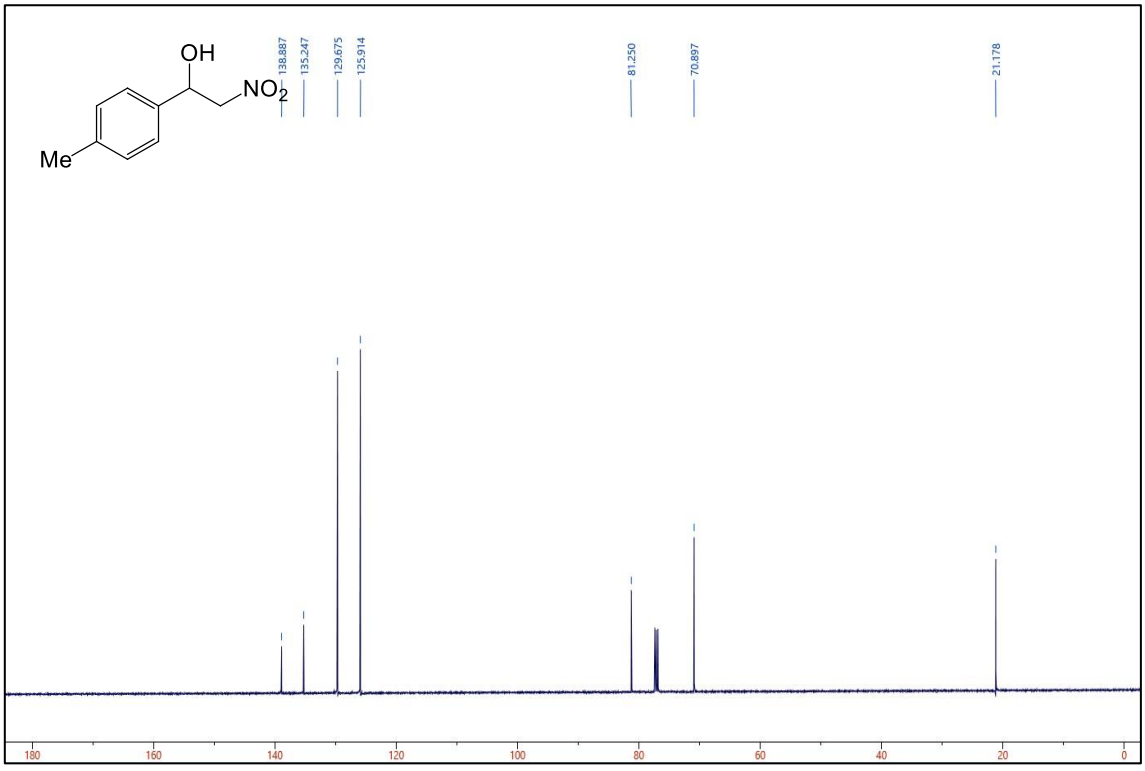


Figure 2.1.12: ^{13}C NMR spectrum of 1-(4-Methylphenyl)-2-nitroethanol

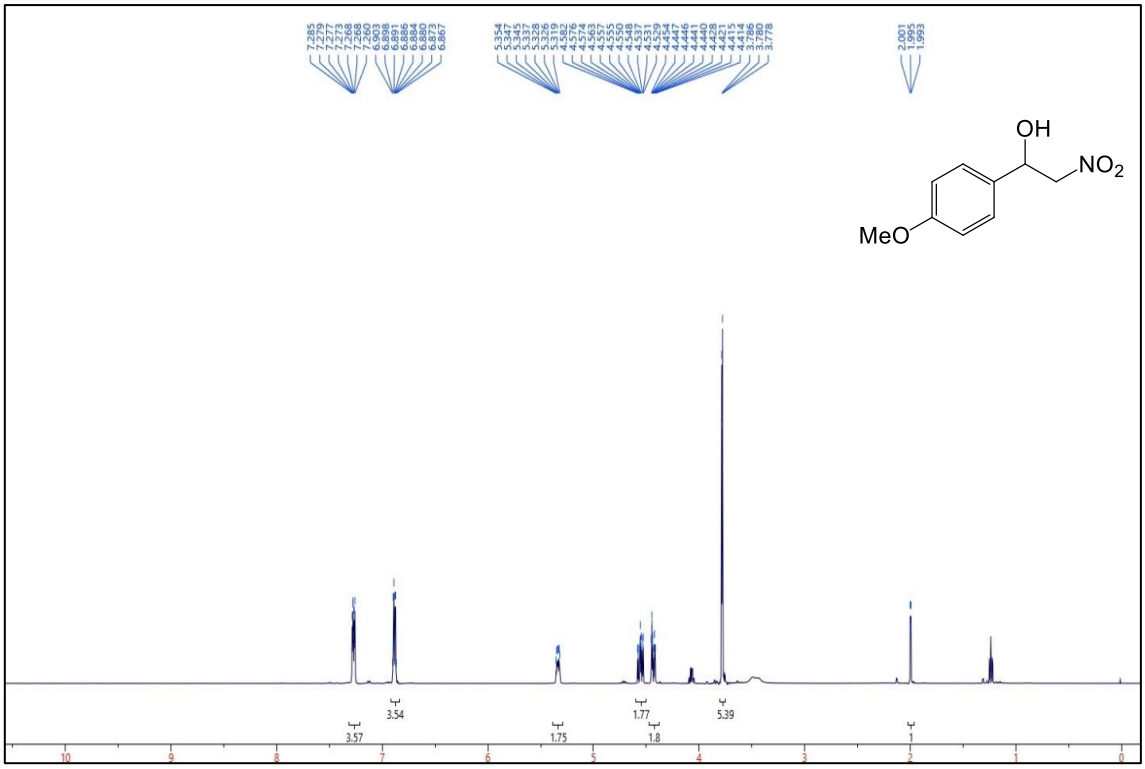


Figure 2.1.13: ^{1}H NMR spectrum of 1-(4-Methoxyphenyl)-2-nitroethanol

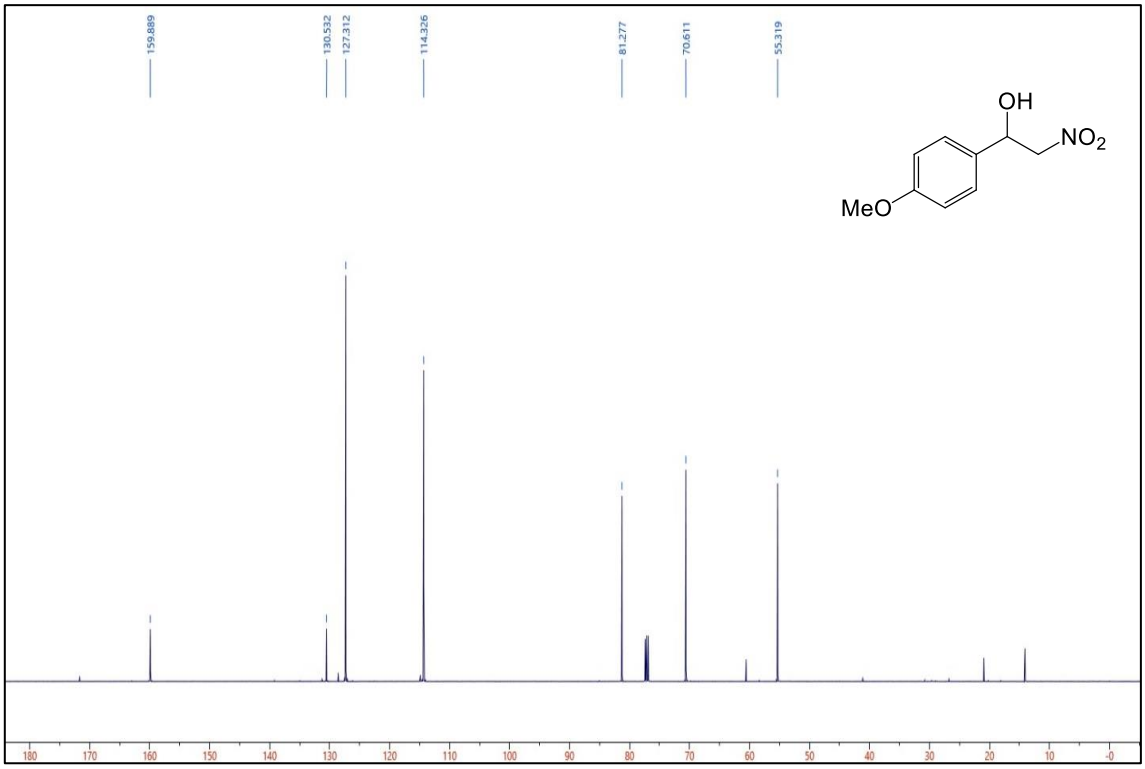


Figure 2.1.14: ^{13}C NMR spectrum of 1-(4-Methoxyphenyl)-2-nitroethanol

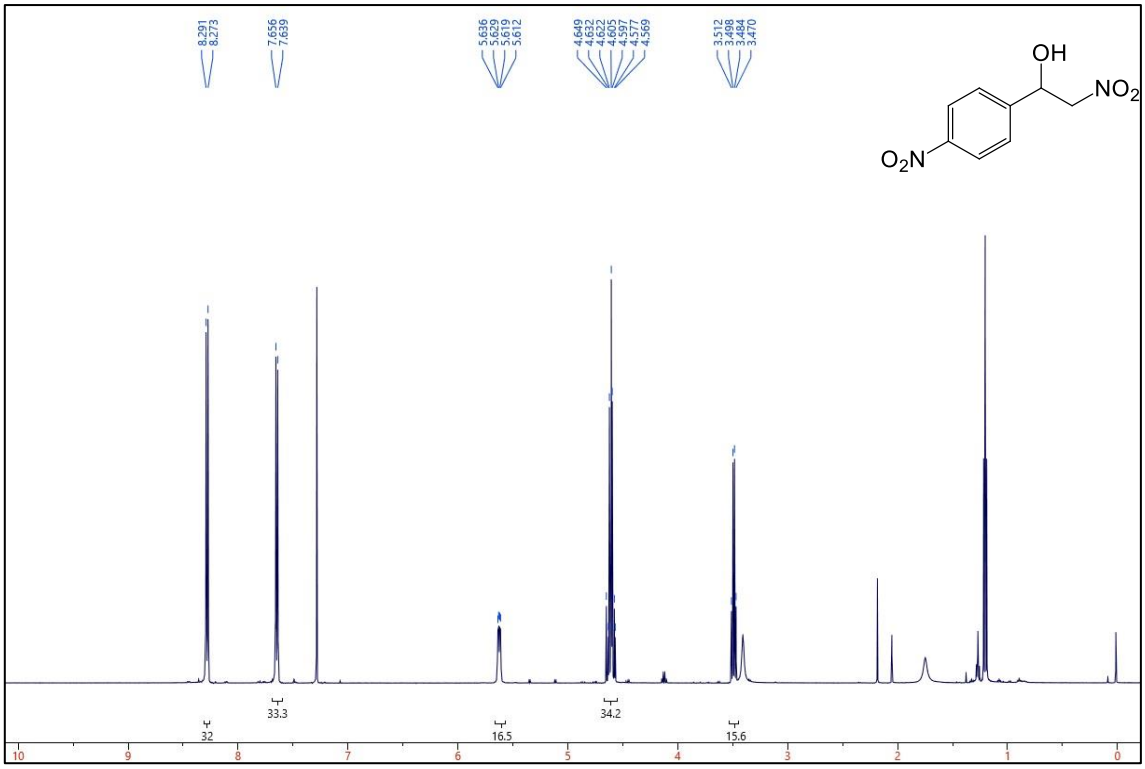


Figure 2.1.15: ^{1}H NMR spectrum of 1-(4-Nitrophenyl)-2-nitroethanol

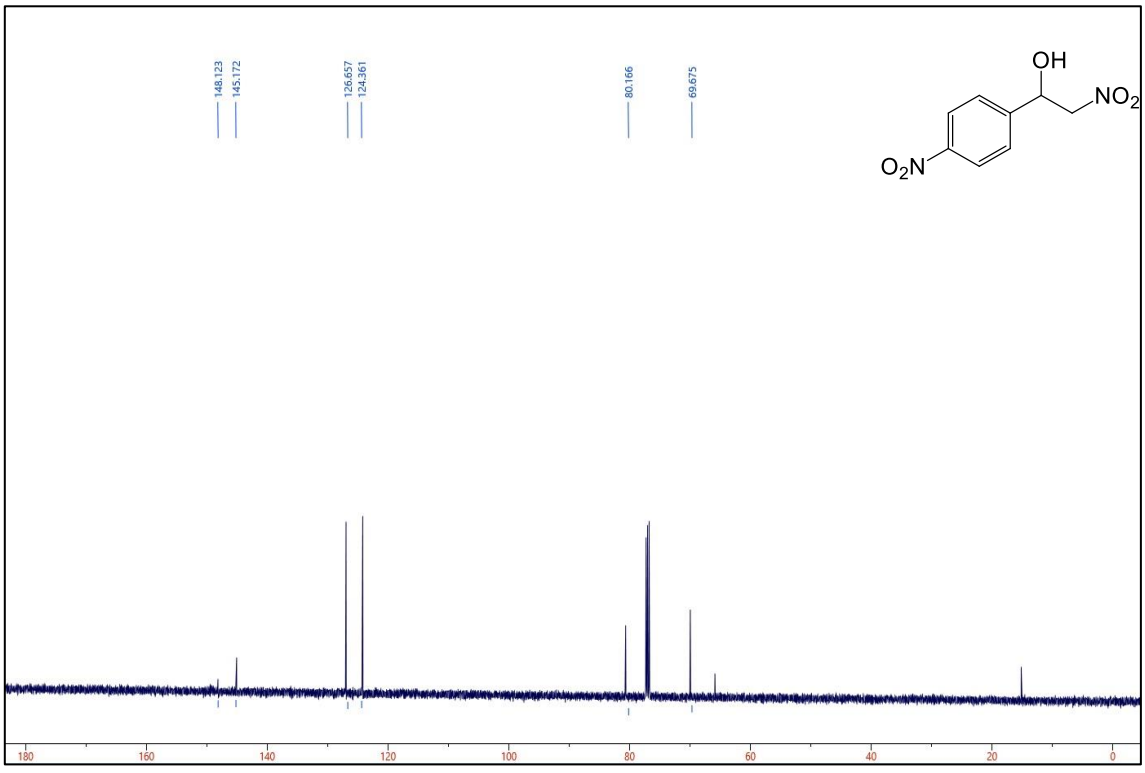


Figure 2.1.16: ^{13}C NMR spectrum of 1-(4-Nitrophenyl)-2-nitroethanol

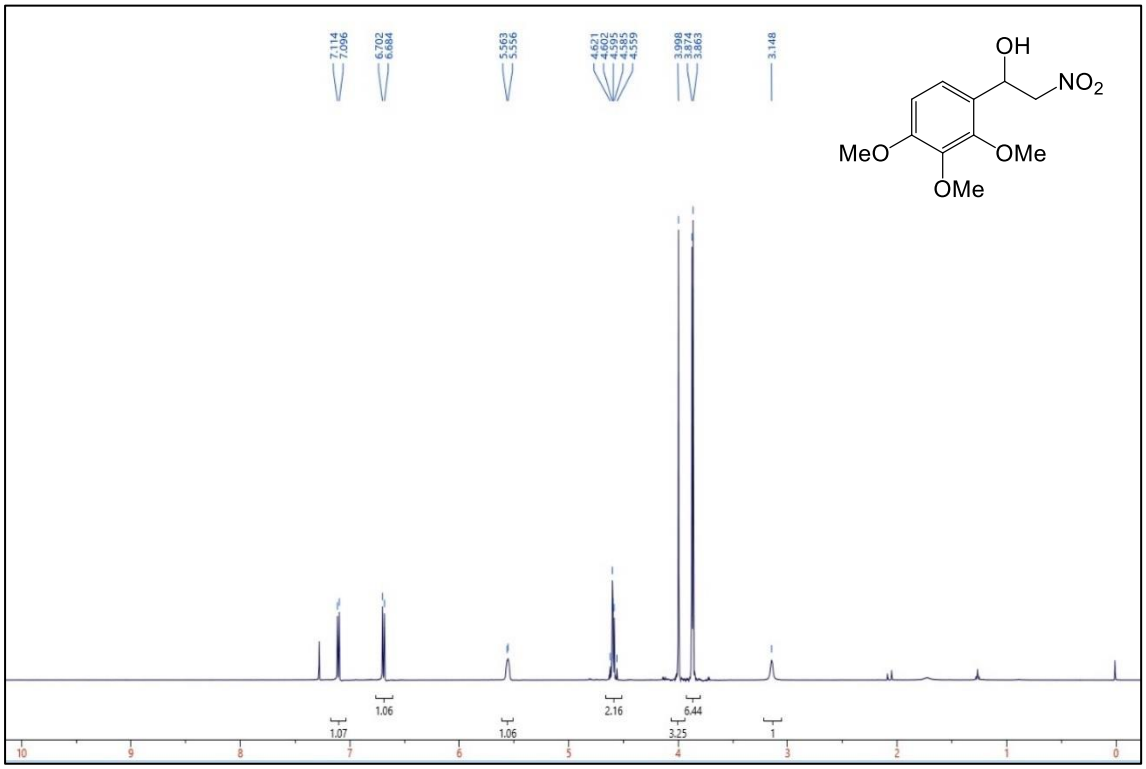


Figure 2.1.17: ^{1}H NMR spectrum of 1-(2,3,4-trimethoxyphenyl)-2-nitroethanol

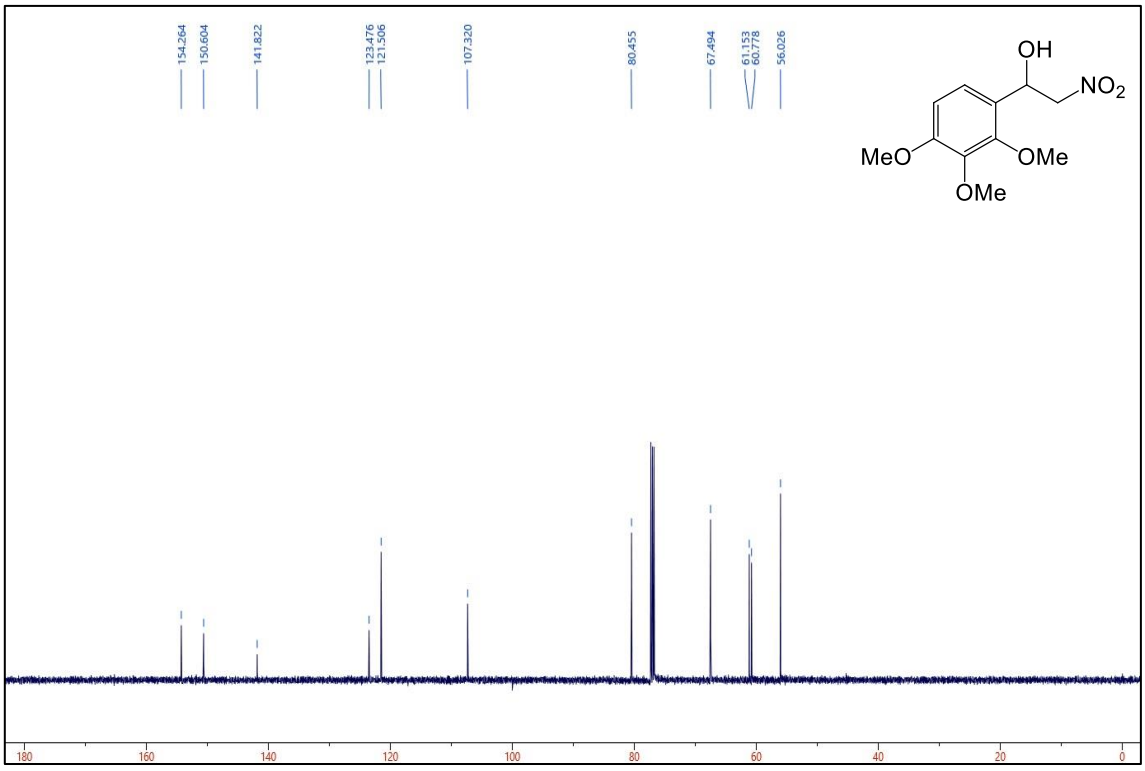


Figure 2.1.18: ^{13}C NMR spectrum of 1-(2,3,4-trimethoxyphenyl)-2-nitroethanol

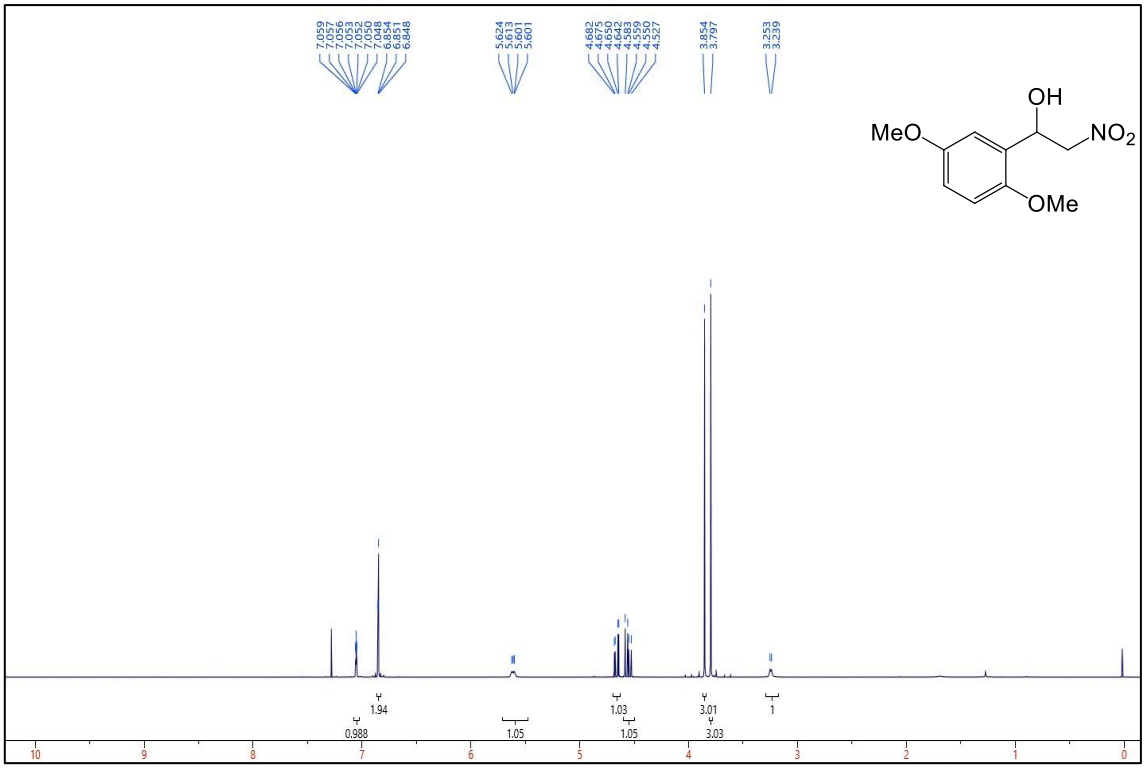


Figure 2.1.19: ^{1}H NMR spectrum of 1-(2,5-dimethoxyphenyl)-2-nitroethanol

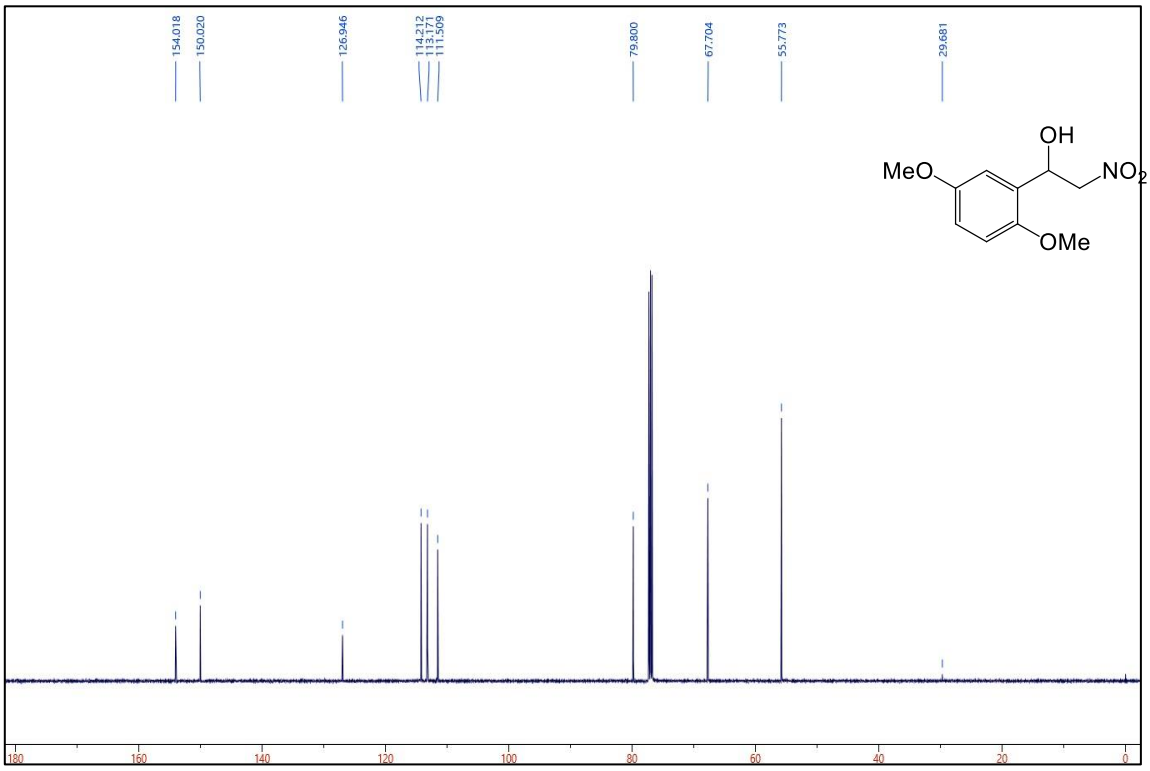
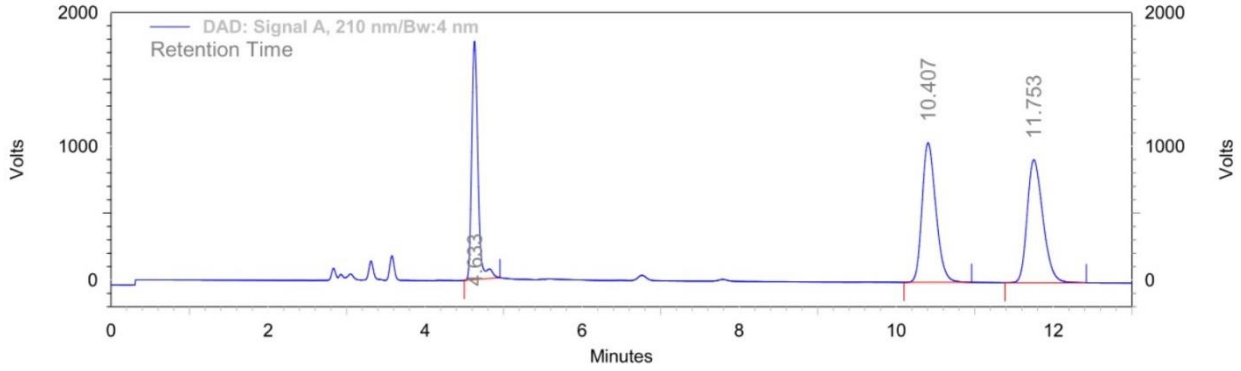
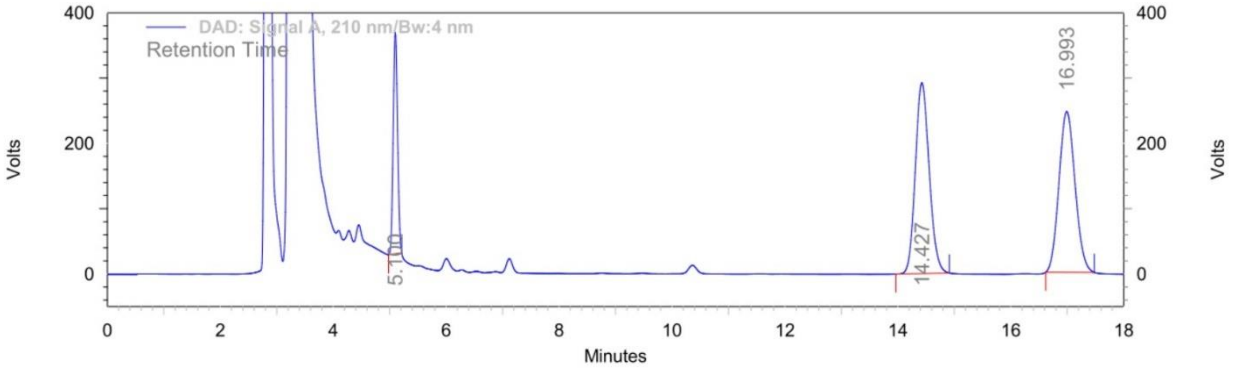


Figure 2.1.20: ^{13}C NMR spectrum of 1-(2,5-dimethoxyphenyl)-2-nitroethanol

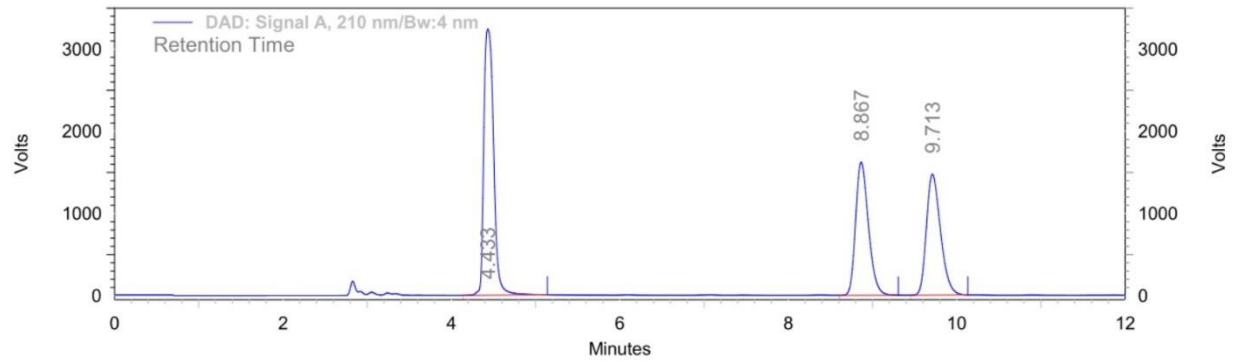
2.4.2. Chiral HPLC resolution of racemic β-nitroalcohols and their corresponding aldehydes



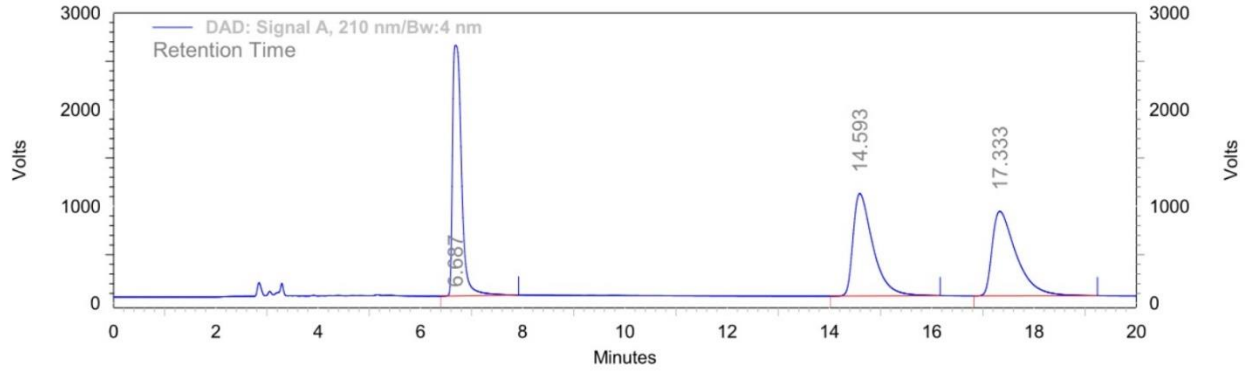
(a)



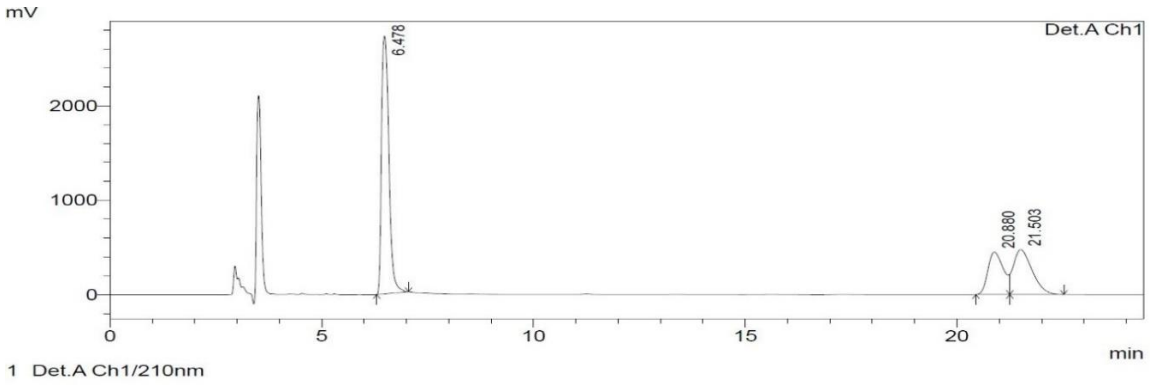
(b)



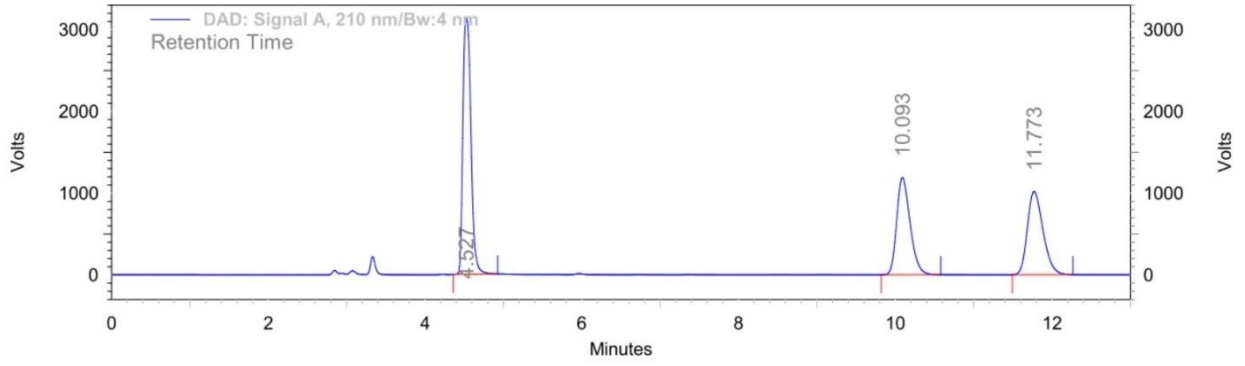
(c)


DAD: Signal A, 210 nm/Bw:4 nm
Retention Time
Volts
Minutes
6.687
14.593
17.333

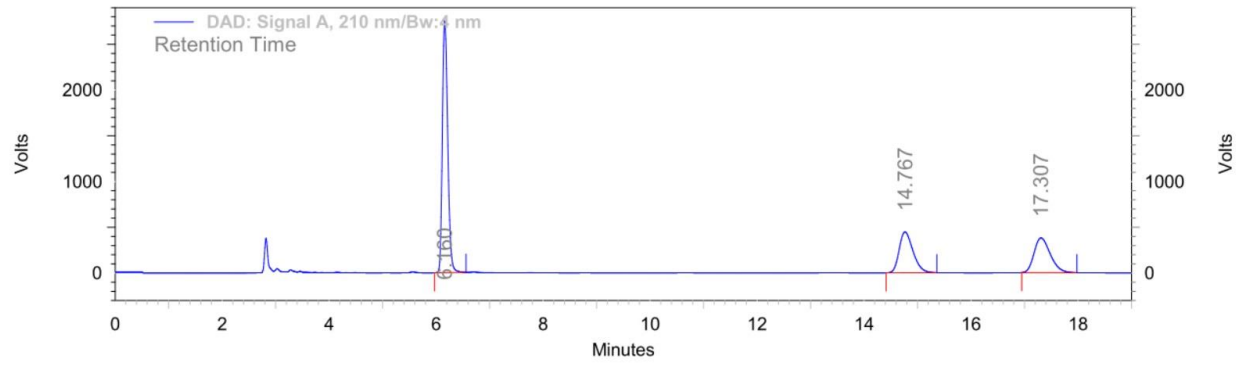
(d)


mV
Det.A Ch1
6.478
20.880
21.503
min
1 Det.A Ch1/210nm

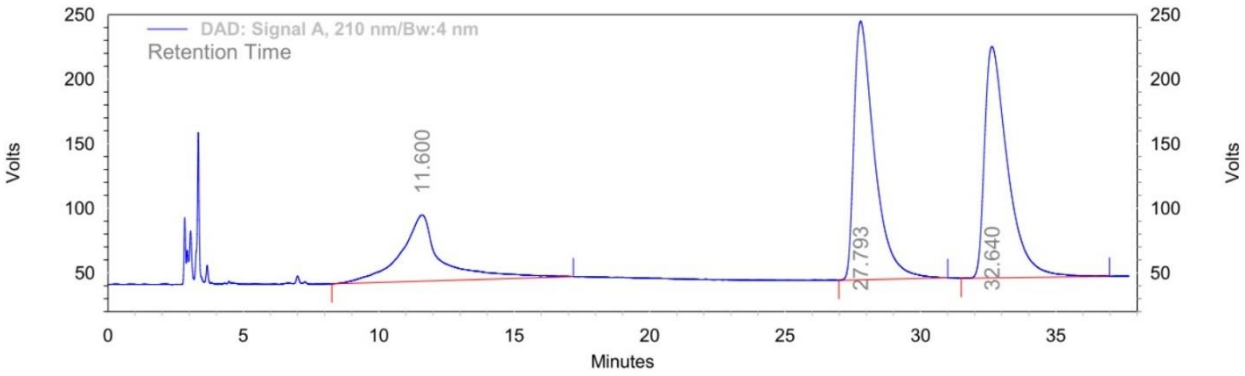
(e)


DAD: Signal A, 210 nm/Bw:4 nm
Retention Time
Volts
Minutes
4.527
10.093
11.773

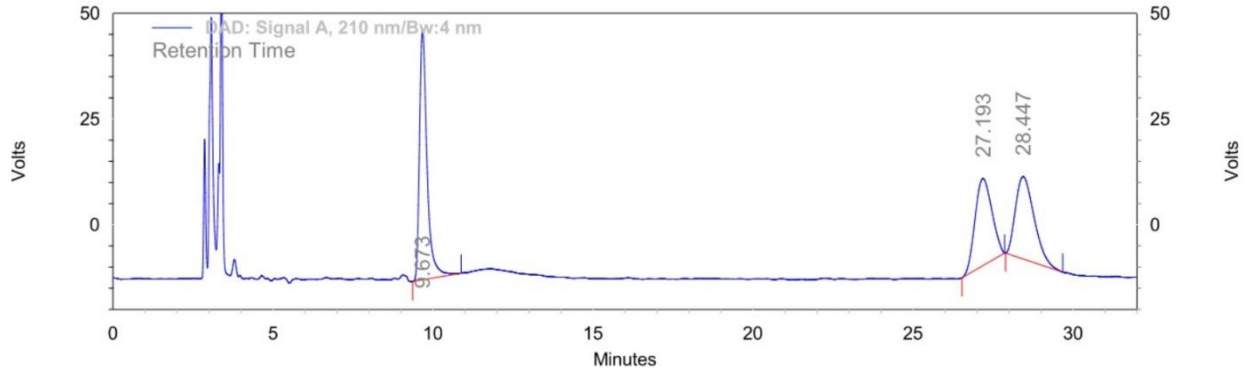
(f)


DAD: Signal A, 210 nm/Bw:4 nm
Retention Time
Volts
2000
1000
0
6.160
14.767
17.307
0
2
4
6
8
10
12
14
16
18
Minutes

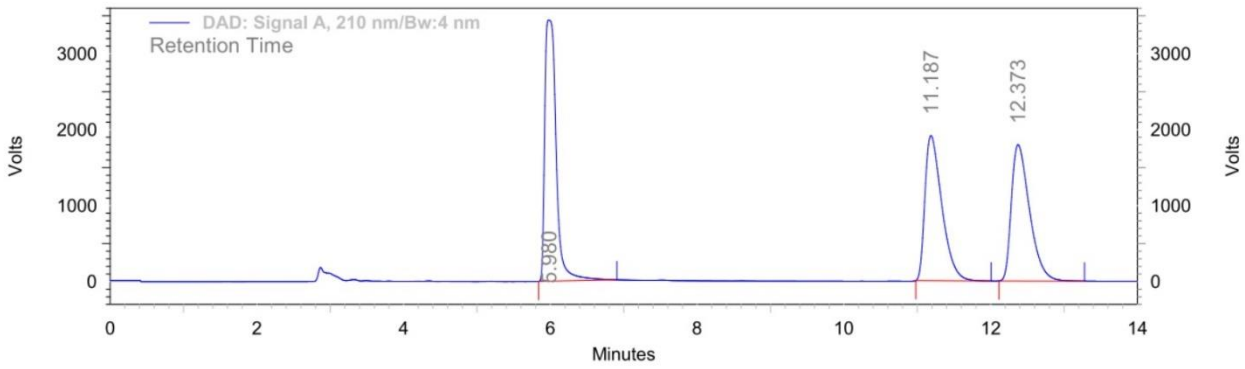
(g)


DAD: Signal A, 210 nm/Bw:4 nm
Retention Time
Volts
250
200
150
100
50
11.600
27.793
32.640
0
5
10
15
20
25
30
35
Minutes

(h)


DAD: Signal A, 210 nm/Bw:4 nm
Retention Time
Volts
50
25
0
9.673
27.193
28.447
0
5
10
15
20
25
30
Minutes

(i)



(j)

Figure 2.2: HPLC chromatograms of chiral resolution of ten different racemic β-nitroalcohols and their corresponding aldehydes. (a) 2-nitro-1-phenylethanol and benzaldehyde, (b) 1-(3-methoxyphenyl)-2-nitroethanol and 3-methoxybenzaldehyde, (c) 1-(3-methylphenyl)-2-nitroethanol and 3-methylbenzaldehyde, (d) 1-(3, 4, 5 - trimethoxyphenyl)-2-nitroethanol and 3,4,5-trimethoxybenzaldehyde, (e)1-(3-hydroxyphenyl)-2-nitroethanol and 3-hydroxybenzaldehyde, (f) 1-(4-methylphenyl)-2-nitroethanol and 4-methylbenzaldehyde, (g) 1-(4-methoxyphenyl)-2-nitroethanol and 4-methoxybenzaldehyde, (h)1-(4-nitrophenyl)-2-nitroethanol and 4-nitrobenzaldehyde, (i) 1-(2, 3, 4 - trimethoxyphenyl)-2-nitroethanol and 2, 3, 4-trimethoxybenzaldehyde, (j) 1-(2, 5 -dimethoxyphenyl)-2-nitroethanol and 2, 5-dimethoxybenzaldehyde.

Table 2.1 represents the details such as retention time of aldehyde, individual enantiomers and the HPLC analysis conditions used for chiral resolution of ten different racemic β-nitroalcohols, that are included in this study.

Column: Chiralpak® IB chiral column. Solvent: *n*-hexane: 2-propanol; flow rate: 1 mL/min; absorbance: 210 nm.

S.No	Substrate	Retention time (min)*	*n*-hexane: 2-propanol (v/v)
1	2-Nitro-1-phenyl ethanol	Benzaldehyde = 4.6 t_R = 10.4, t_s = 11.7	90:10
2	1-(3-Methoxyphenyl)-2-nitroethanol	3-Methoxybenzaldehyde = 5.1, t_R = 14.4, t_s = 16.9	90:10
3	1-(3-Methylphenyl)-2-nitroethanol	3-Methylbenzaldehyde = 4.4, t_R = 8.8, t_s = 9.7	90:10
4	1-(3, 4, 5 - Trimethoxyphenyl)-2-nitroethanol	3,4,5-Trimethoxybenzaldehyde = 6.6, t_R = 14.5, t_s = 17.3.	80:20
5	1-(3-Hydroxyphenyl)-2-nitroethanol	3-Hydroxybenzaldehyde = 6.4, t_R = 20.8, t_s = 21.5.	90:10
6	1-(4-Methylphenyl)-2-nitroethanol	4-Methylbenzaldehyde = 4.5, t_R = 10.0, t_s = 11.7	90:10
7	1-(4-Methoxyphenyl)-2-nitroethanol	4-Methoxybenzaldehyde = 6.1, t_R = 14.7, t_s = 17.3.	90:10
8	1-(4-Nitrophenyl)-2-nitroethanol	4-Nitrobenzaldehyde = 11.6, t_R = 27.7, t_s = 32.6.	90:10
9	1-(2, 3, 4 - Trimethoxyphenyl)-2-nitroethanol	2, 3, 4-Trimethoxybenzaldehyde = 9.6, t_R = 27.1, t_s = 28.4.	90:10
10	1-(2, 5 - Dimethoxyphenyl)-2-nitroethanol	2, 5-Dimethoxybenzaldehyde = 6.9, t_R = 11.1, t_s = 12.3.	90:10

*t_R and t_S are retention time of corresponding (*R*)-β-nitroalcohol, (*S*)-β-nitroalcohol respectively.

2.4.3. SDS-PAGE of purified *At*HNL

*At*HNL purified as described in **section 2.3.2** above was characterized by SDS-PAGE (**Figure 2.3**). All the components such as pellet, supernatant, eluents, and pure protein were analyzed by 12% SDS-PAGE using medium range pre-stained protein marker (BR-BIOCHEM) and stained by Coomassie Brilliant Blue R-250. A clear band at ~28 kDa indicated the good expression and purity of the purified *At*HNL.

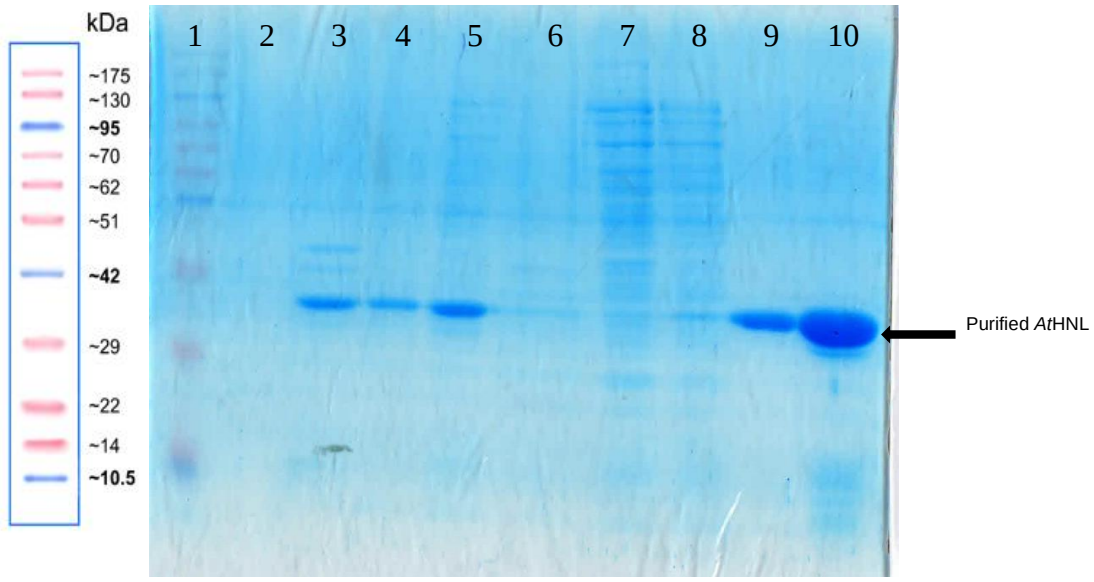


Figure 2.3: SDS-PAGE of NI-NTA purified recombinant *At*HNL. Lanes 1: Protein standard, 2: Sonicated pellet, 3: Sonicated supernatant, 4: Sonicated supernatant (diluted), 5: Ammonium sulphate pellet, 6: Ammonium sulphate supernatant, 7: Binding buffer elution, 8: Washing buffer elution, 9: Elution buffer elution, 10: Concentrated pure protein.

2.4.4. Steady state kinetics of *At*HNL

The kinetic parameters of *At*HNL were determined using mandelonitrile cleavage assay. From the Michaelis-Menten plot (**Figure 2.4**) the kinetic parameters were 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. Similarly, kinetic parameters of *At*HNL were determined for the promiscuous substrate NPE. **Figure 2.5** shows the Michaelis-Menten plot prepared by using NPE cleavage. The kinetic parameters were found to be 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.

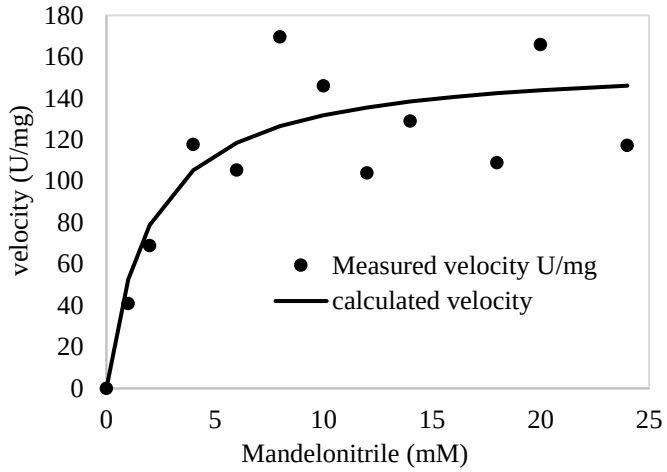


Figure 2.4: Michaelis-Menten plot for the cleavage of racemic mandelonitrile by *At*HNL

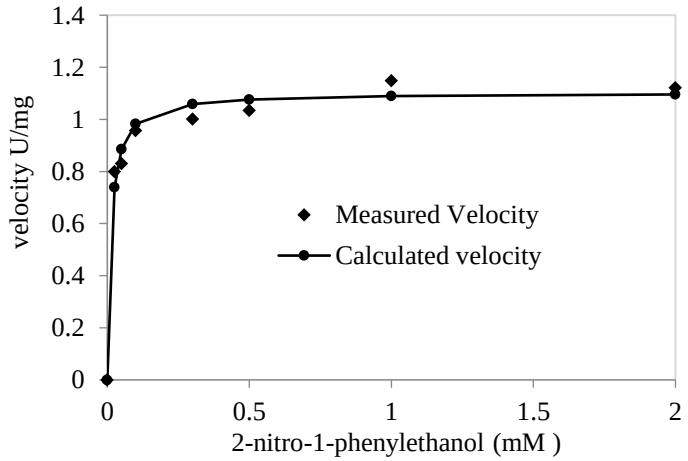


Figure 2.5: Michaelis-Menten plot for the cleavage of racemic NPE by *At*HNL

2.4.5. Optimisation of biocatalytic parameters of retro-Henry reaction in the preparation of (*S*)-β-nitroalcohols

2.4.5.1. Effect of different pH

Finding pH optima of buffer in HNL biocatalysis is essential because it effects on the enantioselectivity of the product. *At*HNL catalysed enantioselective cleavage of racemic NPE at different pH was determined to know the effect of different pH of the buffer in this biotransformation (**Figure 2.6**). The pH optima was selected as 5.0, where 96.54% *ee* and 48.1% conversion of (*S*)-NPE was found.

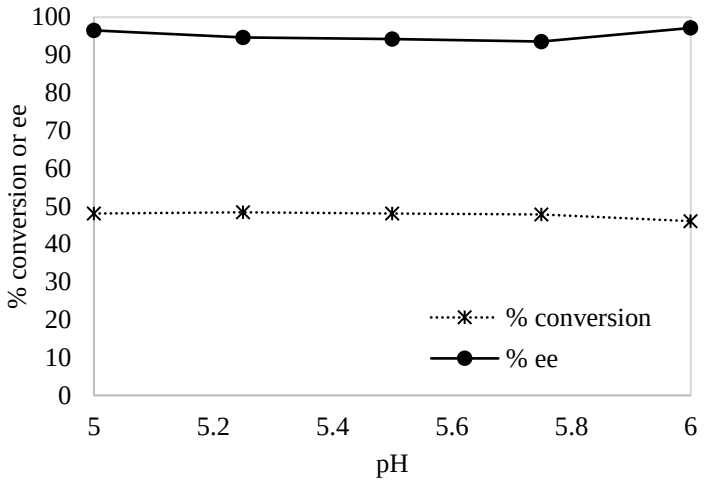


Figure 2.6: Effect of pH on enantioselective cleavage of racemic NPE

2.4.5.2. Effect of different substrate concentrations

The effect of different concentrations of substrate (racemic NPE) on *At*HNL catalysed enantioselective cleavage was investigated. The concentration of NPE was varied from 0.81 to 6.50 mM in the biotransformation (**Figure 2.7**). Based on the highest % *ee* of the product, 3.25 mM of NPE was selected as the optimum substrate concentration, where 93.79% *ee* and 38.94% conversion of (*S*)-NPE was found.

2.4.5.3. Effect of different organic solvents

To elucidate the effect of different organic solvents as biphasic medium in the *At*HNL catalysed retro-Henry reaction, we have selected six of them, i.e., DIPE, hexane, toluene, TBME, THF, and *n*-butyl acetate. Selection of the solvents was done based on their common use in HNL biocatalysis

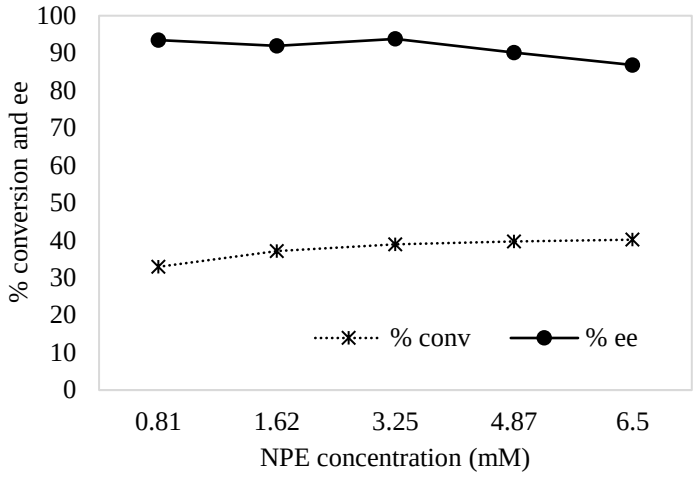


Figure 2.7: Effect of substrate concentration on enantioselective cleavage of rac NPE

in the stereoselective synthesis of cyanohydrins and β-nitroalcohols. Our study has clearly revealed highest *% ee* and conversion of (*S*)-NPE in the case of toluene as the co-solvent in biotransformation (**Figure 2.8**).

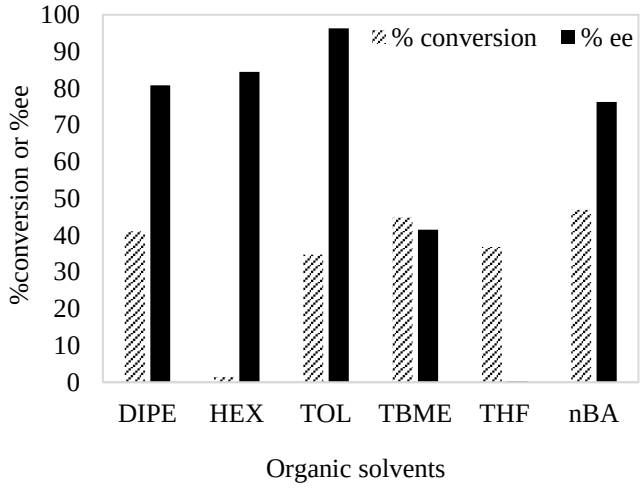


Figure 2.8: Effect of different organic solvents on the enantioselective cleavage of rac NPE. DIPE: diisopropyl ether, HEX: hexane, TOL: toluene, TBME: tert-butyl methyl ether, THF: tetrahydrofuran, nBA: *n*-butyl acetate.

2.4.5.4. Effect of different amount of enzyme

To elucidate the effect of enzyme amount on the enantioselective cleavage of racemic β-nitroalcohol, we have studied five different amounts of *At*HNLs ranging from 0.01 to 0.3 µmol (12.5 to 200 units) (**Figure 2.9**). In case of 0.15 µmol of enzyme, the retro-Henry reaction has

produced 99.73% *ee* and 53.94% conversion of (*S*)-NPE. Beyond which % *ee* of the product did not increase further.

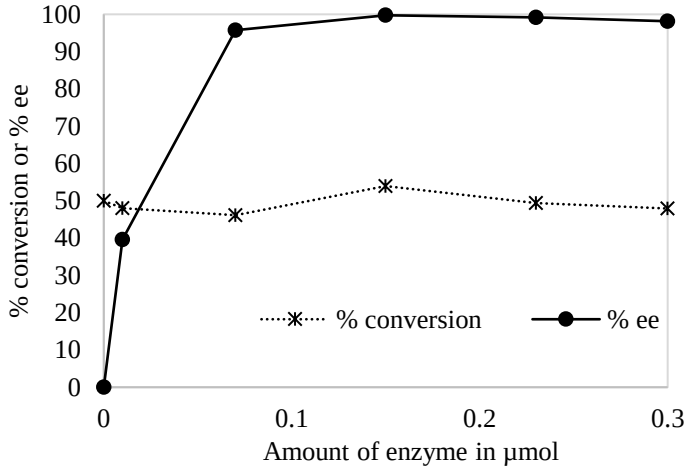


Figure 2.9: Effect of amount of enzyme on the enantioselective cleavage of rac NPE

2.4.5.5. Effect of different % volume of toluene in the preparation of (*S*)-NPE

Among the six organic solvents studied in **24.5.3**, in case of toluene the *At*HNL gave highest enantioselectivity, however, excess solvent could denature the enzyme. Hence the % volume of toluene in the biotransformation was optimised. Toluene % volume was varied from 35 to 65, while another reaction without the solvent was carried out (**Figure 2.10**). Considering the maximum % conversion and *ee* of the product, 65% of toluene was selected for further optimisation experiments.

2.4.5.6. Optimisation of time of the biotransformation

In order to achieve maximum conversion and enantioselectivity in the preparation of (*S*)-NPE, we performed the optimisation of time of biotransformation of the *At*HNL catalysed enantioselective C-C cleavage of racemic NPE. The reaction was monitored at every 30 minute interval until 4 h

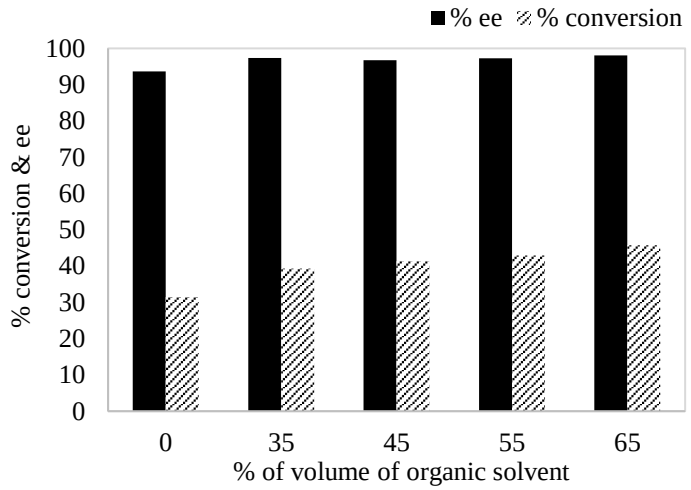


Figure 2.10: Effect of different ratio of toluene on the enantioselective cleavage of rac NPE

(**Figure 2.11**). During the time course study, we have quantified the amount of benzaldehyde formed, (*R*)-NPE, (*S*)-NPE and % *ee* of the (*S*)-NPE. With increase in time, we observed an increase in the formation of benzaldehyde and decrease in the amount of (*R*)-NPE. The amount of (*S*)-NPE remained almost static during the whole study, while % *ee* of (*S*)-NPE gradually increased till 3 hours, where it reached >99.

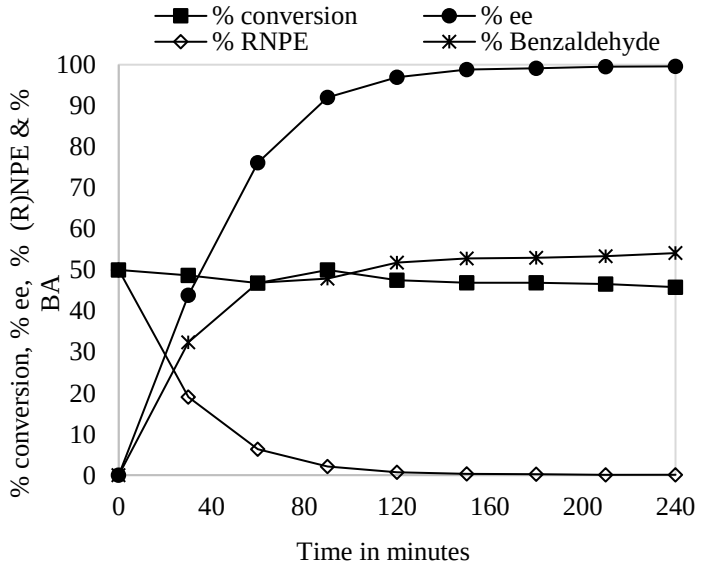
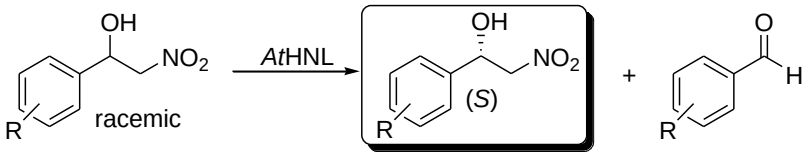


Figure 2.11: Time course of (S)-NPE preparation under optimised reaction conditions.

2.4.5.7. Preparation of different (*S*)-β-nitroalcohols using retro-Henry reaction



Scheme 2.3: *At*HNL catalysed preparation of enantioenriched (*S*)-β-nitroalcohols from corresponding racemic β-nitroalcohols.

Table 2.2: Preparation of (*S*)-β-nitroalcohols by *At*HNL catalysed enantioselective cleavage of corresponding racemic substrates.

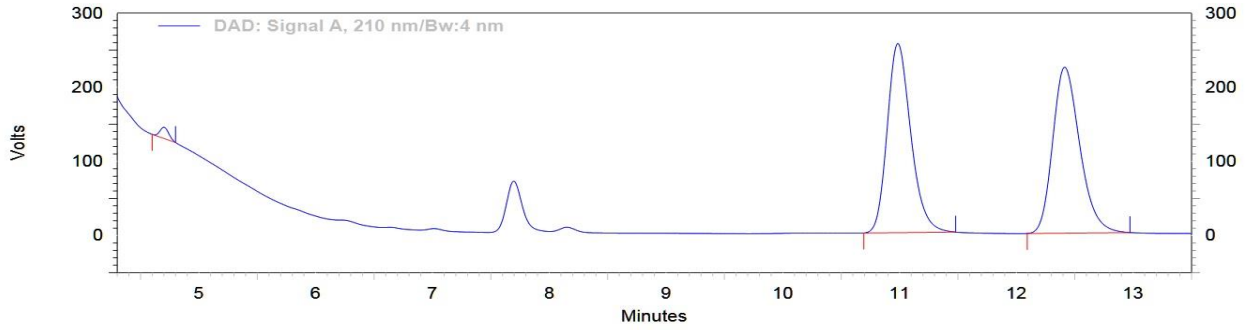
S. No	R	Time (h)	% *ee*[b]	% conver--sion[a]	*% conversion* (*% c*)	*E*[c]
1	H	3	99	47	53	84
2	3-OMe	6	99	41	58	30
3	3-Me	4	99	35	65	20
4	3,4,5-triOMe	6	88	43	54	19
5	3-OH	5	81	19	79	3
6	4-Me	7	85	45	51	25
7	4-OMe	6	44	41	43	6
8	4-NO_2	6	1	47	6	1
9	2,3,4-triOMe	6	3	49	4	5
10	2,5-diOMe	6	22	47	23	7

[a] % conversion or % c = [*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* = [(*S*-*R*)/(*S*+*R*)]*100, [c] For calculating E value we have used Sih's equation[38], $E=\frac{\ln[(1-c)(1-ee)]}{\ln[(1-c)(1+ee)]}$. Here c is the % *conversion* or % *c* = [1– (*R*+*S*)/(R_0+S_0)]*100. R: % area of (*R*)-NPE after reaction, *S*: % area of (*S*)-NPE after reaction; R_o: % area of (*R*)-NPE before reaction, S_o: % area of (*S*)-NPE before reaction. % *ee* is same as given in [b].

Having optimised the biocatalytic reaction parameters of the *At*HNL catalysed retro-Henry reaction, we intended to prove this approach by exploiting its application in the preparation of

a diverse range of (*S*)-β-nitroalcohols (**Scheme 2.3**). Towards this ten different racemic β-nitroalcohols having substitutions at *ortho* or *meta* or *para* or more than one positions of the aromatic ring of the standard NPE structure were chosen (**Table 2.2**). For each substrate the reaction time was varied to obtain highest % *ee* of the corresponding (*S*)-enantiomer of the product. **Table 2.2** shows the % conversion and *ee* of product in each of the ten substrates. The enantioselectivity, *E* value against each reaction was calculated using Sih's equation as described below the table.[38] In the case of *At*HNL catalysed enantioselective cleavage of racemic NPE, the product was produced in >99% *ee* and 47% conversion in 3 hours (**Figure 2.12**). For this transformation, *E* value calculated was found to be 84. The retro-Henry reaction with *meta* substituted substrates, i.e., 3-methoxy and 3-methyl derivatives of NPE resulted in 99% *ee* of (*S*)-enantiomers in both the cases, with 41, 35% conversions in 6 and 4 hours respectively (**Figure 2.13 and Figure 2.14**). When 3,4,5-triOMe derivative of NPE was tested, the wild type *At*HNL gave 88% *ee* of the product which was unexpected due to the bulkiness of the substituents, and 43% conversion in 6 h (**Figure 2.15**). *At*HNL also gave above moderate enantioselectivity towards two other substrates with 3-OH and 4-Me substituted NPE. In the case of racemic 3-OH NPE 81% *ee* of the (*S*)-enantiomer could be found but with only 19% conversion in 5 h, while with 4-Me NPE the product was obtained in 85% *ee* and 45% conversion in 7 h (**Figure 2.16 and Figure 2.17**). We found poor enantioselectivity of the enzyme, when 4-OMe NPE and 2,5-diOMe NPE were subjected in the retro-Henry reaction. These two substrates showed only 44 and 22% *ee* of their corresponding (*S*)-enantiomers in 6 h. Unfortunately, two other substrates, 4-NO_2 NPE and 2,3,4-triOMe NPE were not accepted by the enzymes in the retro-Henry reaction, as in these cases only 1 and 3% *ee* were found respectively in 6 h, which are negligible.

(a) Control:



(b) Reaction:

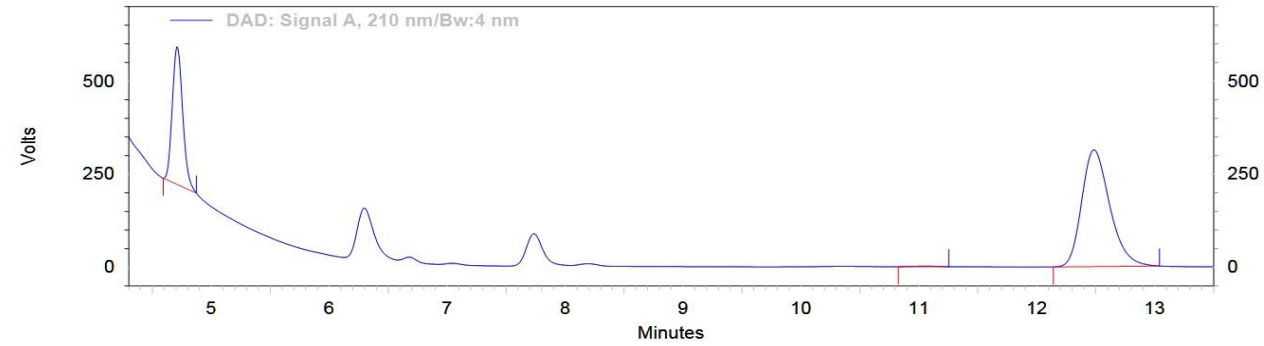
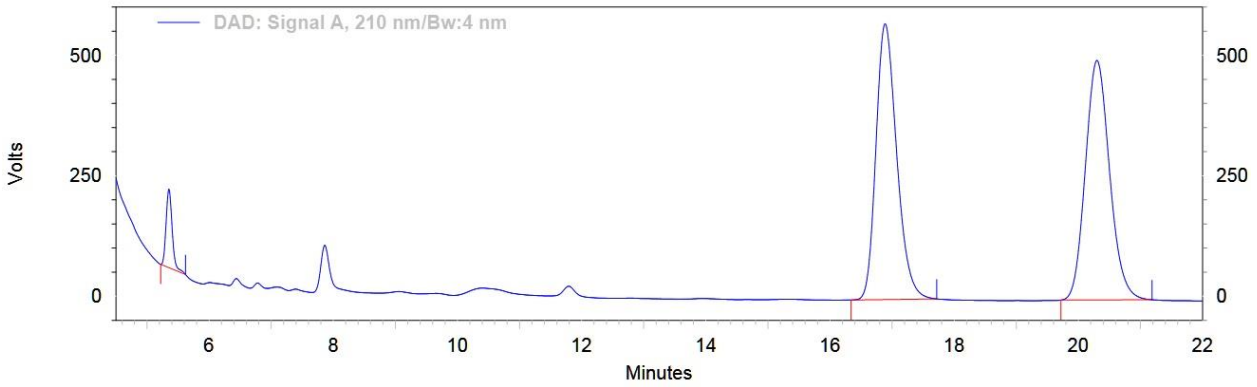


Figure 2.12: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of NPE, (a) control having no enzyme, (b) reaction. Retention times, benzaldehyde: 4.7 min, (*R*)-NPE: 10.9 min and (*S*)-NPE: 12.4 min.

Control:



Reaction:

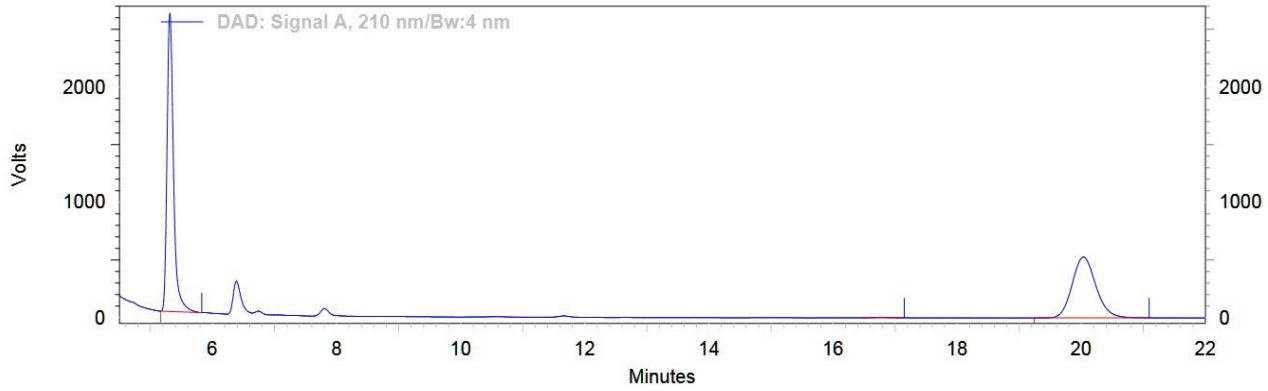
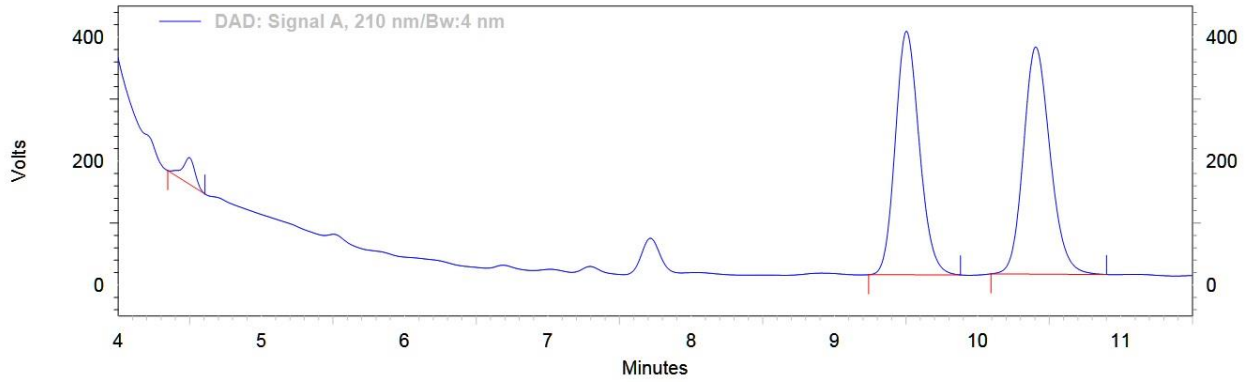


Figure 2.13: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of 1-(3-methoxyphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3-methoxybenzaldehyde: 5.3 min, (*R*)-1-(3-methoxyphenyl)-2-nitroethanol: 16.8 min and (*S*)-1-(3-methoxyphenyl)-2-nitroethanol: 20.3 min.

Control:



Reaction:

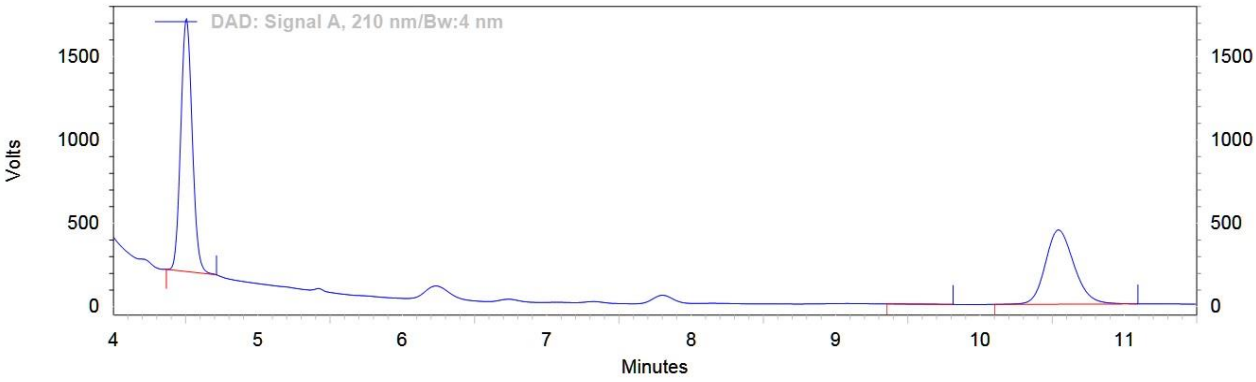
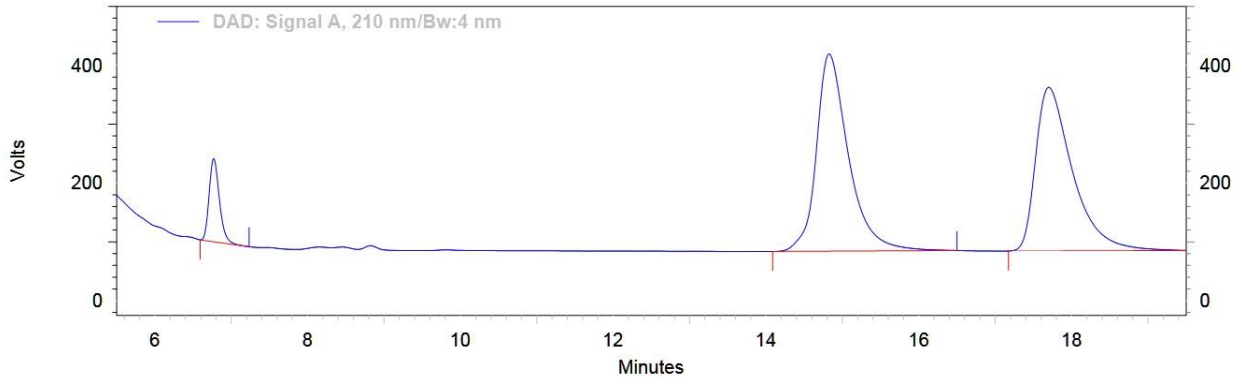


Figure 2.14: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of 1-(3-methylphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3-methylbenzaldehyde: 4.4 min, (*R*)-1-(3-methylphenyl)-2-nitroethanol: 9.5 min and (*S*)-1-(3-methylphenyl)-2-nitroethanol: 10.4 min.

Control:



Reaction:

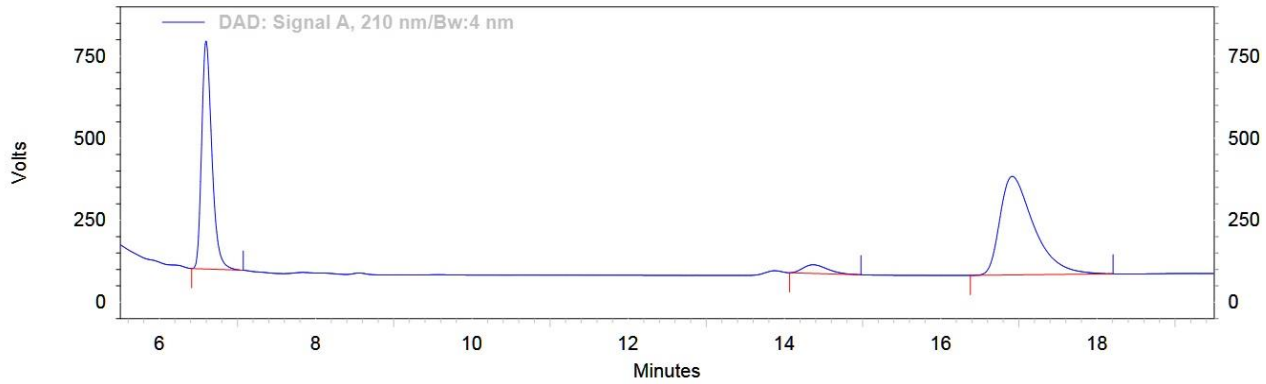
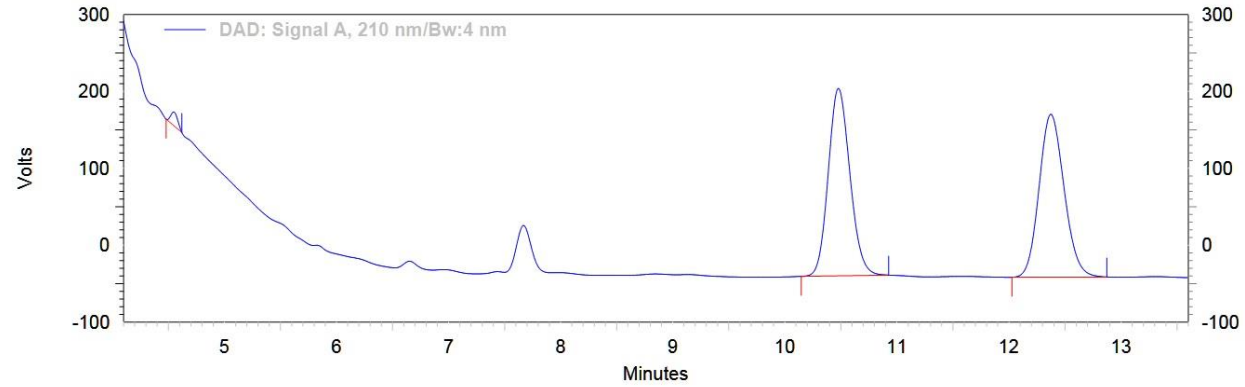


Figure 2.15: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of 1-(3, 4, 5-trimethoxyphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3, 4, 5-trimethoxybenzaldehyde: 6.7 min, (*R*)-1-(3, 4, 5-trimethoxyphenyl)-2-nitroethanol: 14.8 min and (*S*)-1-(3, 4, 5-trimethoxyphenyl)-2-nitroethanol: 17.7 min.

Control:



Reaction:

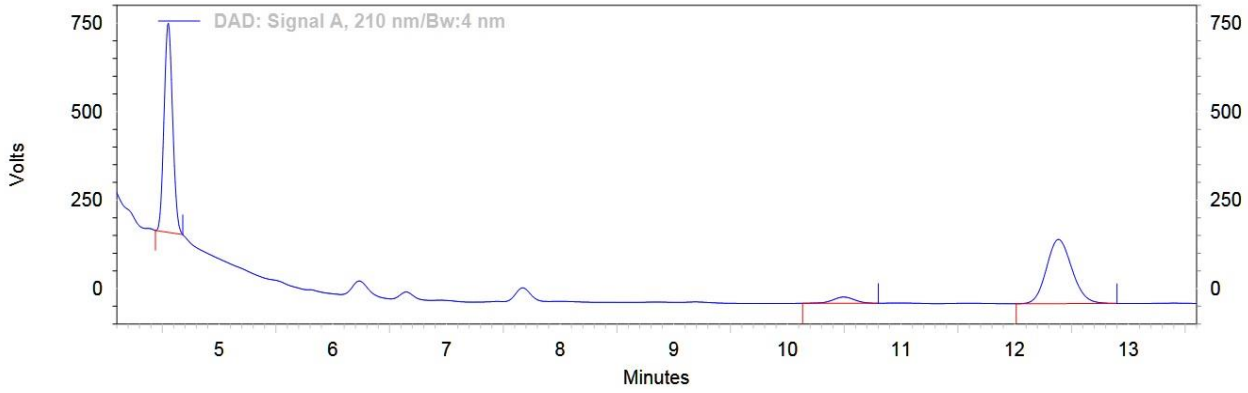
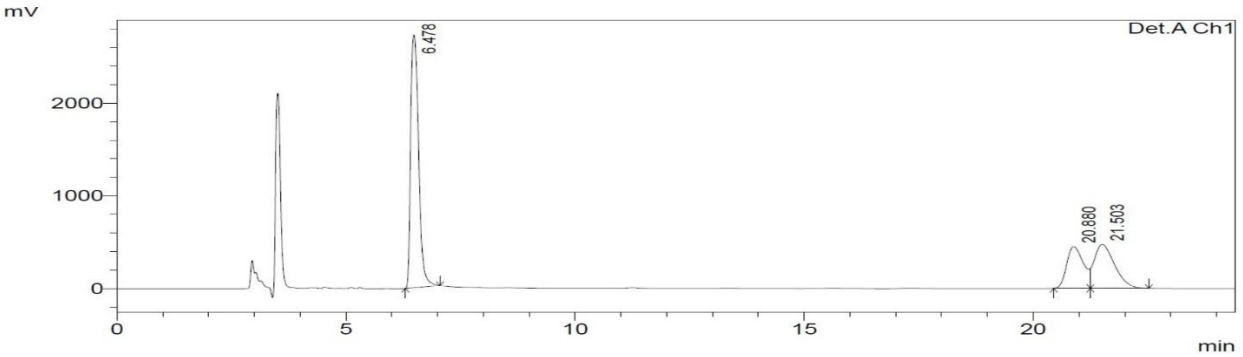


Figure 2.16: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of 1-(4-methylphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 4-methylbenzaldehyde: 4.5 min, (*R*)-1-(4-methylphenyl)-2-nitroethanol: 10.4 min and (*S*)-1-(4-methylphenyl)-2-nitroethanol: 12.3 min.

Control:



Reaction:

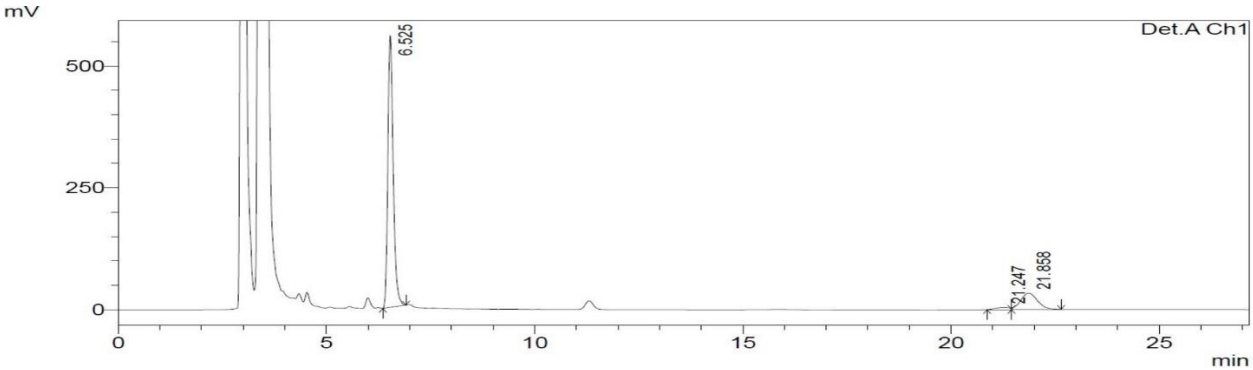


Figure 2.17: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of 1-(3-hydroxyphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3-hydroxybenzaldehyde: 6.5 min, (*R*)-1-(3-hydroxyphenyl)-2-nitroethanol: 21.1 min and (*S*)-1-(3-hydroxyphenyl)-2-nitroethanol: 21.7 min.

2.4.5.8. Preparative synthesis of (*S*)-NPE using retro-Henry reaction

To elucidate the potential of our retro-Henry approach as a plausible synthetic method in the biocatalytic preparation of (*S*)-β-nitroalcohol, we intended to scale up the biotransformation. Preparative scale synthesis of (*S*)-NPE was carried out using purified *At*HNL as described in **2.3.7.**. At the end of 3 h, the biocatalytic product mixture obtained was purified by column chromatography. The product was characterized by ^{1}H and ^{13}C NMR and optical purity was determined by chiral HPLC (**Figure 2.18 - Figure 2.20**). The product (*S*)-NPE was found in 54% yield (with hexane as impurity) and 93% *ee*.

^{1}H NMR of 2-nitro-1-phenylethanol of preparative scale biocatalysis:

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.0 (1H, s), 4.55 (1H, dd, *J* = 3.0, 13.0 Hz), 4.65 (1H, dd, *J* = 10.0, 13.5 Hz), 5.49 (1H, dd, *J* = 3.0, 9.5 Hz), 7.37-7.45 (5H, m).

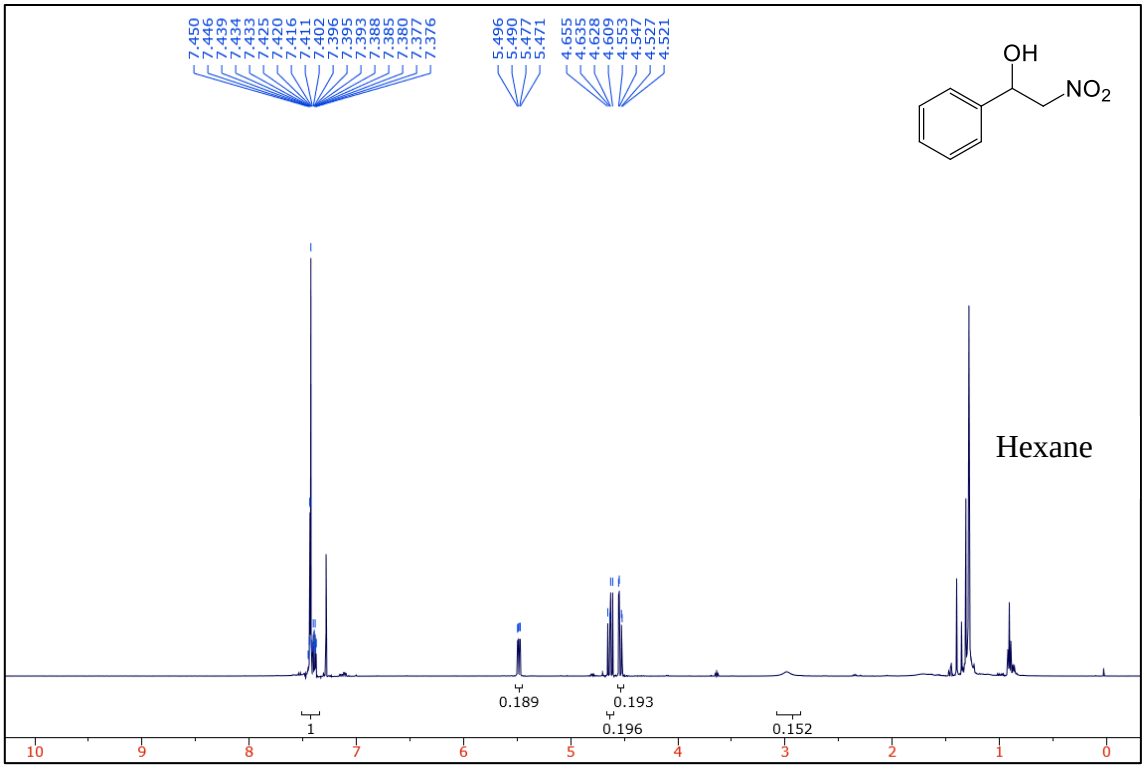


Figure 2.18: ^{1}H NMR of NPE obtained from preparative scale synthesis

^{13}C NMR of 2-nitro-1-phenylethanol of preparative scale biocatalysis:

^{13}C NMR (100 MHz, $CDCl_3$) δ 71.0, 81.2, 125.9 (X2), 129.0, 129.0 (X2), 138.1

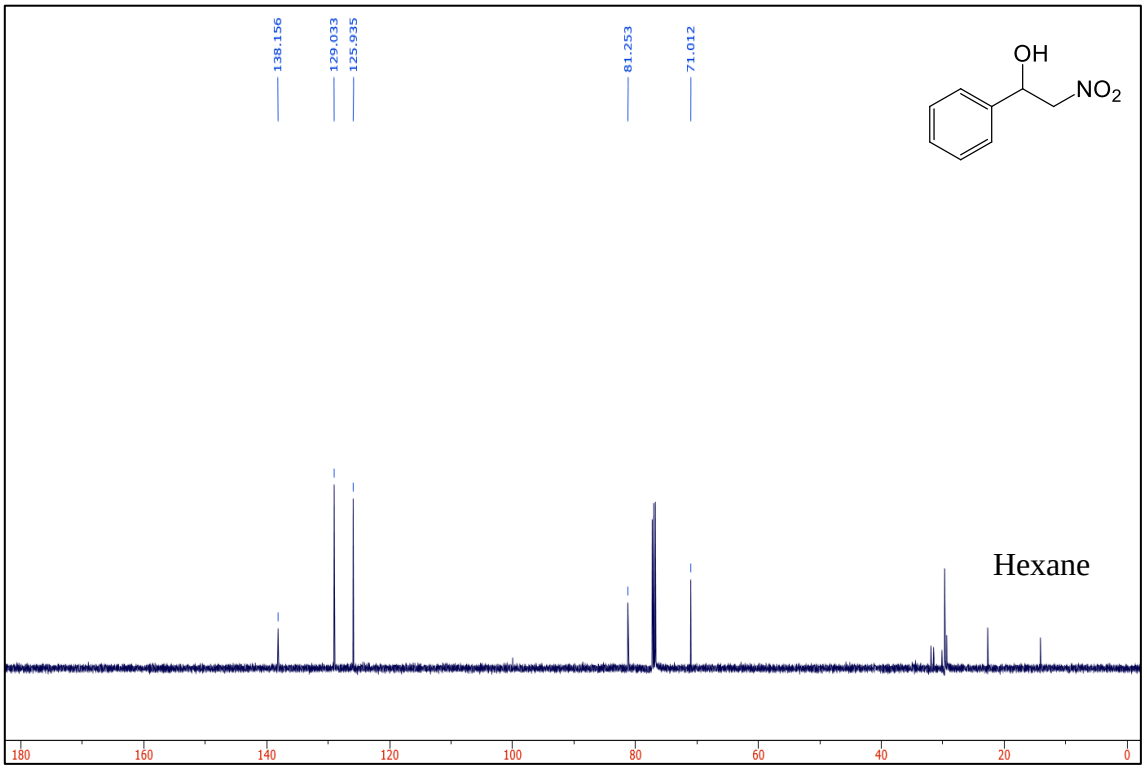
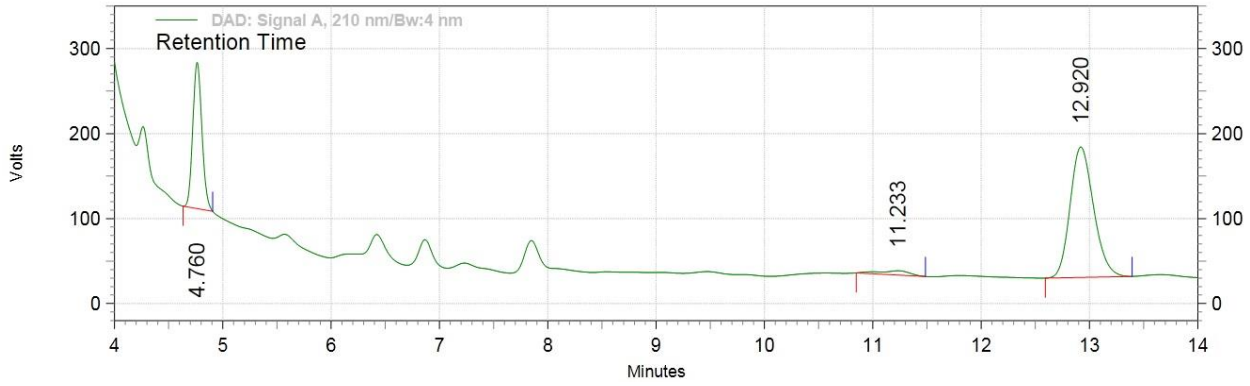
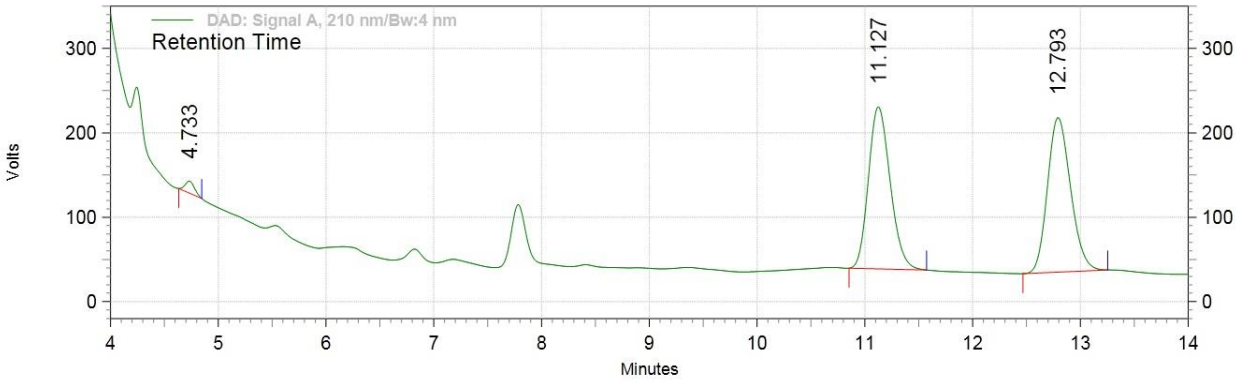


Figure 2.19: ^{13}C NMR of NPE obtained from preparative scale synthesis

(a) Enzymatic reaction:



(b) Control:



(c) Enzymatic reaction, after purification by column chromatography:

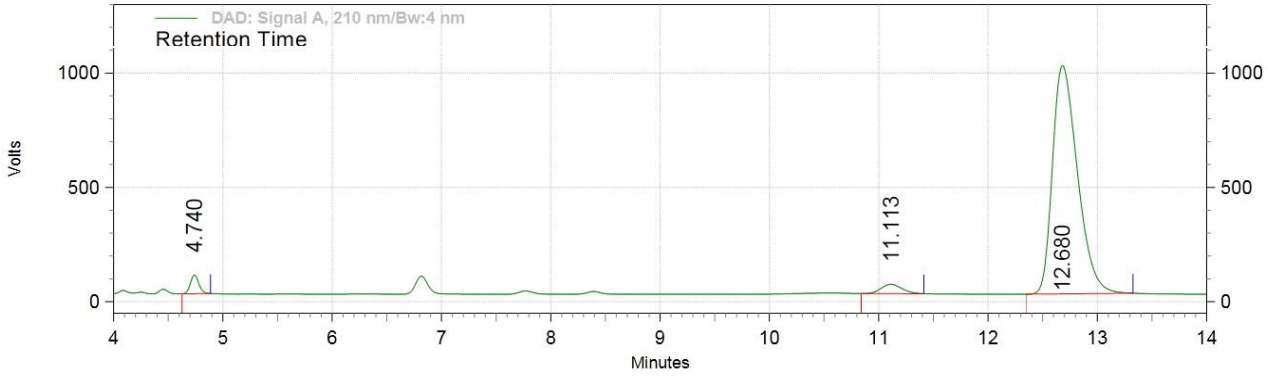


Figure 2.20: HPLC chromatograms of preparative scale biocatalysis (a) before purification by column chromatography, (b) control and (c) after purification by column chromatography.

2.5. Discussions

2.5.1. Conceptualizing the retro-Henry reaction from kinetic studies

Prior to exploring stereoselective C-C bond cleavage by *At*HNL as a method to prepare enantioenriched β-nitroalcohols, 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 enantioselective cleavage of NPE was monitored at 280 nm. Michaelis-Menten plot (**Figure 2.5**) 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 (**Figure 2.4**).

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.[39] 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. The 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.[31] 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 NPE is 0.71 U/mg.[39] As the synthesis reaction is slower than the cleavage one, therefore preparation of (*S*)-β-nitroalcohols by engineered *Hb*HNL would have lower specific activity than 0.71 U/mg. In contrary *At*HNL has shown 1.1 U/mg for the cleavage of racemic NPE. This process produces (*S*)-NPE and hence *At*HNL catalysed enantioselective cleavage appears to be catalytically efficient method for the production of (*S*)-β-nitroalcohols than *Hb*HNL catalysed synthesis. This higher efficiency has motivated us to explore *At*HNL catalysed enantioselective cleavage as a method to prepare (*S*)-β-nitroalcohols.

2.5.2. Optimisation of biocatalytic parameters of retro-Henry reaction in the preparation of (*S*)-β-nitroalcohols

Different biocatalytic parameters for the enantioselective cleavage were optimised using racemic NPE as the substrate to obtain highest % conversion and enantiomeric excess of its (*S*)-enantiomer.

2.5.2.1. Effect of different pH

*At*HNL catalysed 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,[40] 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 (**Figure 2.6**). The optimum pH was taken as 5.0 where 96.54% *ee* and 48.1% conversion of (*S*)-NPE was observed. *At*HNL catalysed synthesis of (*R*)-cyanohydrin has been reported at pH 5.0[41] and (*R*)-β-nitroalcohol at pH 7.[23]

2.5.2.2. Effect of different substrate concentrations

Benzaldehyde being a by-product of retro-Henry reaction, which is also known to inhibit *At*HNL, it has become important to find out the effect of different NPE concentration in the preparation of (*S*)-NPE. Hence, *At*HNL biocatalysis was carried out by varying the NPE concentrations from 0.81 to 6.50 mM in the biotransformation (**Figure 2.7**). 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 catalysed by *Hb*HNL.[32] We have selected 3.25 mM as the optimum substrate concentration where 93.79% *ee* and 38.94% conversion of (*S*)-NPE was found.

2.5.2.3. Effect of different organic solvents

Asano and coworkers reported the effect of different organic solvents in the *At*HNL catalysed synthesis of (*R*)-β-nitroalcohols.[23] They observed highest % *ee* and yield in case of *n*-butyl acetate among diethyl ether, DIPE, TBME, ethyl acetate, hexane, cyclohexane, toluene and xylene. We have selected most of these organic solvents except ethyl acetate which is similar to *n*-butyl acetate, to examine their effect on the *At*HNL catalysed enantioselective cleavage of racemic NPE. The best result was obtained in case of toluene that showed the highest enantiomeric excess, i.e., 96.3% of (*S*)-NPE (**Figure 2.8**). Use of tetrahydrofuran (THF) did not contribute toward the enantioselectivity of the product, while hexane has resulted in poor conversion. DIPE (81%), toluene (96.3%) and *n*-butyl acetate (76.2%) has shown 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-nitroalcohols and (*S*)-1-chloro-3-(4-(2-methoxyethyl)phenoxy) propan-2-ol.[19,42] Biocatalysis without any organic solvent resulted in 93.6% *ee* and 35% conversion of (*S*)-NPE. Thus, comparison of enantioselective 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,[43] 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 **Figure 2.6 and 2.7** showed higher % *ee* of (*S*)-NPE compared to **Figure 2.8**. 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.

2.5.2.4. Effect of different amount of enzyme

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 (**Figure 2.9**). The optimum amount of enzyme was selected as 0.15 µmol where 99.73% *ee* and 53.94% conversion was observed.

2.5.2.5. Effect of different % volume of toluene in the preparation of (*S*)-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% (**Figure 2.10**). Hence 65% of toluene was chosen as optimum solvent content to pursue further optimisation experiments.

2.5.2.6. Optimisation of time of the biotransformation

*At*HNL catalysed enantioselective cleavage of racemic NPE was monitored at different time intervals. An increase in % *ee* of (*S*)-NPE was observed with time (**Figure 2.11**). 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 optimisation 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 catalysed synthesis of (*S*)-β-nitroalcohols,[31,32] 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.

2.5.2.7. Preparation of different (*S*)-β-nitroalcohols using retro-Henry reaction

Using the optimised 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-nitroalcohols 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-nitroalcohols. *At*HNL has been reported to show high % *ee* of product in the synthesis of similar *meta* substituted aromatic β-nitroalcohols from corresponding aldehydes.[23] However with 3,4,5-trimethoxybenzaldehyde and 3-hydroxybenzaldehyde, neither chiral cyanohydrin nor β-nitroalcohol synthesis using *At*HNL has been reported. For these two aldehydes, we report here for the first time *At*HNL catalysed synthesis of (*S*)-2-nitro-1-(3,4,5-trimethoxyphenyl)ethanol, entry **4** of **Table 2.2** in 88% *ee*, 43% conversion and (*S*)-2-nitro-1-(3-hydroxyphenyl)ethanol, entry **5** of **Table 2.2** in 81% *ee*, 19% conversion. Among the *para* substituted aromatic β-nitroalcohols 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*-methoxybenzaldehyde in 68% *ee* but not with *p*-methylbenzaldehyde.[41] Further, *At*HNL has been reported to synthesize (*R*)-β-nitroalcohols of 4-methoxy benzaldehyde in 79% *ee* and 2% yield and 4-methyl benzaldehyde in 94% *ee* and 11% yield.[23] However *At*HNL has not tested in the synthesis of either (*R*)-α-cyanohydrin or (*R*)-β-nitroalcohol 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-nitroalcohols. 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 2.2**) 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-nitroalcohol in high % *ee* with *ortho* substituted aromatic aldehydes.[23,38]

2.6. 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 racemic NPE by *At*HNL has revealed 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. 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. Optimisation of various biocatalytic reaction parameters of the enantioselective C-C bond cleavage by wild type *At*HNL using racemic NPE as the substrate was performed to find out optimal reaction conditions. Under optimised biocatalytic reaction conditions, this transformation resulted in 99% *ee* (*S*) and 47% conversion of the NPE, with *E* value of 84. Ten racemic β-nitroalcohols having substituents at different positions of the aromatic ring were used to prepare their corresponding (*S*)-β-nitroalcohols 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 catalysed route known so far to synthesize a series of (*S*)-β-nitroalcohols. Along with this method, *At*HNL now can be used not only to synthesize (*R*)- but also (*S*)-β-nitroalcohols starting with appropriate substrate. This method of retro-Henry reaction to prepare opposite enantioselective products can be extrapolated to other HNLs.

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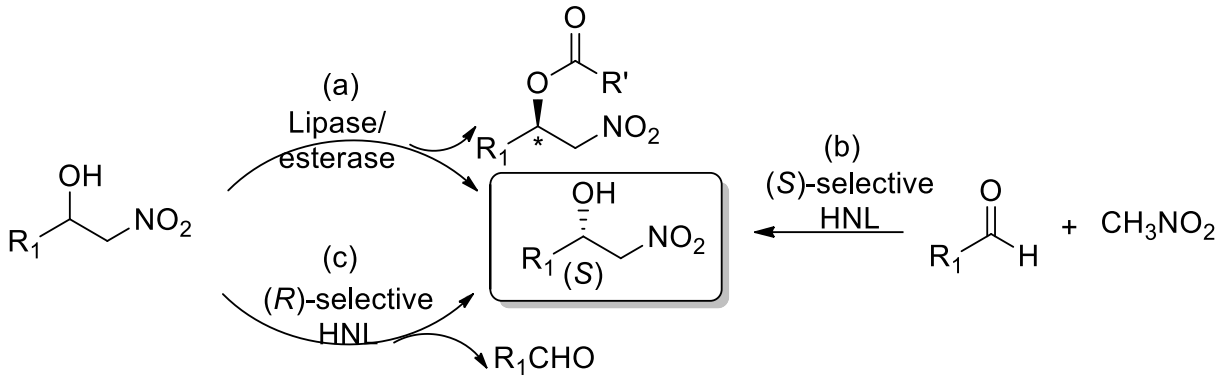
Chapter 3

Immobilized *At*HNL catalysed retro-Henry reaction in the preparation of (*S*)-β-nitroalcohols

3.1. Introduction

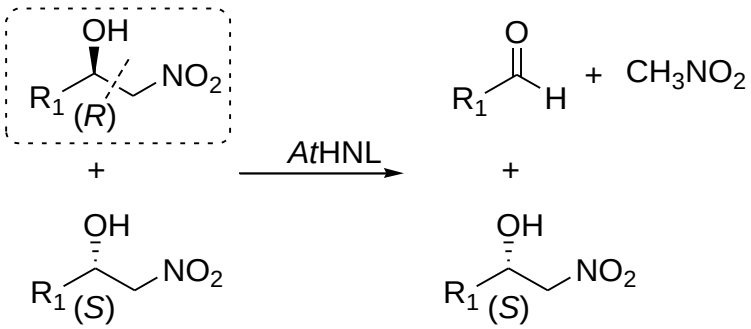
Biocatalytic syntheses of chiral molecules is gaining great importance in recent years due to their high selectivity e.g., chemo, regio, stereo and environmentally friendly nature of the catalysts. Enantiopure β-nitroalcohols are important chiral synthons being used in the synthesis of chiral β-amino alcohols, pharmaceuticals e.g., β-blockers, antibiotics such as chloramphenicol and several bioactive molecules.[1–4] Syntheses of chiral β-nitroalcohols using chemical catalyst include metals, transition metal complexes, and organo catalysts.[5–7] Major routes of biocatalytic synthesis of (*S*)-β-nitroalcohols are (a) lipase catalysed hydrolysis of an acyl derivative of racemic β-nitroalcohol or transesterification of a racemic β-nitroalcohol,[8–14] (b) hydroxynitrile lyase (HNL) catalysed C-C bond formation between electrophilic aldehyde and a nucleophilic nitromethane,[15,16] and (c) HNL catalysed retro-Henry reaction (**Scheme 3.1**).[17] The lipase/esterase catalysed biocatalytic synthesis of chiral β-nitroalcohols is a kinetic resolution which is limited by (i) long reaction time, (ii) maximum 50% theoretical yield, and (iii) involves deprotection of acyl derivatives of the corresponding products in case of acylated (*S*)-β-nitroalcohols. HNL catalysed synthesis of chiral β-nitroalcohols is considered as efficient biocatalytic methods than their counter biocatalytic approaches.



Scheme 3.1: Biocatalytic routes for synthesis of (*S*)-β-nitro alcohols

HNLs are mostly found in plants. Their natural function is to catalyze the cyanogenesis of α-cyanohydrins. It is a process by which plants cleave a cyanohydrin, a hydrolysis product of plant secondary metabolites e.g., cyanogen glycosides[18] and use the resulted toxic HCN for their defense mechanism. Synthetic efficacy of HNLs has been largely proved in the preparation of several chiral cyanohydrins.[19–24] A few HNLs catalyze the synthesis of Henry products by stereoselective nitromethane addition to an aldehyde.[15,16,25,26] *Hevea brasiliensis* HNL (*Hb*HNL) is the first and only HNL reported so far to catalyze the synthesis of (*S*)-β-nitroalcohols from its corresponding aldehydes, a promiscuous enzymatic activity. *Hb*HNL catalysed synthesis of (*S*)-β-nitroalcohols however suffers with long reaction time (48 h). Further, the enzyme shows moderate enantioselectivity i.e., maximum 92% *ee* of (*S*)-2-nitro-1-phenyl ethanol (NPE) at pH 7.0 could be produced.[15,16] Improve in *% ee* of the (*S*)-β-nitroalcohols by using low pH buffer i.e., 5.5 has shown decrease in yield of the products. Subsequently *Arabidopsis thaliana* HNL (*At*HNL), an (*R*)-selective α/β hydrolase fold HNL, was reported to catalyze the synthesis of (*R*)-β-nitroalcohols from corresponding aldehydes.[25] Metal-dependent bacterial HNLs with a cupin fold, *Acidobacterium capsulatum* (*Ac*HNL) and *Granulicella tundricula* (*Gt*HNL) were reported to catalyze (*R*)-selective nitroaldol reaction.[26] Engineered variants of *Ac*HNL and *Gt*HNL were exploited in the stereoselective synthesis of four different (*R*)-β-nitroalcohols in high conversion and ee. Recently we have demonstrated

retro-Henry reaction approach and synthesized ten different (*S*)-β-nitroalcohols using *At*HNL in up to 99% *ee* and *E* up to 84 [17] (**Scheme 3.2**).



Scheme 3.2: *At*HNL catalysed retro-Henry reaction in the enantioselective cleavage of racemic β-nitroalcohols

HNL catalysed retro-Henry reaction has multiple advantages despite of its theoretical 50% yield. For example, an HNL could produce enantiopure β-nitroalcohols of complementary stereo preference (**Scheme 3.2**). The substrate preference of a HNL could be exploited in the retro-Henry method to prepare a series of opposite stereoisomers of β-nitroalcohols, as compared to the stereopreference of the HNL in the Henry reaction. The catalytic efficiency of a HNL for its retro-Henry reaction is higher than the reverse, i.e. Henry reaction. Kinetic studies of *Hb*HNL catalysed Henry reaction reported k_{cat} of 0.013 s^{-1} while 0.16 s^{-1} towards retro-Henry reaction.[27] The catalytic efficiency and specific activity of *At*HNL for nitroaldol synthesis by retro-Henry reaction is much higher than the similar reaction by *Hb*HNL.[17] *Hb*HNL on retro-Henry reaction produces (*R*)-β-nitroalcohols, so when we compare toward the synthesis of (*S*)-β-nitroalcohols, it is the comparison between *Hb*HNL catalysed Henry reaction vs. *At*HNL catalysed retro-Henry reaction. For a HNL, retro-Henry reaction catalysis is a favorable process than Henry reaction.[27] Although retro-Henry reaction is an efficient biocatalytic approach, but the earlier study of *At*HNL catalysed retro-Henry reaction used 0.2 µmol of purified enzyme to convert 4 µmol racemic NPE with 47% conversion, which

correspond to a total turnover number (TTN) of only 9.4.[17] Apart from low TTN, and use of purified enzyme, the above process is limited by (i) poor enzymatic stability, (ii) short storability of the enzyme and (iii) lack of enzyme re-usability. Enzyme immobilization is a probable solution to overcome these limitations. Earlier studies of *At*HNL immobilized on celite R-633 has been reported in the synthesis of enantiopure mandelonitrile.[28] We intended to immobilize *At*HNL and explore its catalytic potential in the retro-Henry reaction catalysed preparation of (*S*)-β-nitroalcohols.

3.2. Objectives

1) To optimise the process of immobilization, prepare and characterize the immobilized *At*HNL
2) To investigate the substrate scope of immobilized *At*HNL in retro-Henry reaction catalysed preparation of (*S*)-β-nitroalcohols

3.3. Materials and Methods

3.3.1. Chemicals and materials

Chemicals such as aldehydes, nitromethane and mandelonitrile, were purchased from Sigma Aldrich, AVRA, SRL, and Alfa-Aesar and used without purification. HPLC grade solvents were purchased from RANKEM, Molychem, FINAR, and SRL, India. Biochemicals such as buffers, culture media and ampicillin were procured from HiMedia laboratory Pvt. Ltd, India. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from BR-BIOCHEM Pvt. Ltd, India. The recombinant *At*HNL gene in pET28a plasmid was synthesized and obtained from Abgenex Pvt. Ltd, India. Celite 545 was purchased from SRL chemicals, India.

3.3.2. Expression and purification of *At*HNL

Expression and purification of *At*HNL was performed as described in section **2.3.2** of **Chapter 2**.

3.3.3. Mandelonitrile and NPE cleavage assay of *At*HNL

HNL activity of both immobilized and pure *At*HNL was measured by monitoring the continuous formation of benzaldehyde from racemic mandelonitrile or NPE, at 280 nm in a spectrophotometer. Activity was determined using racemic mandelonitrile and NPE as substrates, separately. In case of celite *At*HNL, the activity was measured using a discontinuous assay. The reaction was performed in a 1.5 mL micro centrifuge tube. The reaction mixture consisted of citrate phosphate buffer, (pH 5.5, 50 mM, 160 µL) celite *At*HNL (20 µL, suspended in 20 mM KPB pH 7.0) and mandelonitrile (67 mM, 20 µL) or NPE (pH 3.15, 20 mM, 20 µL) solution prepared in citrate phosphate buffer (pH 3.15, 5 mm). The reaction mixture was incubated at room temperature for 1 min and centrifuged at 10000 rpm for 2 minutes to stop the reaction. A 100 µL of aliquot from the reaction mixture was taken in a 96 well plate, and analyzed in a spectrophotometer for increase in absorbance due to benzaldehyde. The activity was calculated by using the molar extinction coefficient of benzaldehyde (1376 $M^{-1}cm^{-1}$). One unit of HNL activity is defined as the amount of enzyme required to produce 1 µmol of benzaldehyde from mandelonitrile or NPE per minute. All measurements were performed in triplicates. The control experiment had all the reaction components except that the celite *At*HNL was replaced by celite alone. HNL activity of the free enzyme (pure *At*HNL) was measured using mandelonitrile and NPE as substrates separately, by performing continuous assay.[29] Briefly, the assay was performed in a 96 well plate. The reaction mixture contained citrate phosphate buffer (pH 5.5, 50 mM, 160 µL), purified *At*HNL (1 mg mL^{-1}, 20 µL) and mandelonitrile solution (67 mM, 20 µL) or NPE (20 mM, 20 µL) prepared in citrate phosphate buffer (pH 3.15, 5 mM). The control experiment had all the

reaction components except that enzyme was replaced by its corresponding buffer. All measurements were performed in triplicates. Control experiment results were subtracted from the enzymatic reaction results.

3.3.4. Synthesis of racemic β-nitroalcohols

Racemic β-nitroalcohols were synthesized using a literature known method.[30] Briefly, a mixture of aldehyde (1 mmol), nitroalkane (10 mmol), and $Ba(OH)_2$ (5 mol%) in H_2O (3 mL) was taken in a round-bottomed flask and stirred at room temperature for 30 to 60 min. The reaction mixture was extracted three times with ethyl acetate. The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The obtained residue was purified by silica gel column chromatography (eluents: hexanes/ethyl acetate), followed by ^{1}H and ^{13}C NMR characterization.

3.3.5. Preparation of celite immobilized *At*HNL by optimisation of enzyme to celite ratio

Celite *At*HNL was prepared by following the protocol of Torrelo *et al.*[31] Purified *At*HNL (15 mg/mL) and celite®545 were mixed in different w/w ratios i.e., 1:5 to 1:30. For proper mixing, the mixture was placed on a rocker for 50-60 min at 4 ℃. Each ratio of enzyme celite mixture was freeze dried in a lyophilizer for 16 h at –90 ℃. Each mixture was washed with 20 mM potassium phosphate buffer (KPB) pH 7 and centrifuged for 2 min at 2000 rpm 4 ℃ to remove the unbound protein. The preparation was dried in a lyophilizer overnight at –90 ℃ to produce the celite immobilized *At*HNL. The activity of celite-*At*HNL of each ratio (1:5 to 1:30) was determined using mandelonitrile and NPE separately.

Using optimised *At*HNL: celite i.e., 1:20, the celite-*At*HNL was prepared in a preparative scale as per the above described protocol. The prepared celite *At*HNL contained 0.038 mg of *At*HNL/mg celite (or 1 mg of *At*HNL/26 mg of celite) was stored at 4 ℃. Mass of celite carrier used was 1320 mg.

3.3.6. Effect of different pH on celite-*At*HNL catalysed retro-Henry reaction

A reaction mixture containing 27 units of celite-*At*HNL in 0.35 mL of 50 mM citrate phosphate buffer (CPB) of varied pH ranging from 4.0 to 7.0, 1.5 mM NPE and 0.65 mL of toluene (65% v/v) was shaken at 1200 rpm at 30 ℃ in an incubator shaker for 6 h. A 50 µL of aliquot from the organic layer was taken and added to 150 µL of hexane/2-propanol = 9:1, centrifuged at 15000*g* at 4 ℃ for 5 min. A 20 µL 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, 11.6, and 13.1 min respectively.

3.3.7. Effect of different organic solvents on celite-*At*HNL catalysed retro-Henry reaction

A set of reaction mixtures containing 27 units of celite-*At*HNL in 0.35 mL of 50 mM CPB of pH 6.0, 1.5 mM NPE, and 0.65 mL of an organic solvent (65% v/v) were prepared. Separate reactions were carried out with different organic solvents, such as diethyl ether, *tert*-butyl methyl ether (TBME), disopropyl ether, *n*-butyl acetate, and toluene. Another similar reaction was done without using any organic solvent (only buffer). Each reaction mixture was shaken at 1200 rpm, 30 ℃ in an incubator shaker for 6 h. Aliquot extraction and HPLC analysis were carried out according to the method described above.

3.3.8. Effect of different v/v ratios of *n*-butyl acetate on celite-*At*HNL catalysed retro-Henry reaction

Each reaction mixture contained 27 units of celite-*At*HNL in 0.35 mL of 50 mM CPB of pH 6.0, 1.5 mM NPE, and *n*-butyl acetate whose volume was varied from 35 to 85% v/v for a set of experiments. Reaction mixture was shaken at 1200 rpm at 30 ℃ in an incubator shaker for 6 h. Aliquot extraction and HPLC analysis were done as per the methods described above.

3.3.9. Effect of different substrate concentration on celite-*At*HNL catalysed retro-Henry reaction

Optimisation of substrate concentration in the celite-*At*HNL catalysed retro-Henry reaction was carried out by varying the racemic NPE concentration from 1.5 to 25 mM. The reaction mixture contained 27 units of celite-*At*HNL in 0.45 mL of 50 mM CPB of pH 6.0, 0.55 mL of *n*-butyl acetate (55% v/v) along with varied concentration of racemic NPE. Reaction mixture was shaken at 1200 rpm at 30 ℃ in an incubator shaker for 6 h. Aliquot extraction and HPLC analysis were performed as described above.

3.3.10. Celite-*At*HNL catalysed synthesis of (*S*)-β-nitroalcohols by retro-Henry reaction under optimised conditions and their chiral analysis

A typical biocatalysis with celite-*At*HNL used 27 units of the immobilized enzyme in 0.45 mL of 50 mM CPB of pH 6.0, 6.0 mM racemic β-nitro alcohol, and 0.55 mL of *n*-butyl acetate (55% v/v). The mixture was shaken at 1200 rpm at 30 ℃ in an incubator shaker. The reaction was monitored at different time intervals. A 50 µL of aliquot from the organic layer was added to 150 µL of hexane/2-propanol = 9:1, centrifuged at 15000*g* at 4 ℃ for 5 min. A 20 µL of the organic layer was analyzed in a HPLC using Chiralpak® IB chiral column under the HPLC conditions described above.

3.3.11. Reusability of celite-*At*HNL in the retro-Henry reaction

Reusability of celite-*At*HNL in the retro-Henry reaction was determined by repeated use of the immobilized enzyme in the optimised reaction conditions as described above. After completion of the reaction, the reaction mixture was centrifuged to stop the reaction, organic and aqueous fractions were decanted. The celite-*At*HNL remained in the pellet of the centrifuge tube was washed with *n*-butyl acetate twice and used in the subsequent cycle. In the next cycle of reaction, fresh reactants were added as mentioned in the optimised conditions above. Likewise, four cycles of reaction were performed. After the completion of each cycle 50 µL of aliquot

from the organic layer was added to 150 µL of hexane/2-propanol = 9:1, centrifuged at 15000*g* at 4 °C for 5 min. A 20 µL of the organic layer was analyzed in a HPLC using Chiralpak® IB chiral column under the HPLC conditions described above.

3.3.12. Characterization of celite-*At*HNL by scanning electron microscope

Scanning electron microscope images of celite-*At*HNL were taken by using ESEM - Environmental Scanning Electron Microscope, model - XL30, make - FEI/Philips. Celite-*At*HNL dried powder was placed on a carbon tape which is sticked to the metal stub. Prior to SEM imaging, sample was coated with gold nanoparticles under vacuum. Micrographs were obtained for each sample at 1000X magnification.

3.4. Results

3.4.1. NMR characterization of racemic β-nitroalcohols

Numbers after the product name corresponds to the serial number in **tables 3.1 and 3.3**

NMR characterization of **2-nitro-1-phenylethanol 1**, **1-(3-methoxyphenyl)-2-nitroethanol 2, 1-(3-methylphenyl)-2-nitroethanol 3,** and **1-(4-Methylphenyl)-2-nitroethanol 6** and their corresponding spectra are presented in section **2.4.1** of **Chapter 2.**

1-(4-Fluorophenyl)-2-nitroethanol 4[32]

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.195-3.199 (1H, d, *J* = 2, Hz), 4.49-4.62 (2H, m), 5.454-5.473 (1H, d, *J* = 9.5 Hz), 7.087-7.122 (2H, t, *J* = 8.5 Hz), 7.387-7.417 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.3, 81.1, 115.8, 116.1, 127.7, 127.8,133.9, 134.0, 161.6, 164.1.

1-(3-Chlorophenyl)-2-nitroethanol 5[32]

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.17 (1H, brs), 4.50-4.60 (2H, m), 5.43-5.46 (1H, dd, *J* = 3.5, 9.5 Hz), 7.27-7.29 (1H, m), 7.34-7.35 (2H, m), 7.430-7.437(1H, m); ^{13}C NMR (120 MHz, $CDCl_3$) δ 70.2, 80.9, 124, 126.2, 129 (X2),130.3, 135, 140.1.

(*E*)-1-nitro-4-phenylbut-3-en-2-ol 7[32]

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.807 (1H, brs), 4.50-4.56 (2H, m), 5.05-5.09 (1H, m), 6.14-6.18 (1H, dd, *J* = 6.0, 15.5 Hz), 6.78-6.82 (1H, dd, *J* = 1.0, 15.5 Hz), 7.28-7.41(5H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 69.64, 79.9, 124.9, 126.7, 128.5, 128.7, 133.7, 135.5.

1-(4-Chlorophenyl)-2-nitroethanol 8[32]

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.04 (1H, brs), 4.48-4.61 (2H, m), 5.44-5.47 (1H, dd, *J* = 3.2, 9.2 Hz), 7.34-7.41 (4H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.3, 80.9, 127.3, 129.2, 134.8, 136.5

1-(4-(allyloxy)phenyl)-2-nitroethanol 9

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.871 (1H, s), 4.47-4.63 (4H, m), 5.30-5.33 (1H, m), 5.40-5.45 (2H, m), 6.02-6.10 (1H, m), 6.94-6.96 (2H, d, *J* = 8.5 Hz), 7.31-7.33 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 68.8, 70.6, 81.2, 115.1, 117.9, 127.2, 130.3, 132.9, 159.0.

1-(2-Chlorophenyl)-2-nitroethanol 10[32]

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.44-4.49 (1H, m), 4.66-4.70 (1H, dd, *J* = 2.5, 14 Hz), 5.84-5.87 (1H, m), 7.28-7.40 (3H, m), 7.66-7.68 (1H, dd, *J* = 1.5, 7.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 67.8, 79.3, 127.5, 127.6, 129.7, 129.2, 131.4,135.5

1-(3,5-Dimethoxyphenyl)-2-nitroethanol 11[32]

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.80 (6H, s), 4.48-4.61 (2H, m), 5.37-5.40 (1H, dd, *J* = 2.5, 9.5 Hz), 6.43-6.44 (1H, t, *J* = 2.5), 6.54-6.55 (2H, d, *J* = 2.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.4, 71.0, 81.2, 100.6, 103.8, 140.6, 161.2.

1-(3-Nitrophenyl)-2-nitroethanol 12[32]

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.80 (6H, s), 4.48-4.61 (2H, m), 5.37-5.40 (1H, dd, *J* = 2.5, 9.5 Hz), 6.43-6.44 (1H, t, *J* = 2.5), 6.54-6.55 (2H, d, *J* = 2.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.4, 71.0, 81.2, 100.6, 103.8, 140.6, 161.

^{1}H NMR and ^{13}C NMR spectra of racemic β-nitroalcohols

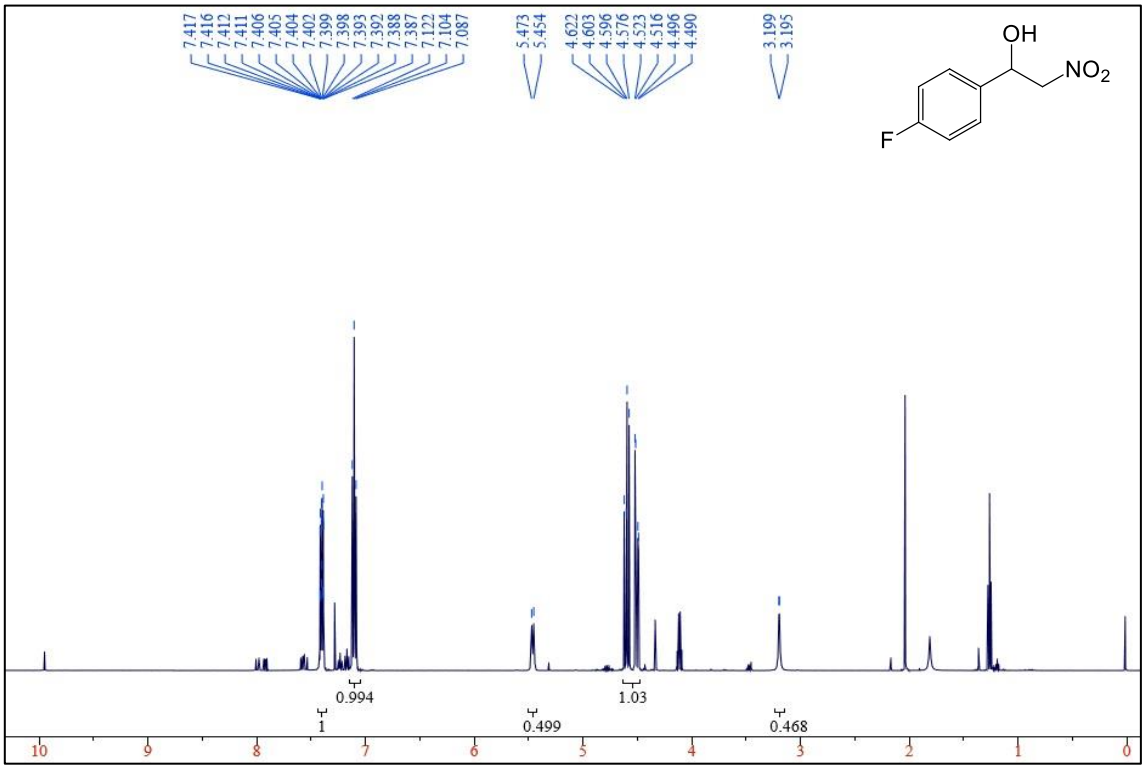


Figure 3.1.1: ^{1}H NMR spectrum of 1-(4-fluorophenyl)-2-nitroethanol

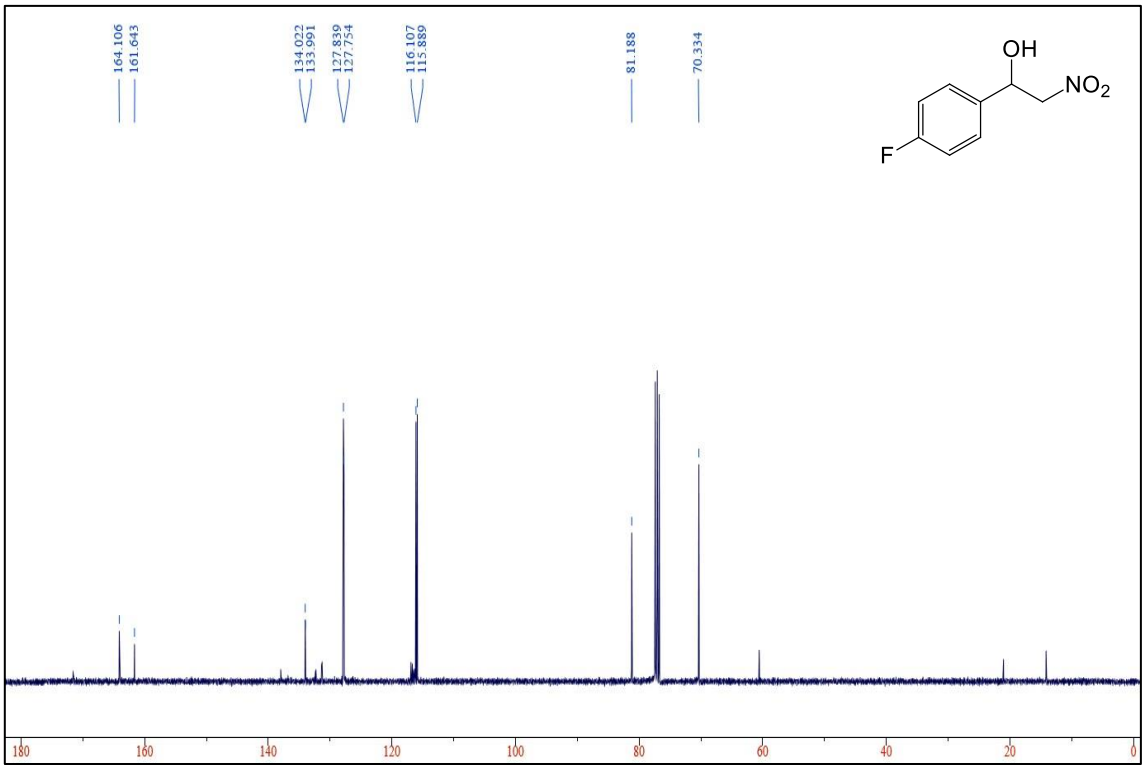


Figure 3.1.2: ^{13}C NMR spectrum of 1-(4-fluorophenyl)-2-nitroethanol

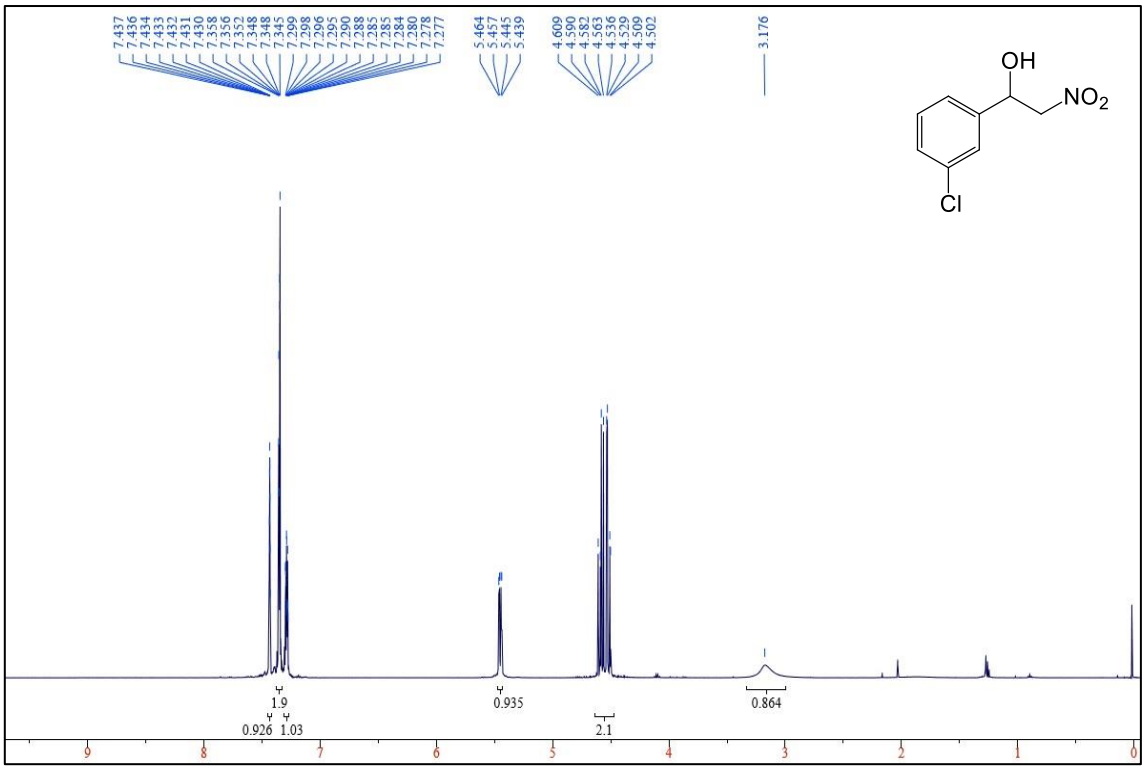


Figure 3.1.3: ^{1}H NMR spectrum of 1-(3-chlorophenyl)-2-nitroethanol

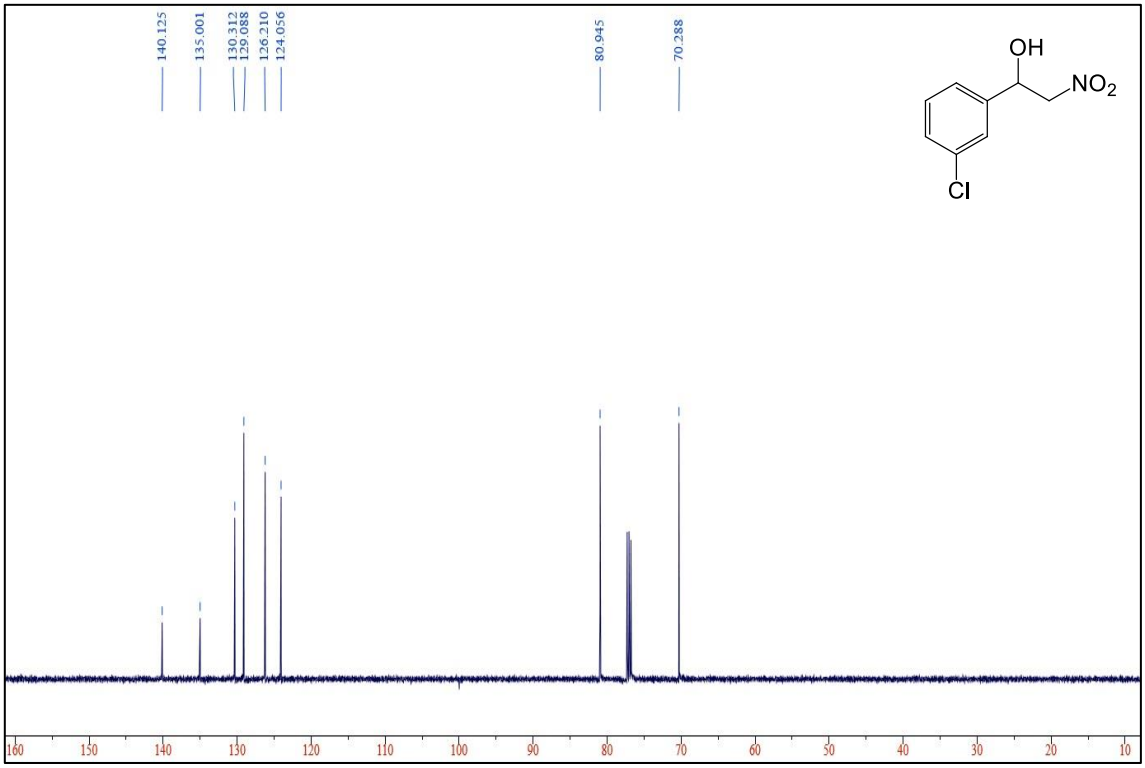


Figure 3.1.4: ^{13}C NMR spectrum of 1-(3-chlorophenyl)-2-nitroethanol

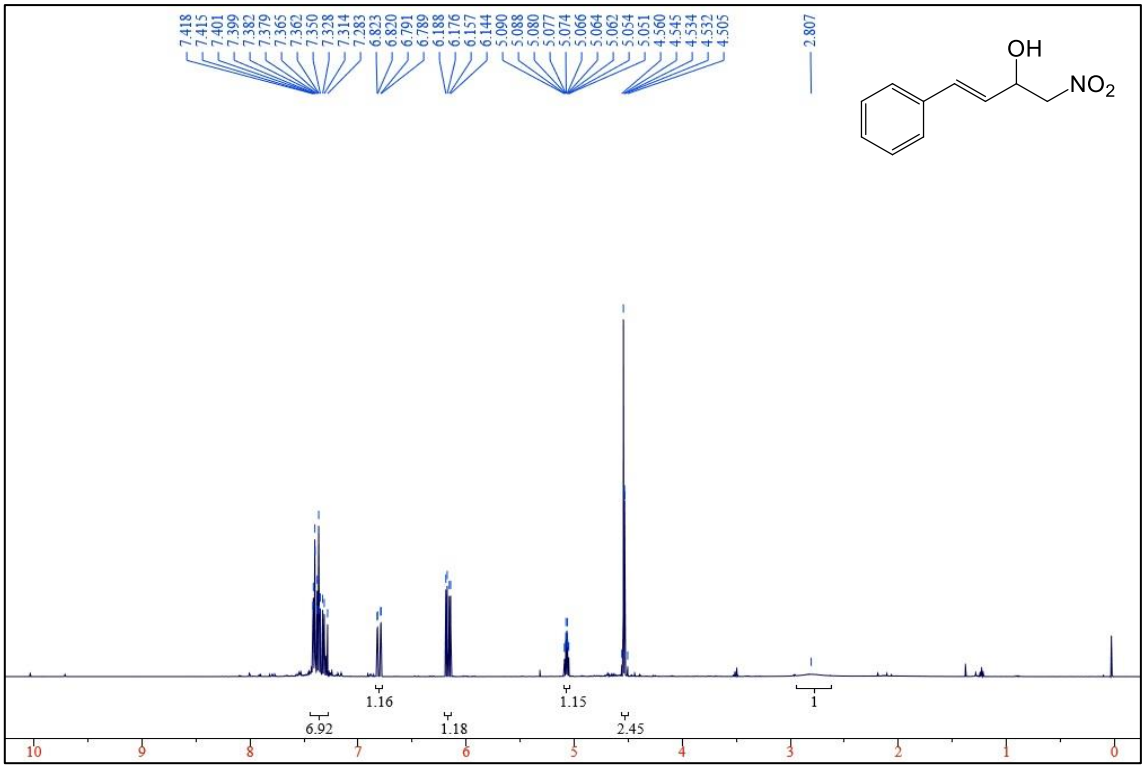


Figure 3.1.5: ^{1}H NMR spectrum of (*E*)-1-nitro-4-phenylbut-3-en-2-ol

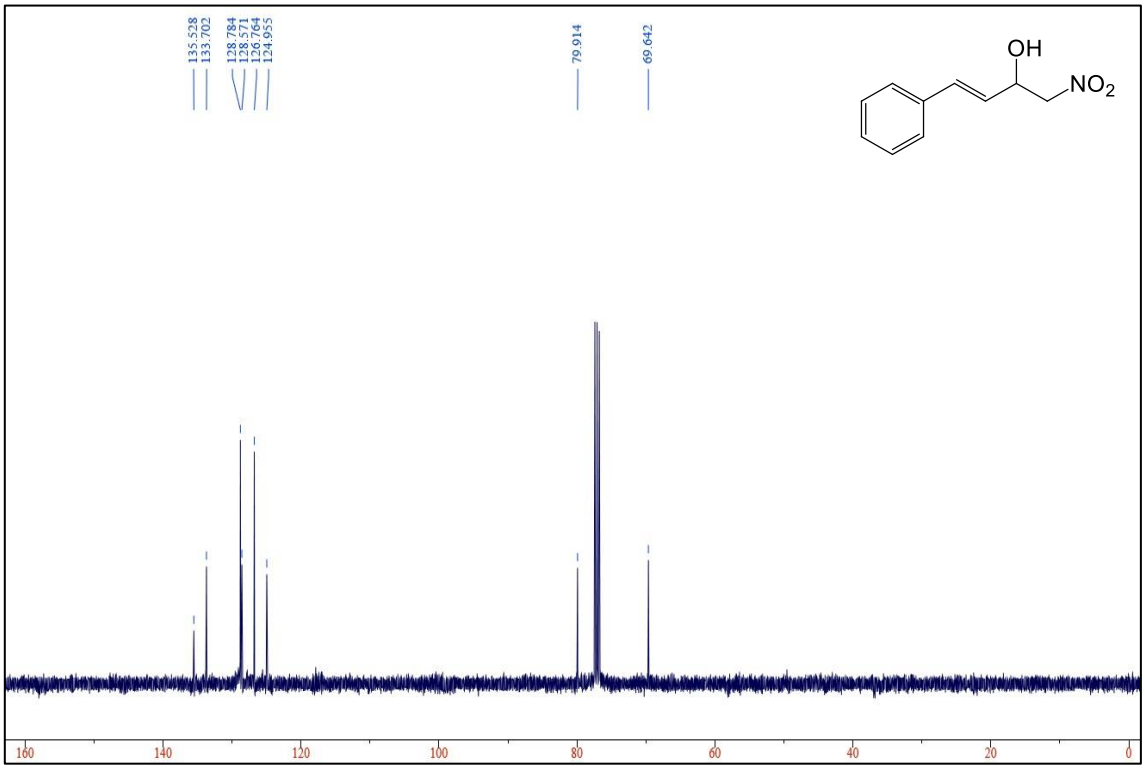


Figure 3.1.6: ^{13}C NMR spectrum of (*E*)-1-nitro-4-phenylbut-3-en-2-ol

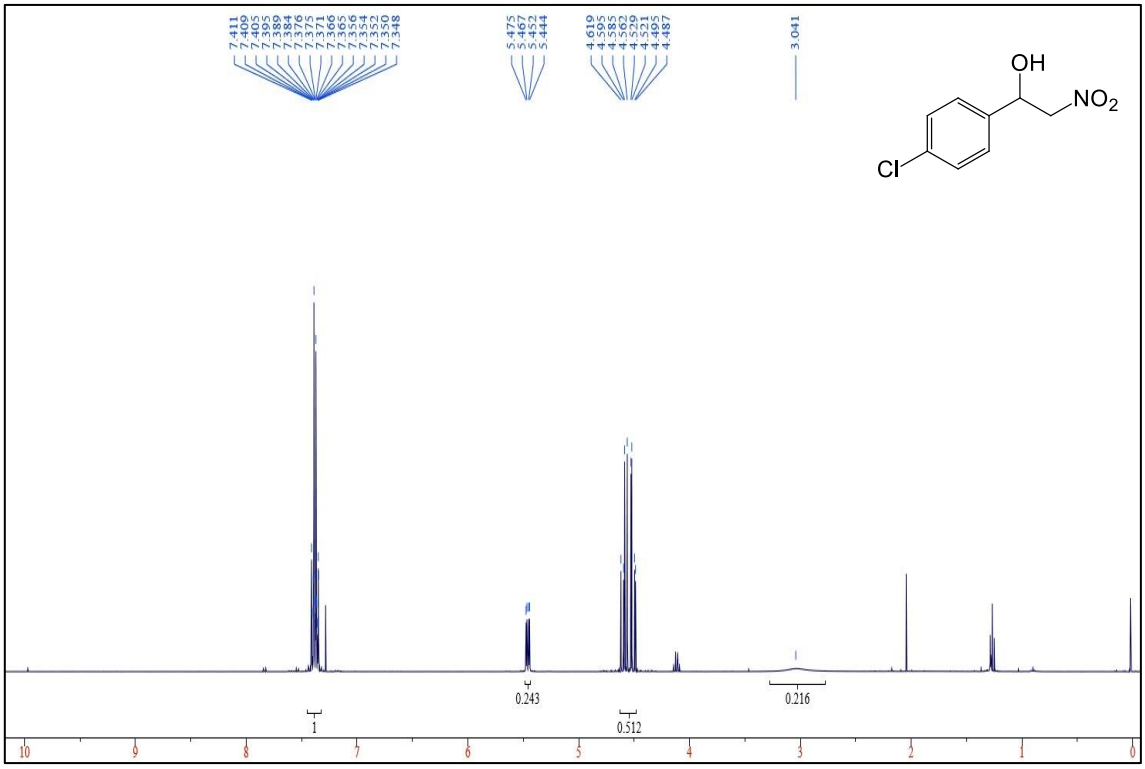


Figure 3.1.7: ^{1}H NMR spectrum of 1-(4-chlorophenyl)-2-nitroethanol

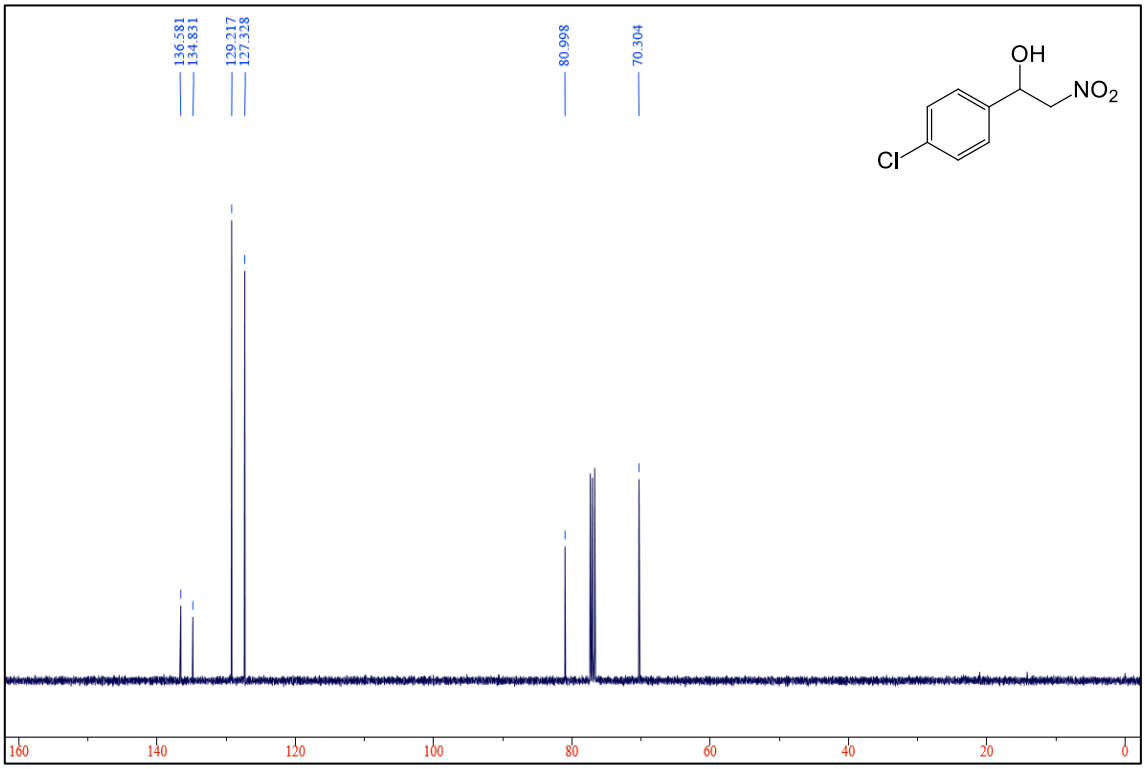


Figure 3.1.8: ^{13}C NMR spectrum of 1-(4-chlorophenyl)-2-nitroethanol

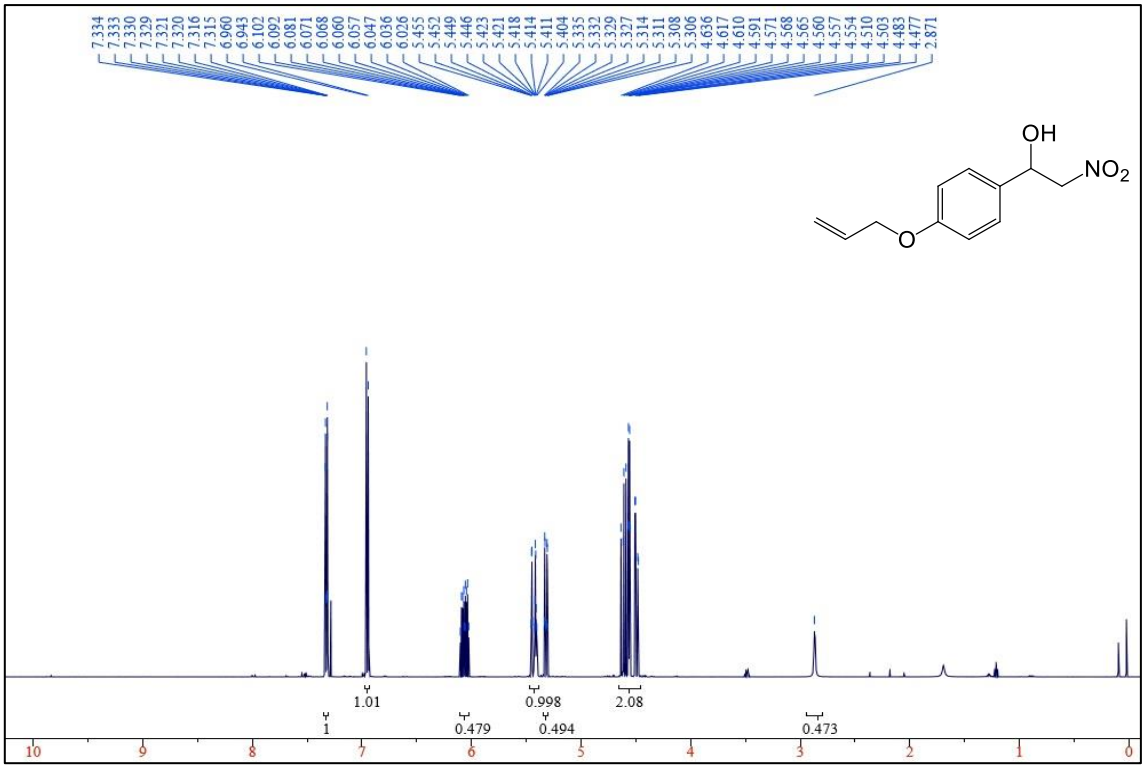


Figure 3.1.9: ^{1}H NMR spectrum of 1-(4-(allyloxy)phenyl)-2-nitroethanol

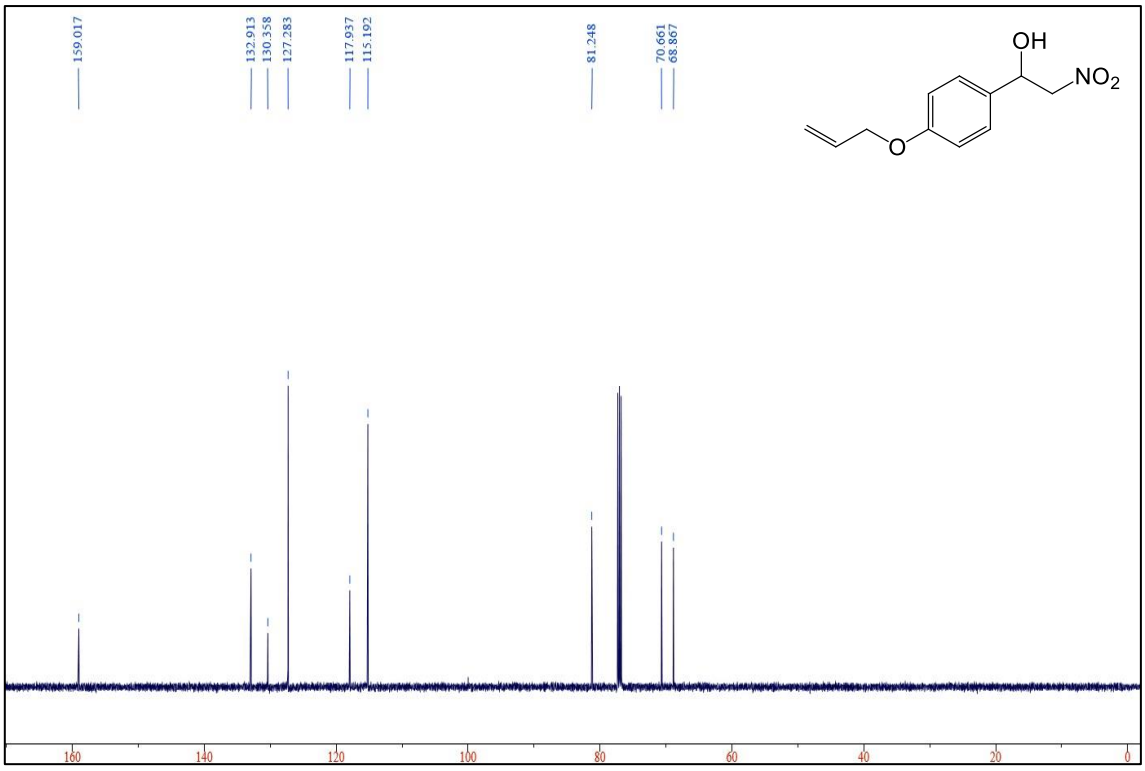


Figure 3.1.10: ^{13}C NMR spectrum of 1-(4-(allyloxy)phenyl)-2-nitroethanol

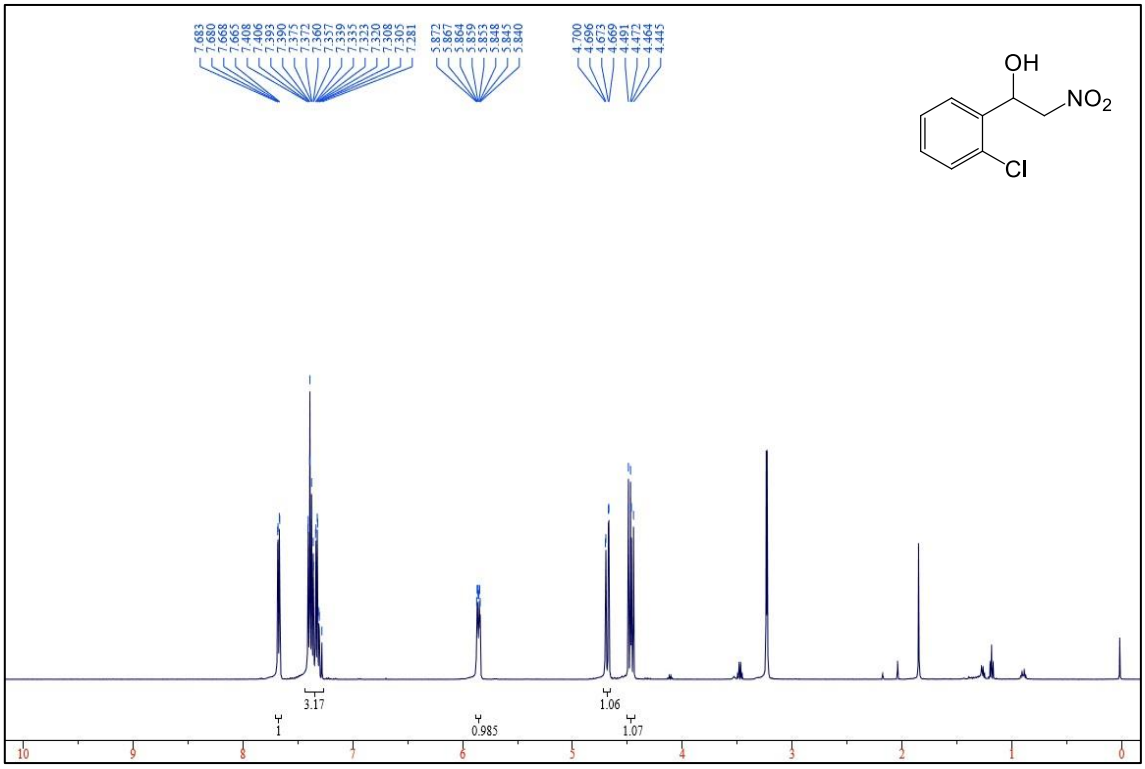


Figure 3.1.11: ^{1}H NMR spectrum of 1-(2-chlorophenyl)-2-nitroethanol

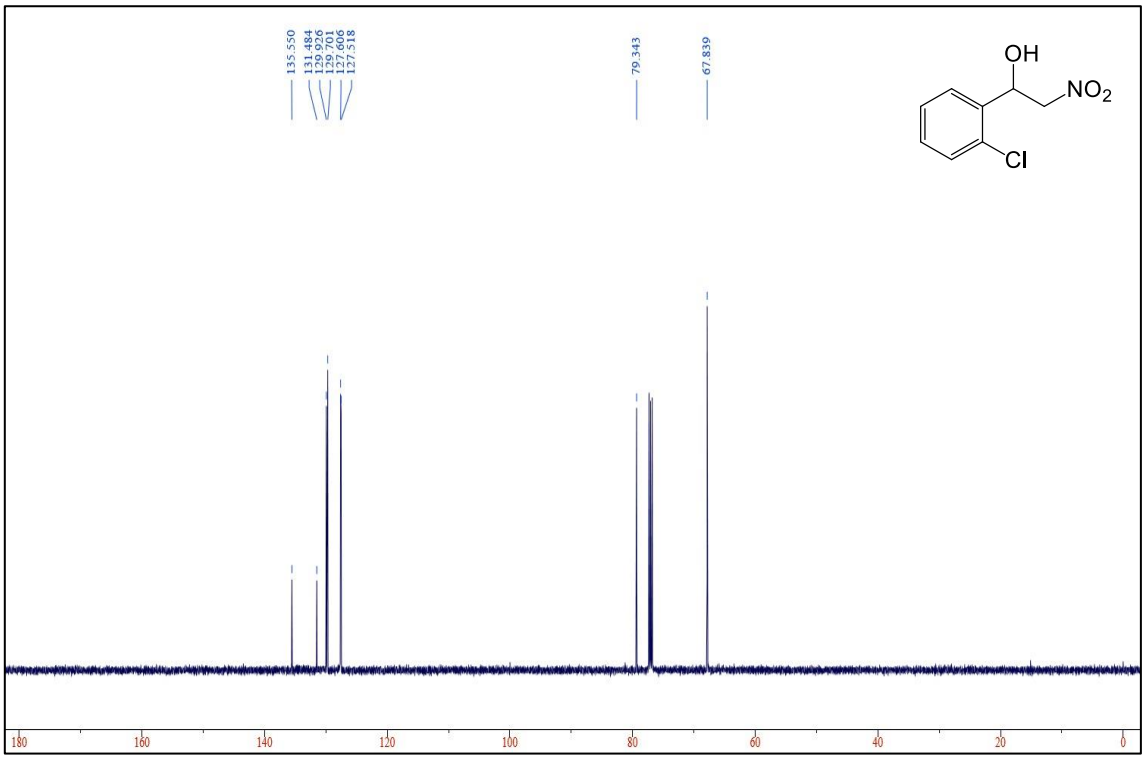


Figure 3.1.12: ^{13}C NMR spectrum of 1-(2-chlorophenyl)-2-nitroethanol

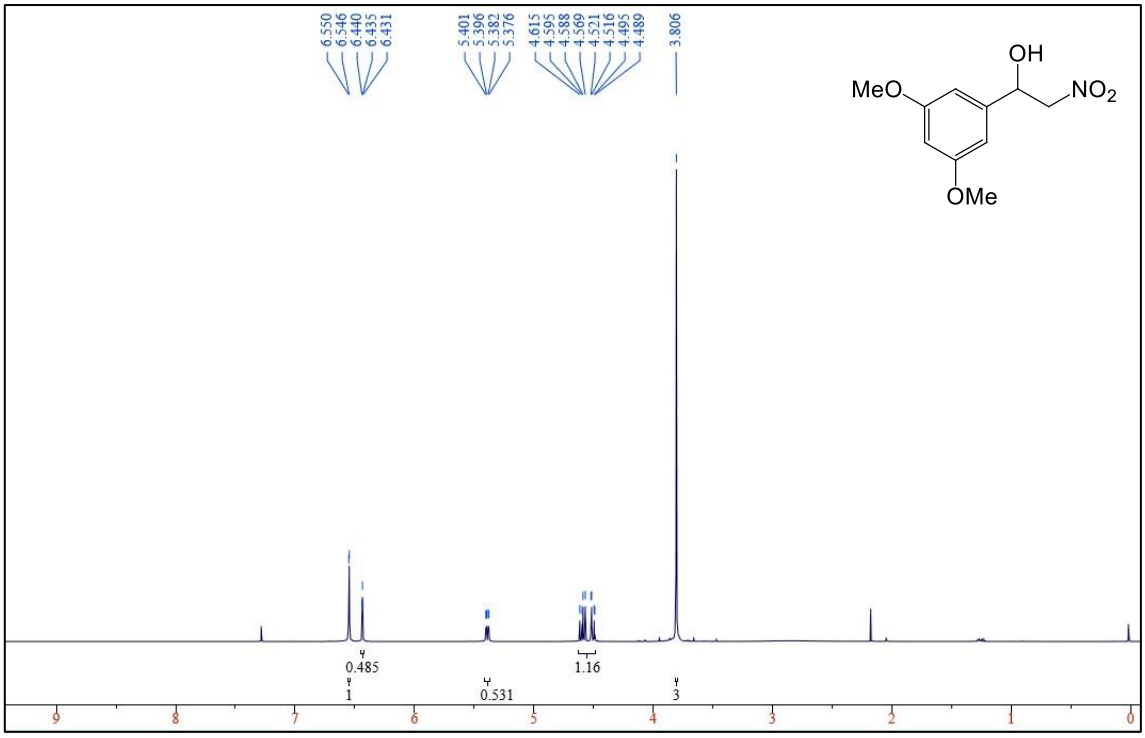


Figure 3.1.13: ^{1}H NMR spectrum of 1-(3,5-dimethoxyphenyl)-2-nitroethanol

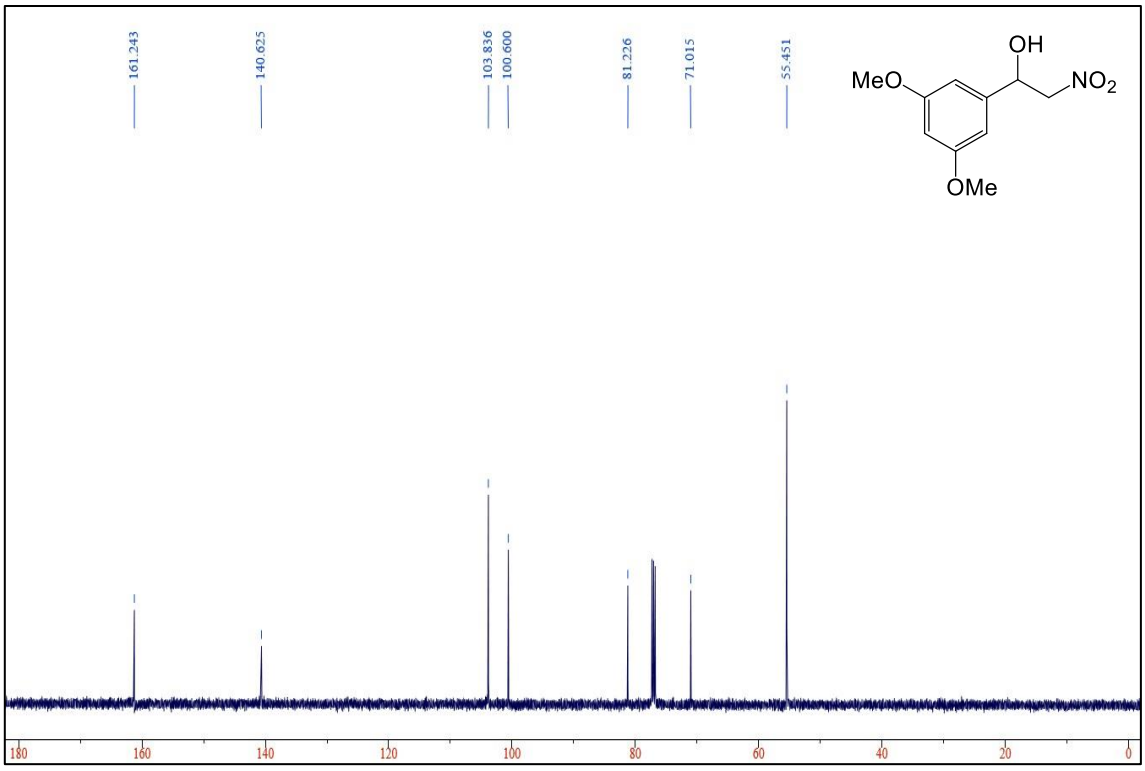


Figure 3.1.14: ^{13}C NMR spectrum of 1-(3,5-dimethoxyphenyl)-2-nitroethanol

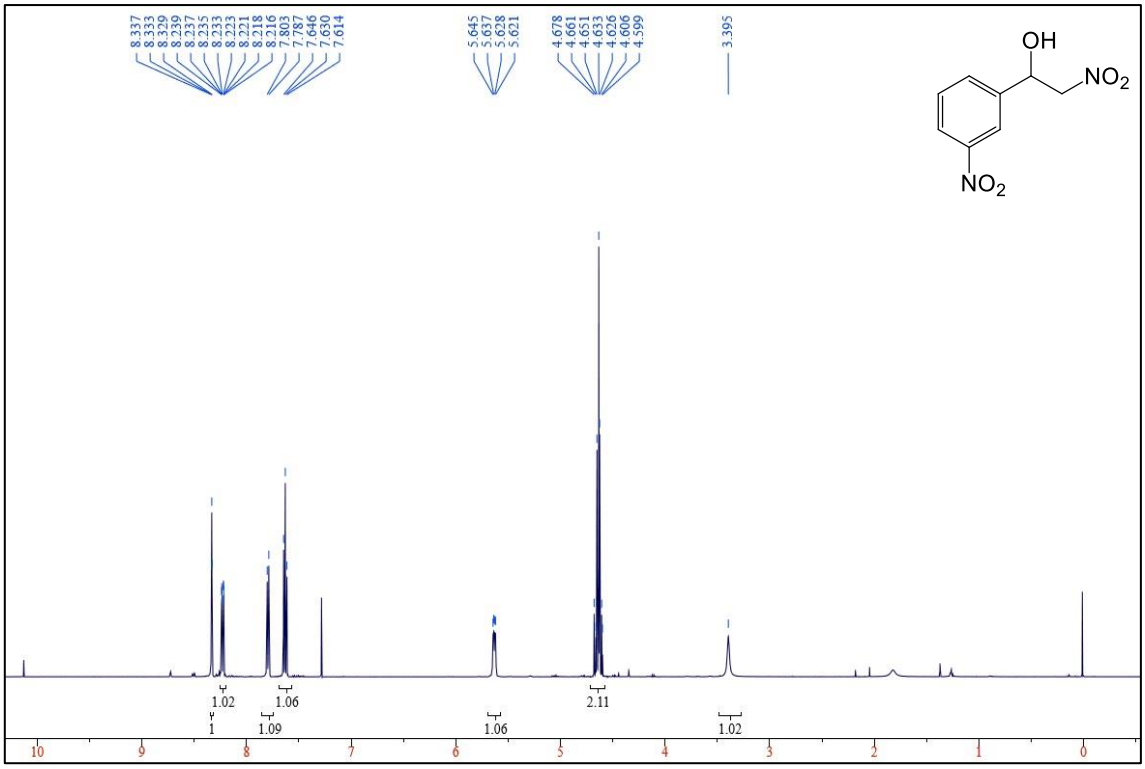


Figure 3.1.15: ^{1}H NMR spectrum of 1-(3-nitrophenyl)-2-nitroethanol

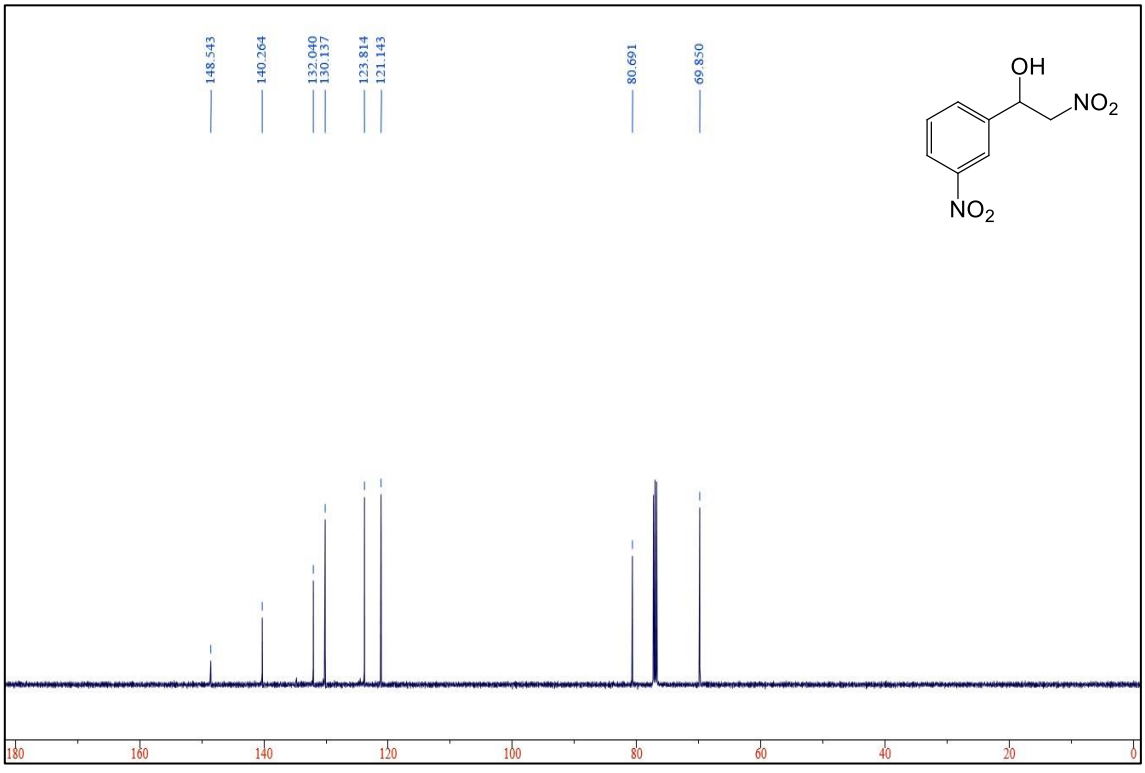


Figure 3.1.16: ^{13}C NMR spectrum of 1-(3-nitrophenyl)-2-nitroethanol

Table 3.1 represents the details such as retention time of aldehyde, individual enantiomers and the HPLC analysis conditions used for chiral resolution of twelve different racemic β-nitroalcohols that are included in this study.

S. No	Substrate	Retention time (min)*	*n*-hexane: 2-propanol (v/v)	Flow rate (mL/ min)
1	2-Nitro-1-phenylethanol	Benzaldehyde = 4.8 t_R = 11.4, t_S = 13.0	90:10	1
2	1-(3-Methoxyphenyl)-2-nitroethanol	3-Methoxybenzaldehyde = 5.3, t_R = 16.7, t_S = 20.2	90:10	1
3	1-(3-Methylphenyl)-2-nitroethanol	3-Methylbenzaldehyde = 4.6, t_R = 9.6, t_S = 10.6	90:10	1
4	1-(4-Fluorophenyl)-2-nitroethanol	4-Fluorobenzaldehdye = 4.9, t_R = 10.5, t_S = 11.8.	90:10	1
5	1-(3-Chlorophenyl)-2-nitroethanol	3-Chlorobenzaldehyde = 4.9, t_R = 11.0, t_S = 12.8.	90:10	1
6	1-(4-Methylphenyl)-2-nitroethanol	4-Methylbenzaldehyde = 4.7, t_R = 10.9, t_S = 12.9	90:10	1
7	(*E*)-1-nitro-4-phenylbut-3-en-2-ol	Cinnamaldehyde = 7.1, t_S = 26.8, t_R = 29.6.	90:10	1
8	1-(4-Chlorophenyl)-2-nitroethanol	4-Chlorobenzaldehyde = 5.1, t_R = 11.6, t_S = 13.7.	90:10	1
9	1-(4-(Allyloxy)phenyl)-2-nitroethanol	4-Allyloxybenzaldehyde = 5.9, t_R = 13.3, t_S = 15.1.	90:10	1
10	1-(2-Chlorophenyl)-2-nitroethanol	2-Chlorobenzaldehyde = 6.2, t_R = 38.1, t_S = 40.3.	97.5:2.5	0.8
11	1-(3,5-Dimethoxyphenyl)-2-nitroethanol	3,5-Dimethoxybenzaldehyde = 5.7, t_S = 16.8, t_R = 21.1.	90:10	1
12	1-(3-Nitrophenyl)-2-nitroethanol	3-Nitrobenzaldehyde = 10.1, t_S = 18.2, t_R = 22.0.	90:10	1

Column: Chiralpak® IB chiral column. Solvent: *n*-hexane: 2-propanol; absorbance: 210 nm. *t_R and t_S are retention time of corresponding (*R*)-β-nitroalcohol, (*S*)-β-nitroalcohol respectively.

3.4.2. Celite-*At*HNL preparation

3.4.2.1. Optimisation of enzyme to celite ratio

In order to find out the ratio of *At*HNL: celite at which the immobilized enzyme show optimum activity, different ratios of *At*HNL: celite (w/w) ranging from 1:5 to 1:30 were tested towards racemic mandelonitrile and NPE cleavage activity (**Figure 3.2**). Specific activities of the resulted celite-*At*HNLs were obtained and subsequently residual activity i.e., % recovery of enzyme activity after immobilization, was determined. At 1:5, the recovery of celite-*At*HNL activity with respect to NPE and mandelonitrile cleavage was 19 and 12% respectively. With increase in celite ratio, gradual increase in recovery of activity was observed, except an unusual trend at 1:15 ratio. At 1:20, maximum activity recovery was observed towards both NPE and mandelonitrile i.e. 24 and 14% respectively. The residual activity was almost maintained even in case of 1:30 but with 1:25, a decrease in activity recovery was witnessed. Therefore 1:20 enzyme:celite ratio was choosen as optimum ratio in the preparation of celite-*At*HNL.

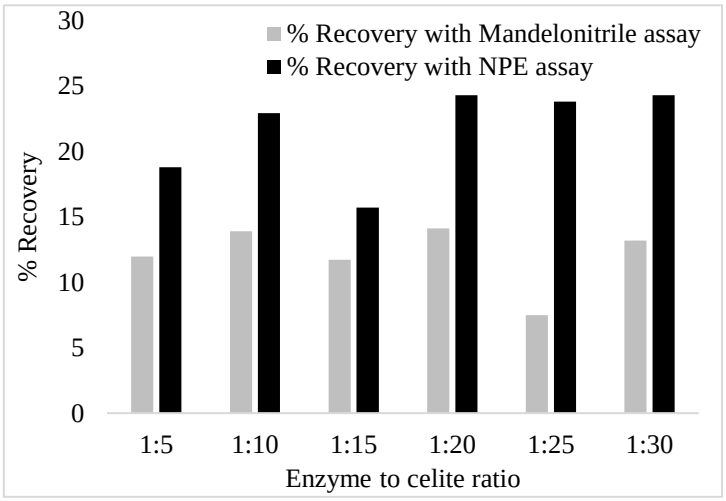


Figure 3.2: Effect of different *At*HNL: celite towards mandelonitrile and NPE cleavage activity

3.4.2.2. Characterization of celite-*At*HNL by scanning electron microscopy (SEM)

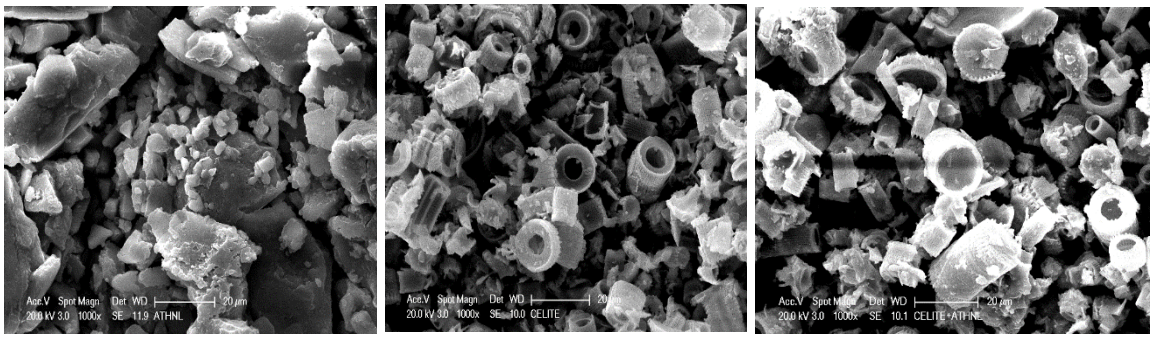


Figure 3.3: Scanning Electron Microscope images of free *At*HNL, celite alone and celite-*At*HNL from left to right.

The photomicrographs of free *At*HNL, celite alone and celite-*At*HNL were observed by SEM as presented in **Figure 3.3**. Free *At*HNL powder was visualized as small flakes, celite particles were observed as spherical shaped bodies and celite-*At*HNL was observed as spherical shaped bodies coated with enzyme. The celite-*At*HNL exhibited considerable alterations in morphology.

3.4.2.3. Calculation of efficiency, yield and recovery of celite-*At*HNL

Immobilization yield, the immobilization efficiency and the activity recovery are the three terms most often used to determine the success of an enzyme immobilization process. From the preparative scale experiment, these parameters were calculated by using the following formulae and given in **Table 3.2**.[33] All activities were based on racemic mandelonitrile cleavage assay.

Immobilized activity = total starting activity – supernatant activity = 1386 – 292.6 = 1093.4 U

$$\text{Efficiency (\%)} = \frac{\text{observed activity}}{\text{immobilized activity}} * 100 \;=\; (475.2/\,1093.4) * 100 \;=\; 43.4\%$$

$$\text{Yield (\%)} \;= \frac{\text{immobilized activity}}{\text{starting activity}} * 100 \;=\; (1093.4\,/\,1386) * 100 \;=\; 78.8\%$$

$$\text{Recovery (\%)} = \frac{\text{observed activity}}{\text{starting activity}} * 100 \; = \; (475.2/\,1386) * 100 \; = \; 34.28\%$$

Table 3.2 Total activity of purified *At*HNL, celite-*At*HNL and the supernatant

Enzyme or supernatant	Total amount (mg)	Specific activity (U/mg)	Total activity (U)
Purified enzyme	66	21	1386
Celite-*At*HNL	44	10.8	475.2
Supernatant	22	13.3	292.6

3.4.3. Optimisation of biocatalytic parameters for celite-*At*HNL catalysed retro-Henry reaction in the synthesis of enantiopure 2-nitro-1-phenylethanol

3.4.3.1. Effect of different pH

In order to find out the optimum pH of the celite-*At*HNL catalysed retro-Henry reaction, the biocatalysis was carried out at different pH ranging from 4.0 to 7.0. Highest *% ee* i.e., 99.6% of (*S*)-NPE was obtained at pH 6.0, hence it was considered as optimum pH (**Figure 3.4**).

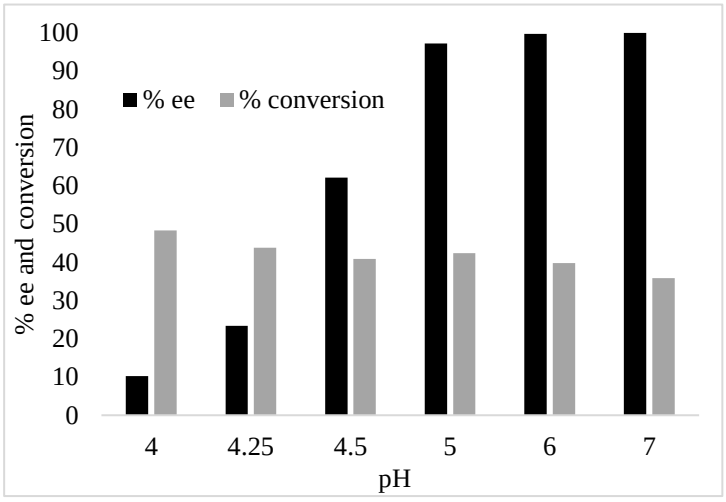


Figure 3.4: Effect of different pH on celite-*At*HNL catalysed retro-Henry reaction

3.4.3.2. Effect of organic solvent

HNL biocatalysis are often carried out in biphasic systems. It is because use of organic solvent helps in minimization of spontaneous formation of racemic products, e.g., cyanohydrin and β-nitroalcohol. Further it helps in easy product extraction. We have selected five different organic solvents that are commonly used in HNL biocatalysis, and studied their effect on celite-*At*HNL catalysed retro-Henry reaction. **Figure 3.5** represents the *% ee* of (*S*)-NPE, and conversion of the different biotransformations containing diverse organic solvents in the biphasic system.

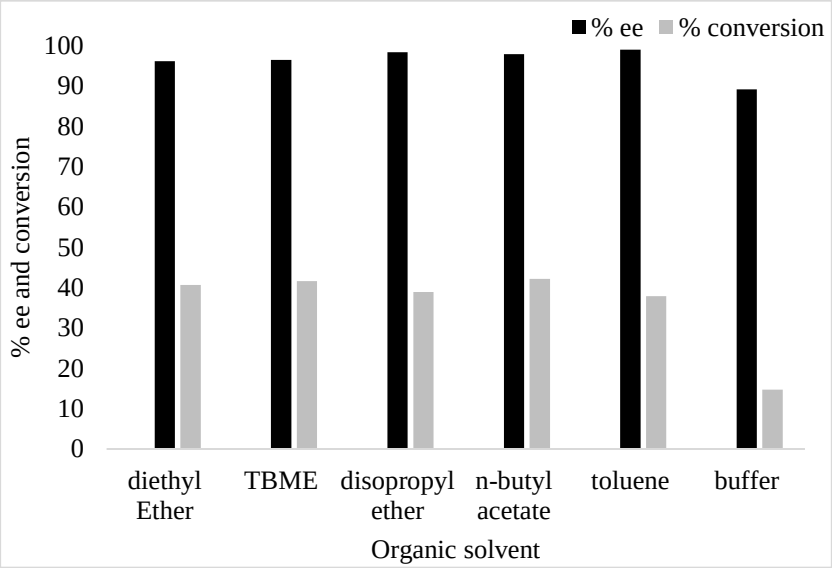


Figure 3.5: Effect of different organic solvents on celite-*At*HNL catalysed retro-Henry reaction

3.4.3.3. Effect of content of *n*-butyl acetate

Optimisation of *n*BuOAc content in the reaction mixture is necessary because excess of organic solvent may denature the enzyme while minimum amount may not provide the biphasic emulsion. We have varied the content of % *n*BuOAc from 35 to 85 (v/v) of the total reaction volume (**Figure 3.6**). Greater than 90*% ee* of (*S*)-NPE was observed in the range of 35-75% of *n*BuOAc. Highest conversion and *% ee* was obtained in case of 55% *n*BuOAc, while it

decreased beyond 75%. In the range of 35 to 75% of *n*BuOAc the *% ee* of product was almost similar.

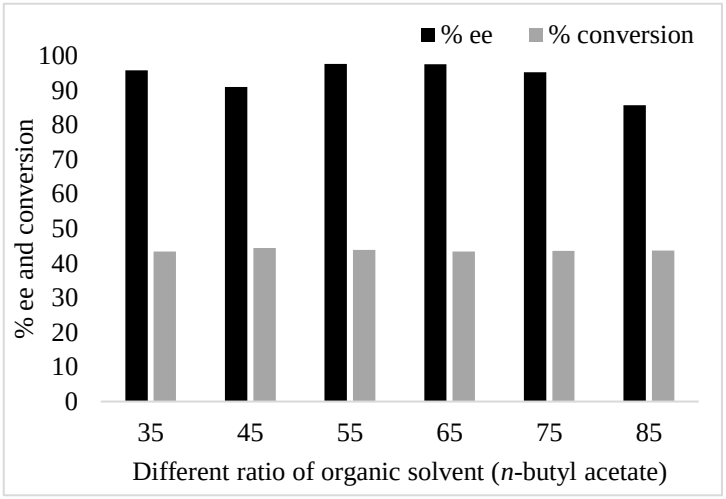


Figure 3.6: Effect of content of *n*BuOAc on celite-*At*HNL catalysed retro-Henry reaction

3.4.3.4. Effect of different substrate concentration

In order to find out the maximum substrate concentration that can be used in the celite-*At*HNL catalysed retro-Henry reaction, we have varied the racemic NPE concentration from 1.5 to 15 mM. Highest *% ee* i.e., 99.6 of (*S*)-NPE was obtained in case of 1.5 mM racemic NPE (**Figure 3.7**).

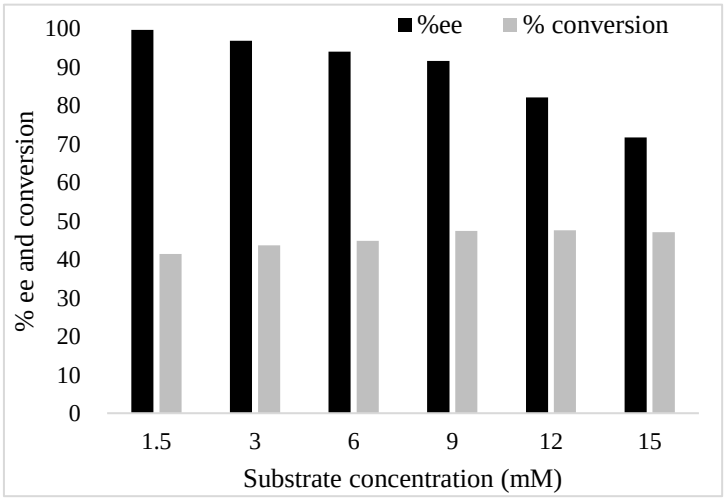


Figure 3.7: Effect of rac NPE concentration on celite-*At*HNL catalysed retro-Henry reaction

3.4.3.5. Reusability of celite-*At*HNL

Reusability is an important property of an immobilized enzyme. We have studied the reusability of celite-*At*HNL for four consecutive cycles (**Figure 3.8**). We observed decrease in *% ee* of (*S*)-NPE in second cycle onwards although the % conversion remained unaffected.

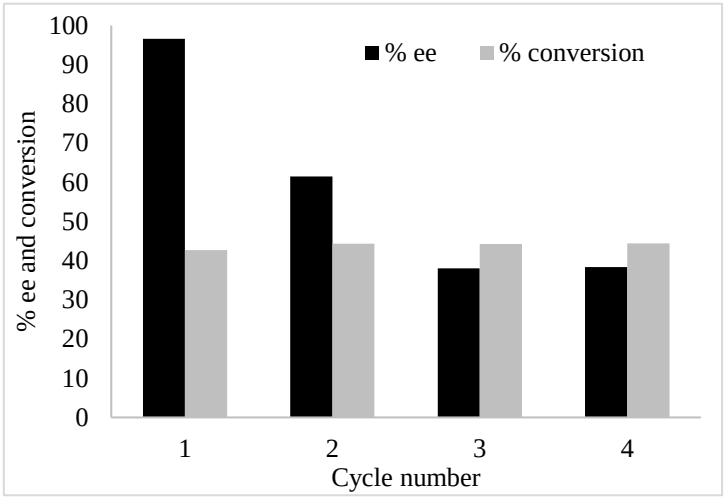
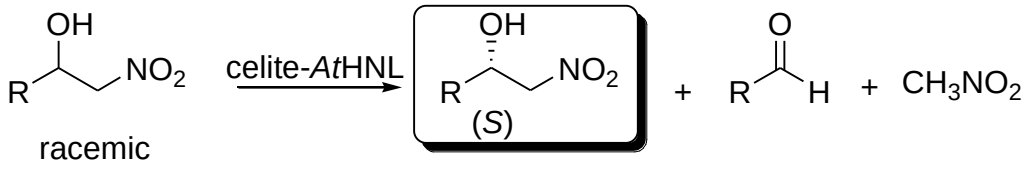


Figure 3.8: Recyclability of celite-*At*HNL in the retro-Henry reaction

3.4.3.6. Preparation of (*S*)-β-nitroalcohols



Scheme 3.3: *At*HNL catalysed retro-Henry reaction in the synthesis of (*S*)-β-nitroalcohols

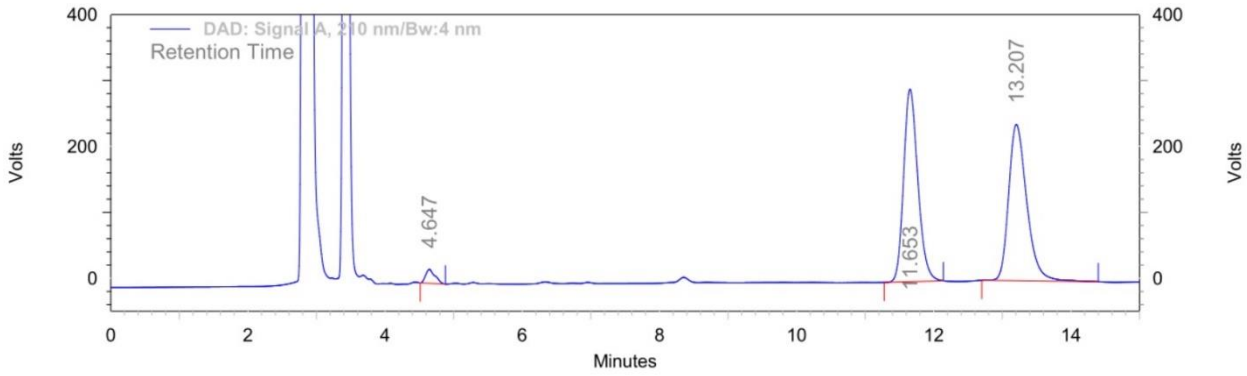
Table 3.3: Celite *At*HNL-catalysed enantioselective cleavage of different racemic β-nitroalcohols under optimised conditions

S. No.	R	Time (h)	*% ee*	% conversion
1	C_6H_5	9	97.3	48.0
2	3-$OMeC_6H_4$	21	97.4	46.3
3	3-MeC_6H_4	19	98.5	44.6
4	4-FC_6H_4	30	74.9	44.2
5	3-ClC_6H_4	20	72.7	54.7
6	4-MeC_6H_4	21	67.6	47.8
7	C_6H_5-CH=CH	9	5.2	46.9
8	4-ClC_6H_4	21	16.4	47.0
9	4-CH_2=CH-CH_2O-C_6H_4	19	16.3	53.9
10	2-ClC_6H_4	20	32.8	48.6
11	3,5-*di* $OMeC_6H_3$	20	48.4	56.3
12	3-$NO_2C_6H_4$	20	32.7	61.3

Celite-*At*HNL catalysed retro-Henry reaction is represented in **Scheme 3.3**. A dozen of chiral (*S*)-β-nitroalcohols were prepared using the optimised biocatalytic parameters (**Table 3.3**), while the reaction time has varied. In the case of NPE, celite-*At*HNL produced (*S*)-NPE in 97.3*% ee* and 48% conversion in 9 hours (**Figure 3.9**). Use of racemic 1-(3-methoxyphenyl)-2-nitro ethanol in the retro-Henry reaction has produced corresponding (*S*)-enantiomer in 97.4*% ee* and 46.3% conversion in 21 hours (**Figure 3.10**). Similarly with racemic 1-(3-methylphenyl)-2-nitroethanol, the product was found in 98.5*% ee* and 44.6% conversion in 19 hours (**Figure 3.11**). When electron withdrawing groups were used in the aromatic ring of the substrate, a decrease in *% ee* of product was observed. Retro-Henry reaction of racemic 1-(4-fluorophenyl)-2-nitroethanol by celite-*At*HNL took 30 hours to produce its corresponding (*S*)-enantiomer in 74.9*% ee* and 44.2% conversion (**Figure 3.12**). Racemic 1-(3-chlorophenyl)-2-nitroethanol as a substrate has resulted in 72.7*% ee* and 54.7% conversion in 20 h (**Figure 3.13**). Celite-*At*HNL in the retro-Henry reaction of racemic 1-(4-methylphenyl)-2-nitroethanol has shown 67.6*% ee* and 47.8% conversion to its (*S*)-enantiomer in 21 hours (**Figure 3.14**). Poor enantioselectivity, i.e., 5.2, 16.4, and 16.3*% ee* was observed in the case of racemic substrates (*E*)-1-nitro-4-phenylbut-3-en-2-ol, 1-(4-chlorophenyl)-2-nitroethanol, and 1-(4-

(allyloxy)phenyl)-2-nitroethanol respectively, in the retro-Henry reaction, with the conversions ranged between 46.9-53.9% (**Figures 3.15, 3.16, 3.17**). Three other substrates, racemic 1-(2-chlorophenyl)-2-nitroethanol, 1-(3,5-dimethoxyphenyl)-2-nitroethanol, and 1-(3-nitrophenyl)-2-nitroethanol, in the retro-Henry reaction of celite-*At*HNL has resulted in moderate enantioselectivity, i.e., 32.8, 48.4, and 32.7% *ee* respectively with 48.6-61.3% conversions (**Figure 3.18, 3.19, 3.20**).

Control:



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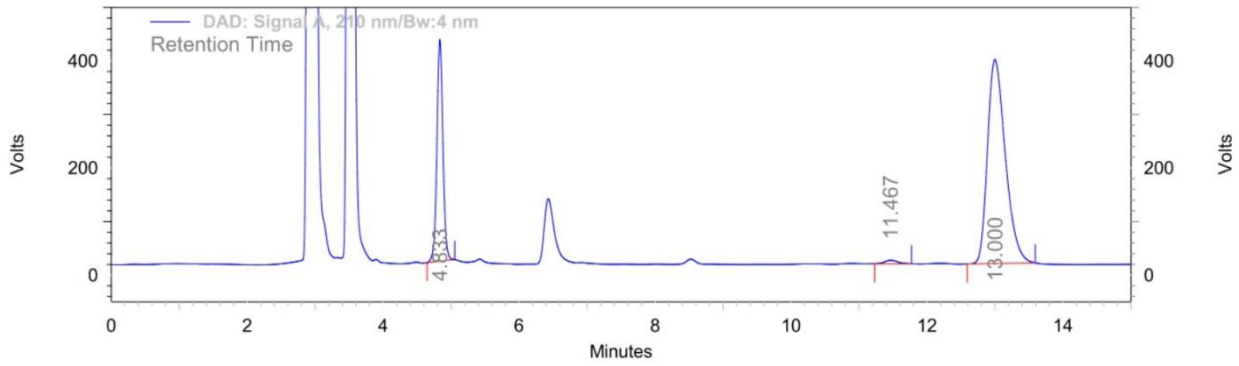
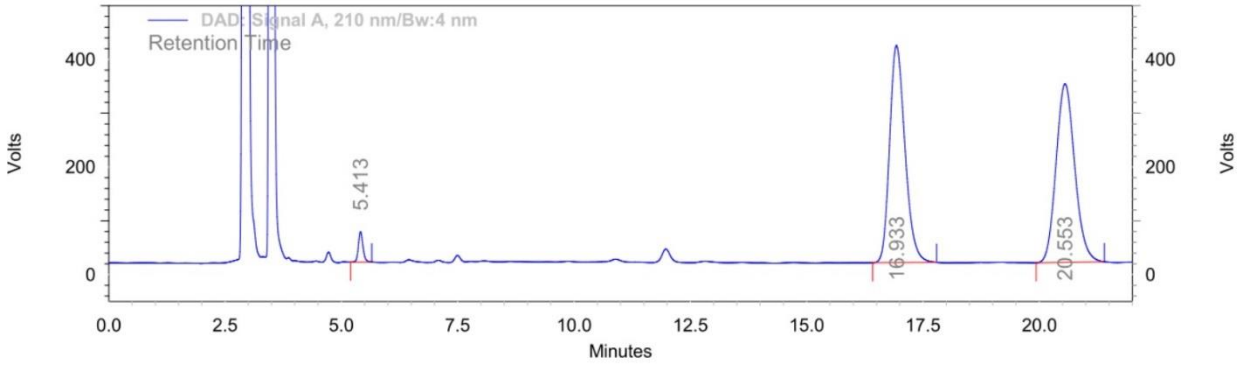


Figure 3.9: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 2-nitro-1-phenylethanol, (a) control having no enzyme, (b) reaction. Retention times, benzaldehyde: 4.8 min, (*R*)-2-nitro-1-phenylethanol: 11.4 min and (*S*)-2-nitro-1-phenylethanol: 13.0 min.

Control:



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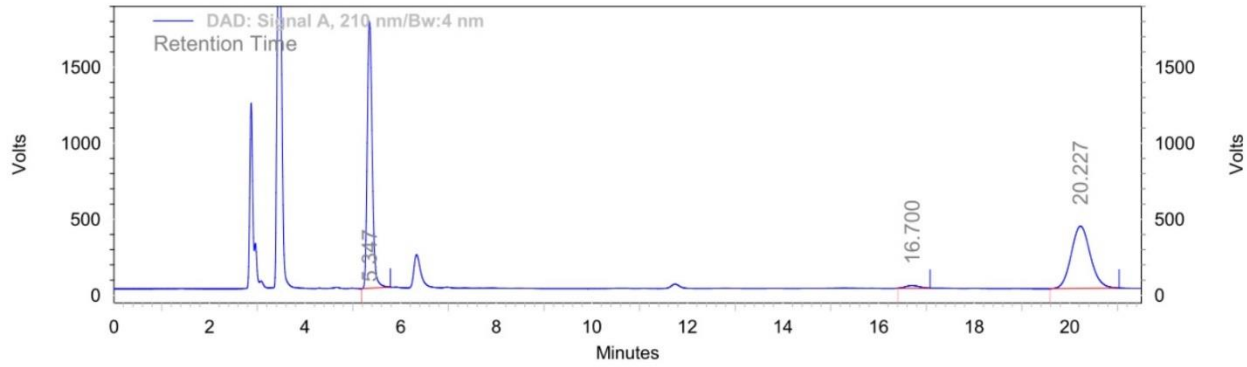

Figure 3.10: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(3-methoxyphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3-methoxybenzaldehyde: 5.3 min, (*R*)-1-(3-methoxyphenyl)-2-nitroethanol: 16.7 min and (*S*)-1-(3-methoxyphenyl)-2-nitroethanol: 20.2 min.

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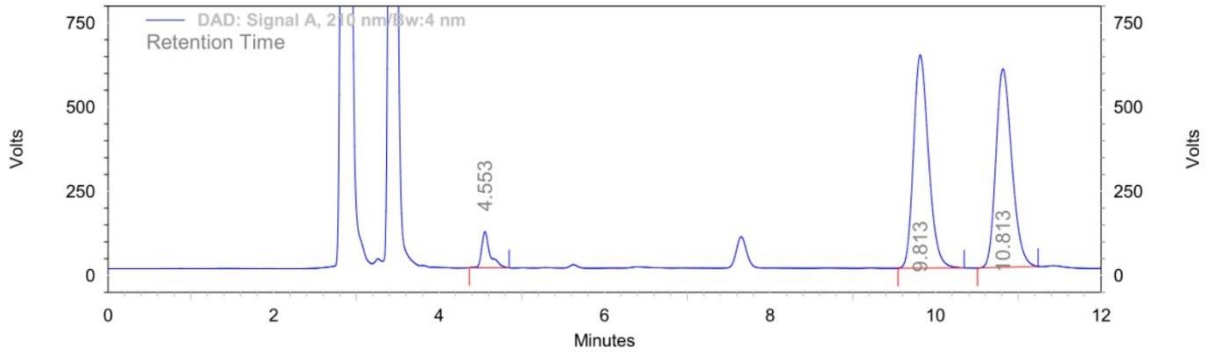

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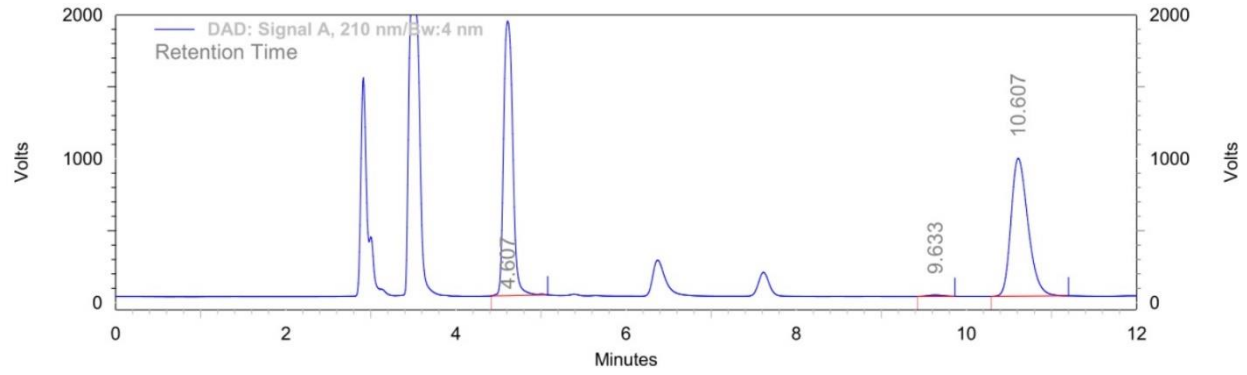

Figure 3.11: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(3-methylphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3-methylbenzaldehyde: 4.6 min, (*R*)-1-(3-methylphenyl)-2-nitroethanol: 9.6 min and (*S*)-1-(3-methylphenyl)-2-nitroethanol: 10.6 min.

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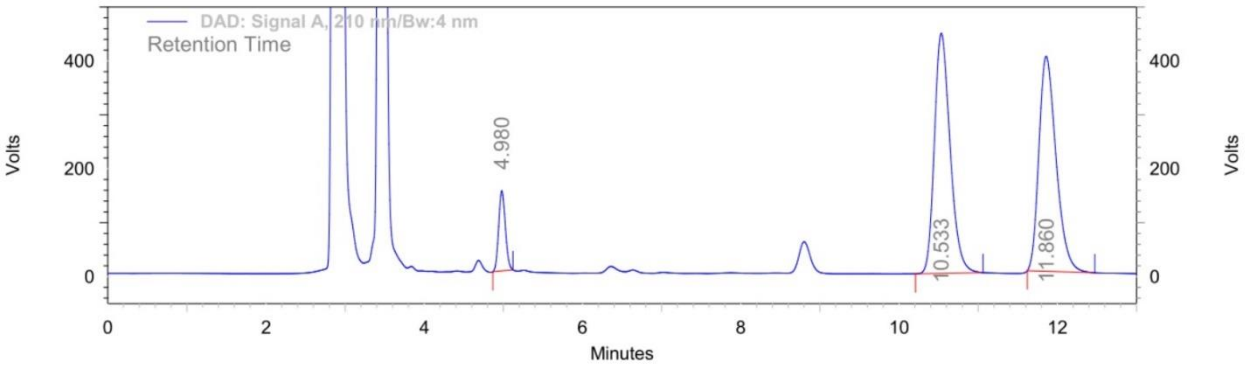

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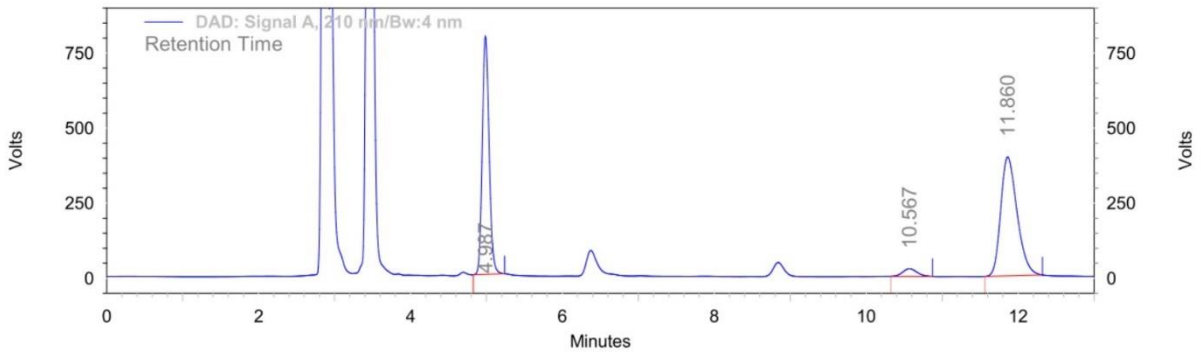

Figure 3.12: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(4-fluorophenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 4-flourobenzaldehyde: 4.9 min, (*R*)-1-(4-fluorophenyl)-2-nitroethanol: 10.5 min and (*S*)-1-(4-fluorophenyl)-2-nitroethanol: 11.8 min.

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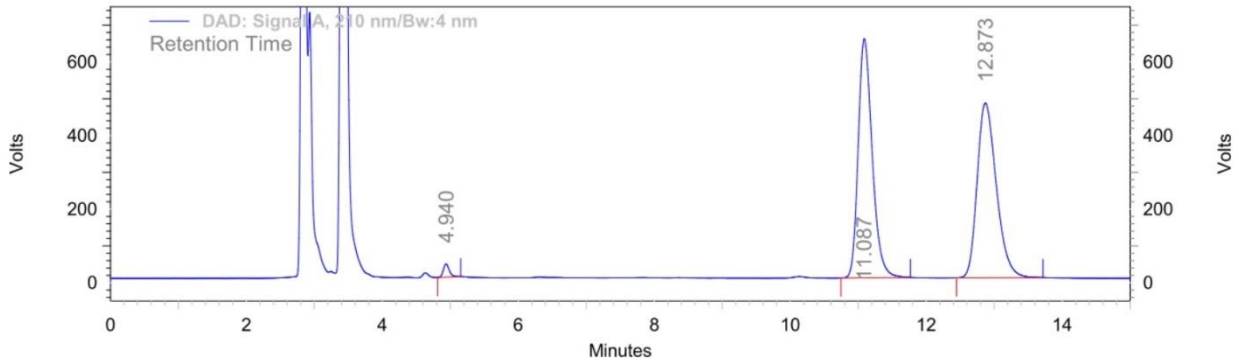

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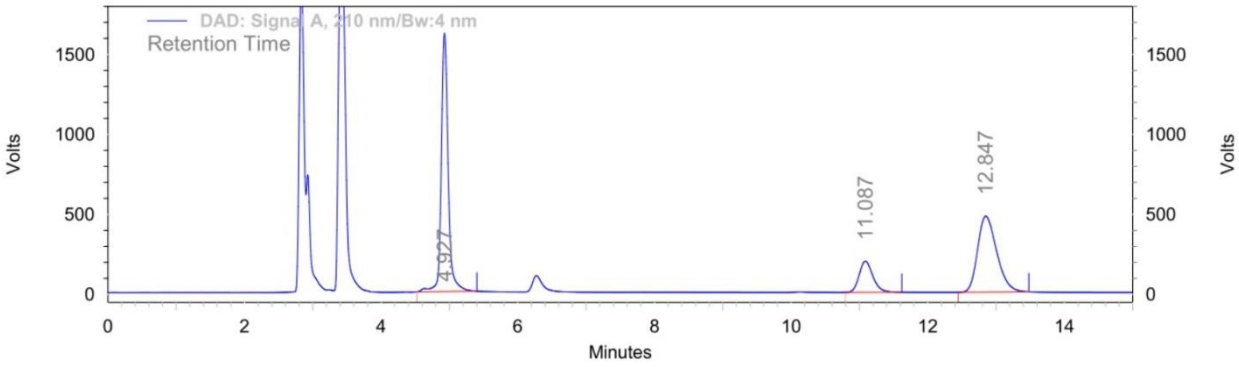
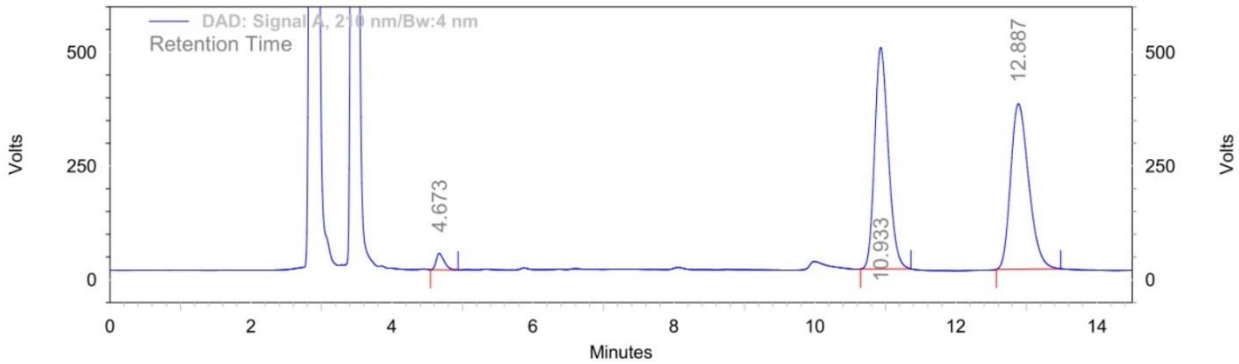


Figure 3.13: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(3-chlorophenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3-chlorobenzaldehyde: 4.9 min, (*R*)-1-(3-chlorophenyl)-2-nitroethanol: 11.0 min and (*S*)-1-(3-chlorophenyl)-2-nitroethanol: 12.8 min.

Control:



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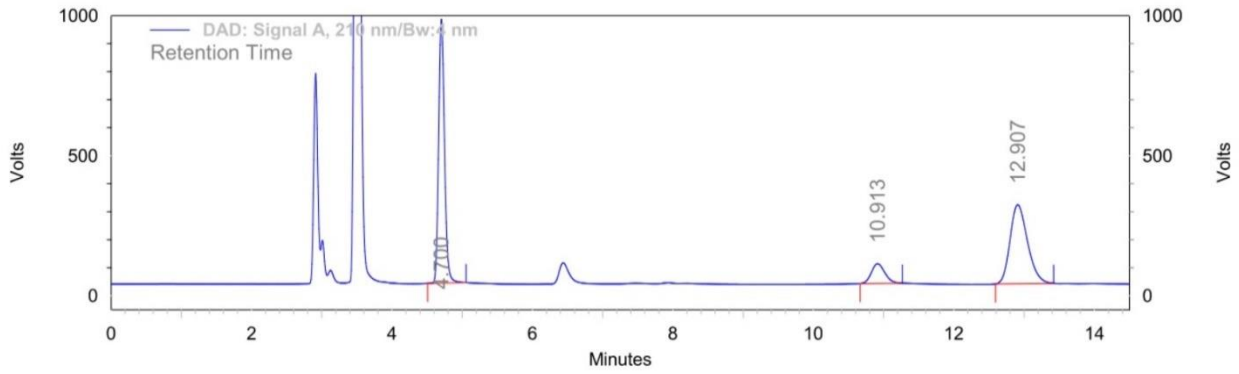
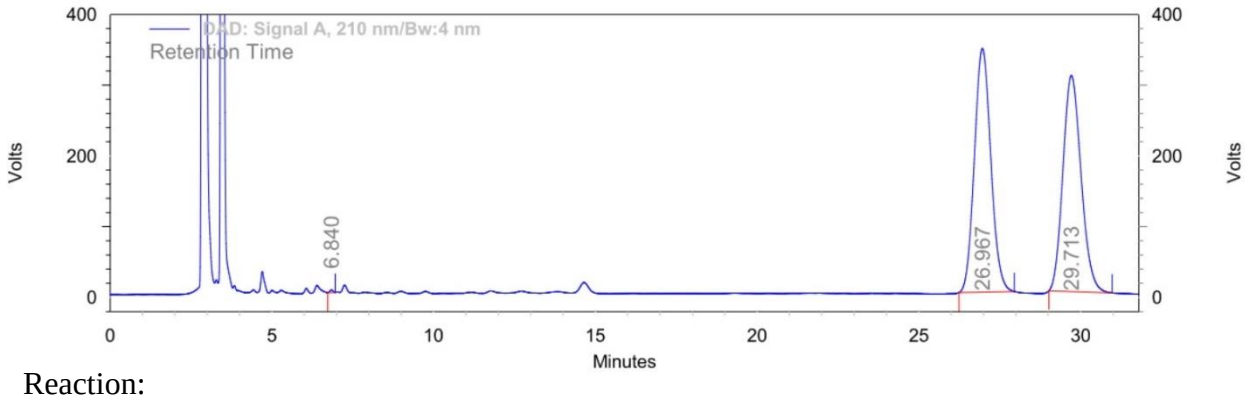


Figure 3.14: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(4-methylphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 4-methylbenzaldehyde: 4.7 min, (*R*)-1-(4-methylphenyl)-2-nitroethanol: 10.9 min and (*S*)-1-(4-methylphenyl)-2-nitroethanol: 12.9 min.

Control:



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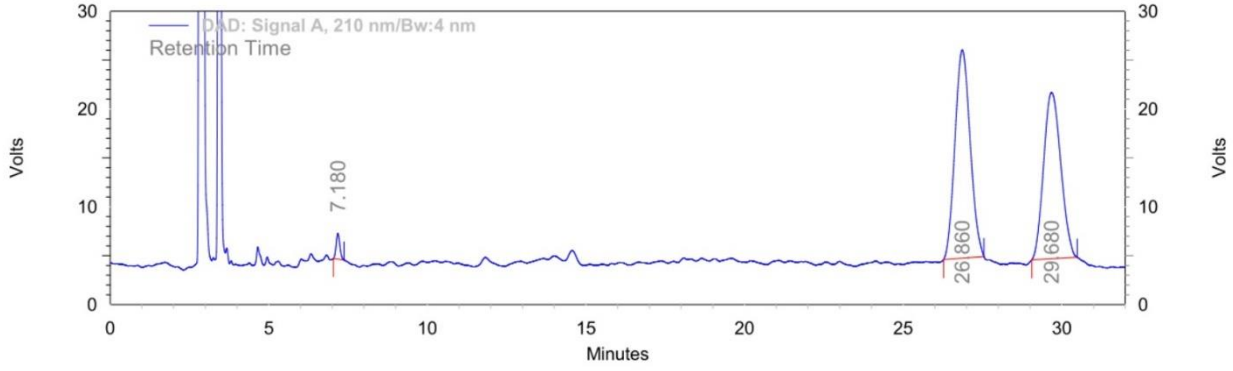
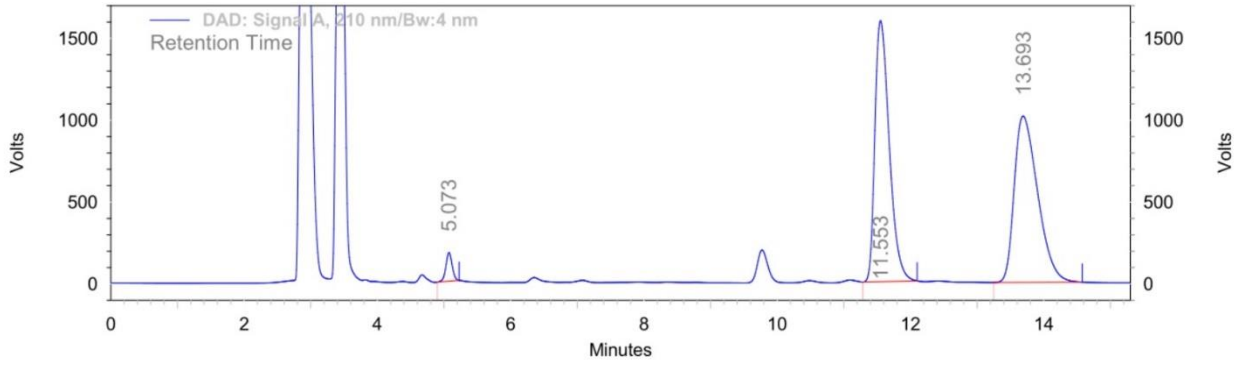


Figure 3.15: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of (*E*)-1-nitro-4-phenylbut-3-en-2-ol, (a) control having no enzyme, (b) reaction. Retention times, cinnamaldehyde: 7.1 min, (*S*)-(*E*)-1-nitro-4-phenylbut-3-en-2-ol: 26.8 min and (*R*)-(*E*)-1-nitro-4-phenylbut-3-en-2-ol: 29.6 min.

Control:



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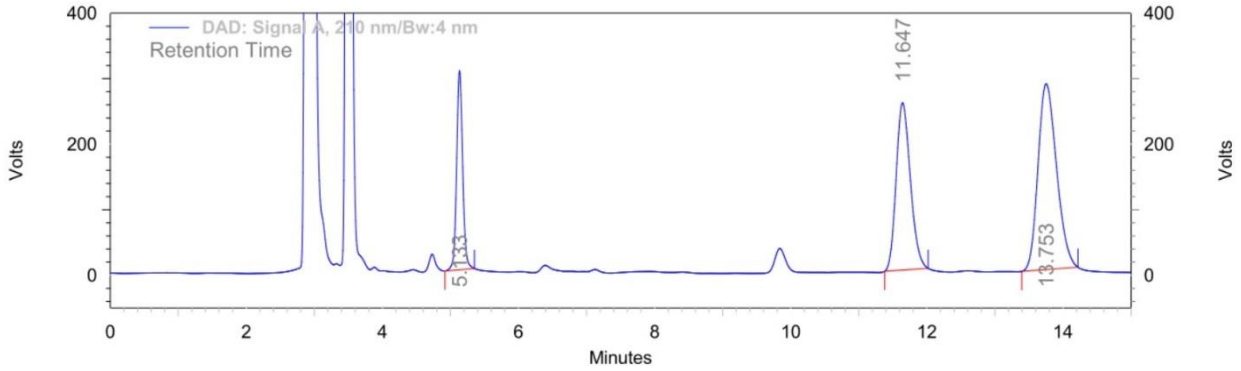
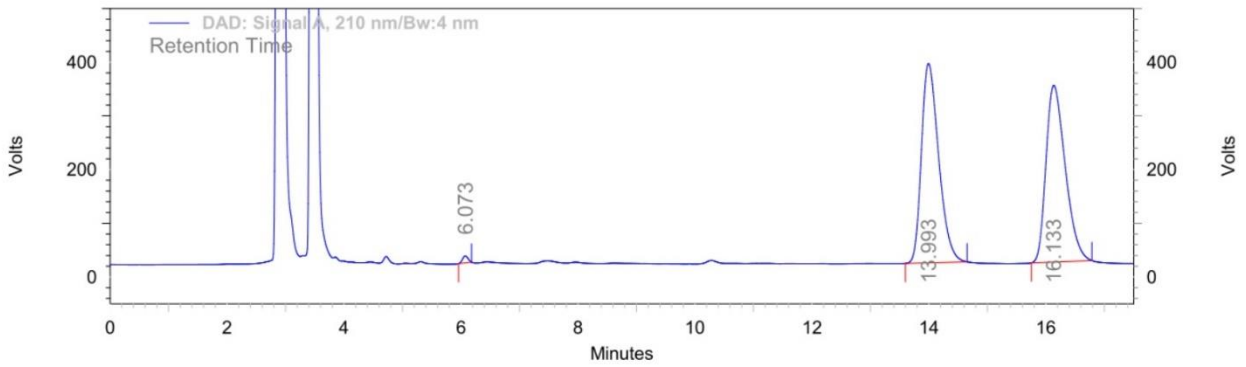


Figure 3.16: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(4-chlorophenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 4-chlorobenzaldehyde: 5.1 min, (*R*)-1-(4-chlorophenyl)-2-nitroethanol: 11.6 min and (*S*)-1-(4-chlorophenyl)-2-nitroethanol: 13.7 min.

Control:



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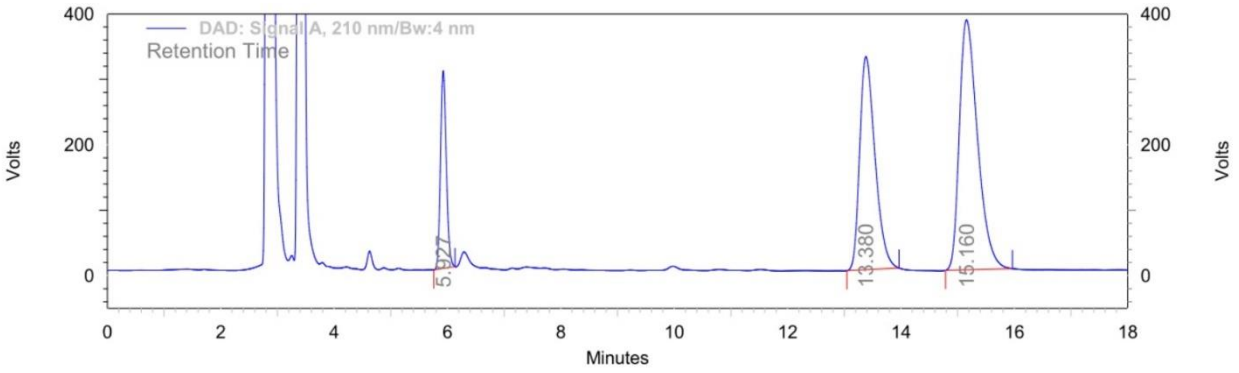

Figure 3.17: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(4-(allyloxy)phenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 4-allyloxybenzaldehyde: 5.9 min, (*R*)-1-(4-(allyloxy)phenyl)-2-nitroethanol: 13.3 min and (*S*)-1-(4-(allyloxy)phenyl)-2-nitroethanol: 15.1 min.

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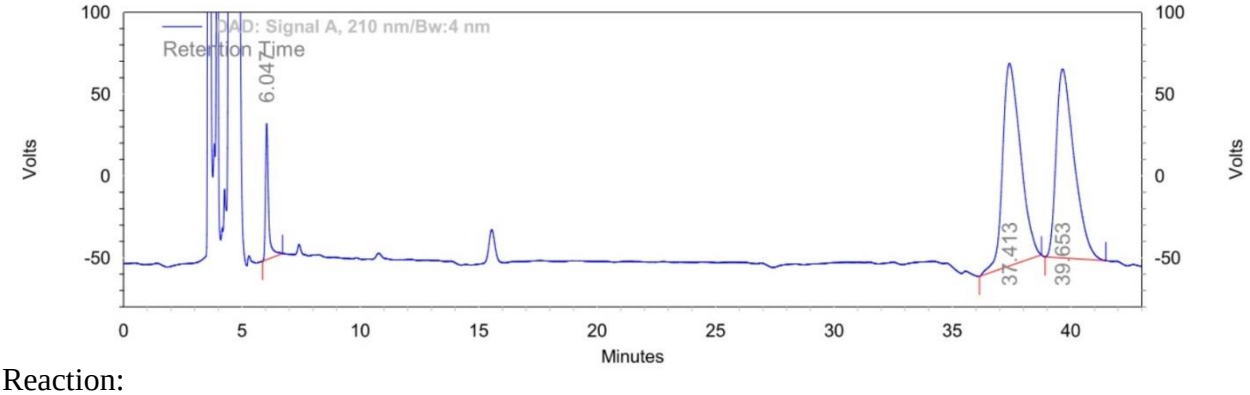

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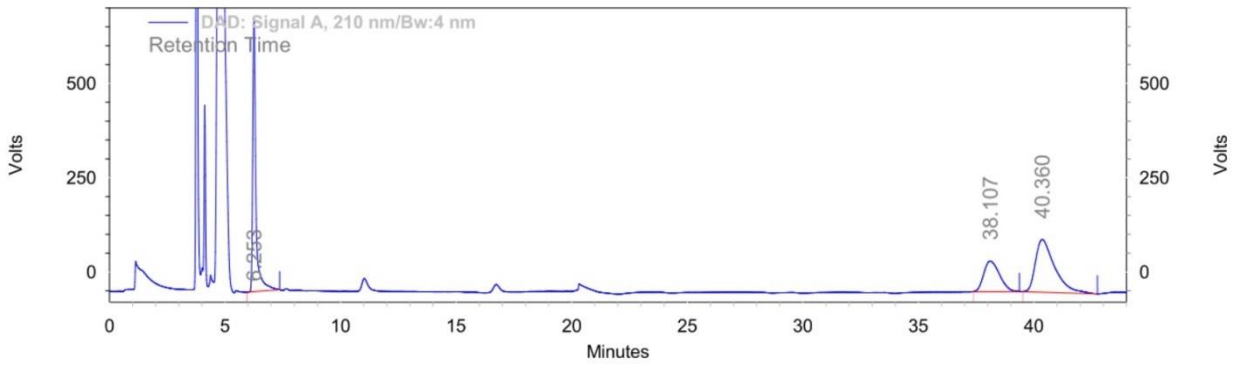

Figure 3.18: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(2-chlorophenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 2-chlorobenzaldehyde: 6.2 min, (*R*)-1-(2-chlorophenyl)-2-nitroethanol: 38.1 min and (*S*)-1-(2-chlorophenyl)-2-nitroethanol: 40.3 min.

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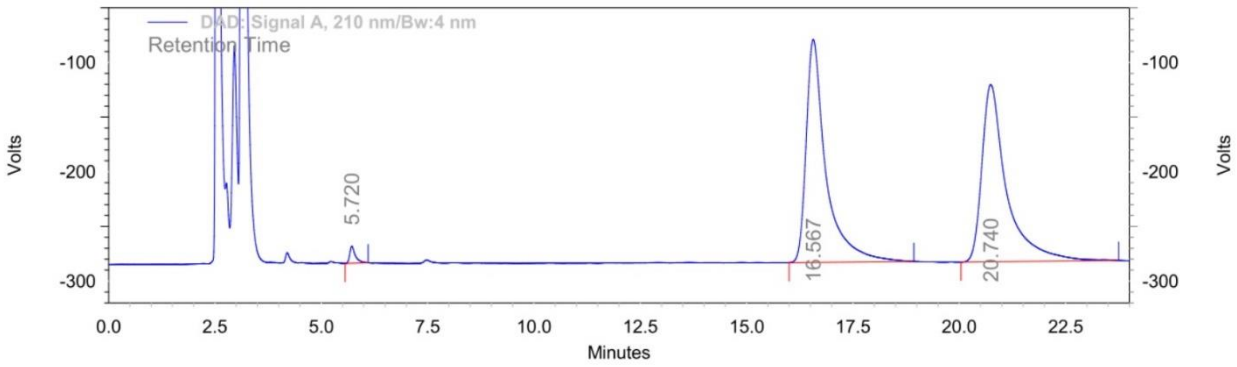

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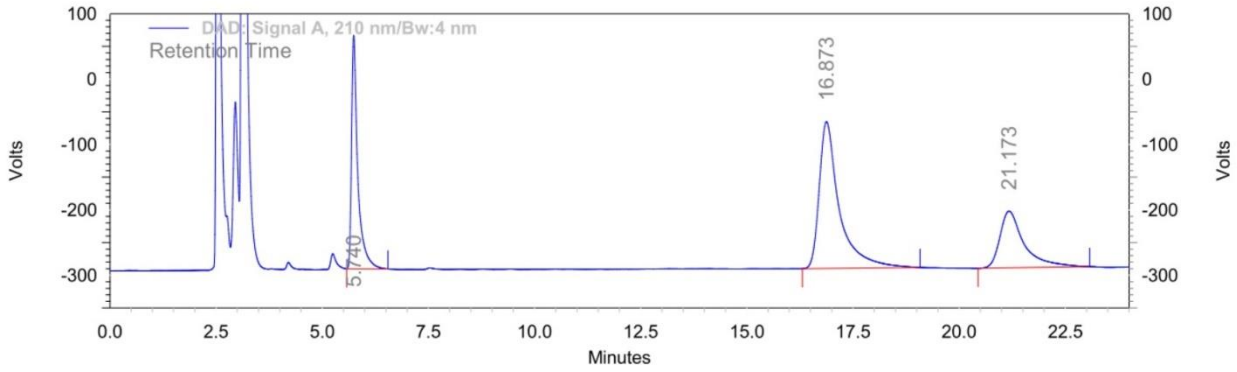
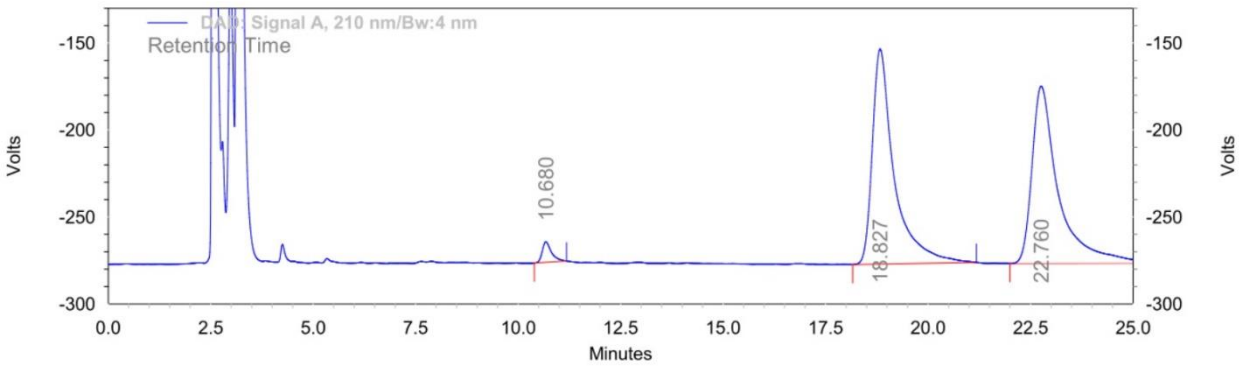


Figure 3.19: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(3,5-dimethoxyphenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3,5-dimethoxybenzaldehyde: 5.7 min, (*S*)-1-(3,5-dimethoxyphenyl)-2-nitroethanol: 16.8 min and (*R*)-1-(3,5-dimethoxyphenyl)-2-nitroethanol: 21.1 min.

Control:



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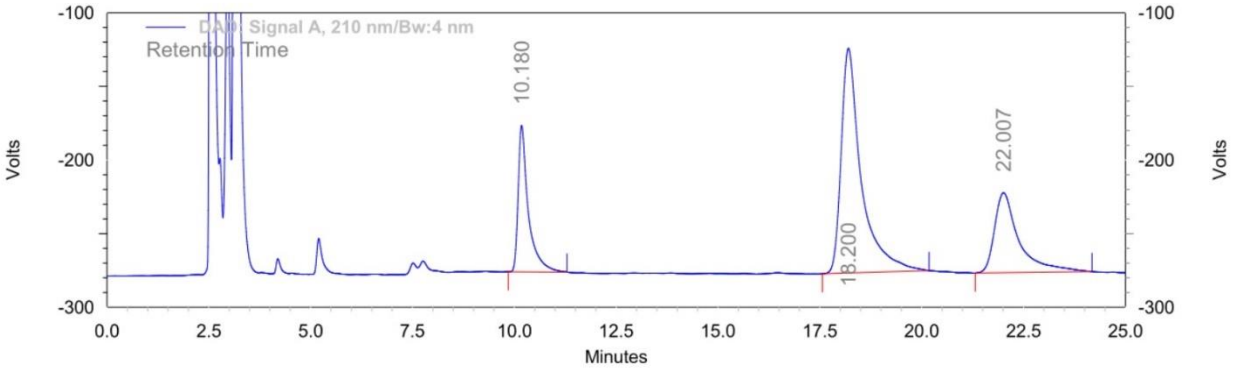

Figure 3.20: HPLC chromatogram of celite *At*HNL catalysed enantioselective cleavage of 1-(3-nitrophenyl)-2-nitroethanol, (a) control having no enzyme, (b) reaction. Retention times, 3-nitrobenzaldehyde: 10.1 min, (*S*)-1-(3-nitrophenyl)-2-nitroethanol: 18.2 min and (*R*)-1-(3-nitrophenyl)-2-nitroethanol: 22.0 min.

3.5. Discussion

3.5.1. Preparation of celite-*At*HNL

According to earlier studies, the moderate hydrophobicity of celite supports leads to a good water transport to the adsorbed enzymes and correspondingly excellent activities inside the micro-aqueous reaction systems.[34,35]

Okrob *et al* reported celite-*At*HNL preparation with enzyme: support varied from 1:4 to 1:50.[28] Finally, they have selected 1:4 ratio for the preparation of the immobilized enzyme that was used in the synthesis of chiral mandelonitrile. However, the basis of their selection of 1:4 ratio was not provided. *Me*HNL (*Manihot esculenta* HNL) has also been immobilized in celite R-633.[31] Preparation of celite-*Me*HNL used a variation of enzyme: support of 1:4 to 1:100, however the optimum ratio has not been discussed. We found maximum recovery with 1:20 ratio of enzyme: support. Even at 1:5 ratio, the % recovery of activity was ~85% of that of the celite-*At*HNL with 1:20. The prepared celite-*At*HNL was characterized by scanning electron microscope prior to its use in biocatalysis.

3.5.2. Optimisation of biocatalytic parameters for celite-*At*HNL catalysed retro-Henry reaction

3.5.2.1. Effect of different pH

In case of HNL biocatalysis, pH plays a significant role, irrespective of cyanohydrin or nitroaldol synthesis.[25,36] *At*HNL has been reported to be almost inactive at pH <5.0.[29] This study reported the celite-*At*HNL catalysed retro-Henry reaction at low pH. Surprisingly at pH 4.5 the immobilized enzyme has showed reasonably good activity with 62*% ee* and 41% conversion. At pH 5.0 very high *% ee* i.e., 97% and 42% conversion of (*S*)-NPE was observed (**Figure 3.4**). Earlier studies reveal that *At*HNL has showed oxynitrilase activity at low pH i.e., 4.5 after enzyme engineering[36] or when fused with florescent proteins,[37] however its nitroaldolase activity at low pH has not been investigated. The celite-*At*HNL has the potential to be used for oxynitrilase activity, where low pH is an essential parameter to minimize the formation of racemic cyanohydrin due to spontaneous hydrocyanation. Our previous study reported optimum retro-Henry reaction activity by purified *At*HNL at pH 5.0.[17] *At*HNL immobilized on celite R-633 has been used in the synthesis of chiral cyanohydrins at pH 5.5.[28] Celite-*Me*HNL has been reported in cyanohydrin synthesis at pH 5.0[31] and celite-*Pa*HNL at pH 5.4.[38]

3.5.2.2. Effect of organic solvent

An aqueous-organic biphasic system is imperative for HNL biocatalysis to avoid the formation of spontaneous racemized product in aqueous solution that leads to decreases in the *% ee* of products.[20,39] Also, organic solvent addition helps improve the solubility of the substrate in biocatalysis and minimizes substrate and/or product inhibition. Even in the case of an acyl-peptidase catalysed Henry reaction, Yu et al have reported the use of organic solvent to be beneficial.[40]

We observed highest *% ee* i.e., 99% and ~38% conversion in toluene, however, in *n*-butylacetate (*n*BuOAc) highest i.e., 42.2% conversion was observed with 98*% ee* of (*S*)-NPE (**Figure 3.5**). Hence *n*BuOAc was selected as the best organic solvent. Clearly in absence of any organic solvent the *% ee* was only 89% and 14.7% conversion. Earlier study on retro-Henry reaction by purified *At*HNL has showed optimal activity in toluene.[17] Asano et al reported optimum Henry reaction activity of *At*HNL in *n*BuOAc. Clearly immobilization in celite surface has also showed organic solvent tolerance.

3.5.2.3. Effect of content of *n*-butyl acetate

The impact of ratio of organic solvent to aqueous content is significantly important for HNL catalysed biotransformation. The organic contents not only help in solubilization of substrate and product which influences the rate of the reaction, but also could make a negative impact such as enzyme denaturation. Hence its optimisation has become important. In our study of *At*HNL catalysed retro-Henry reaction using pure enzyme, we found highest activity with 65% toluene [17]. *At*HNL biocatalysis in synthesis of Henry products has shown highest *% ee* of (*R*)-NPE in 50% *n*BuOAc.[25] *At*HNL immobilized based on (a) fusion with a family 2 carbohydrate-binding module, and (b) catalytically active inclusion bodies, were used in the synthesis of (*R*)-mandelonitrile in an aqueous-MTBE based biphasic medium, where the organic solvent content was not specified.[41,42]

3.5.2.4. Effect of different substrate concentration

In HNL biocatalysis substrate concentration plays an important role. In the case of hydrocyanation as well as nitroaldol reaction, aldehydes are used as substrates and at higher concentrations HNLs such as *Hb*HNL, *Bm*HNL, and *At*HNL exhibit substrate inhibition.[43,25,44] In the case of *At*HNL catalysed retro-Henry reaction, benzaldehyde is a product, which is a known inhibitor of the enzyme. Hence, it has become important to find out the optimal substrate concentration which influences the production of enantiopure β-nitroalcohol. With increasing

substrate concentration, *% ee* of (*S*)-NPE decreased, while % conversion, which is total NPE content has increased. We observed similar trends during purified *At*HNL catalysed retro-Henry reaction.[17] This could be due to incomplete cleavage of the (*R*)-enantiomer of the racemic NPE by *At*HNL, at higher substrate concentration. The reason for incomplete cleavage possibly due to product inhibition, i.e., benzaldehyde.[29] With the intention to use maximum substrate concentration, we have selected 6 mM racemic NPE to use in further biotransformations. In case of 6 mM rac-NPE, 94*% ee* of product was obtained with ~45% conversion (**Figure 3.7**).

3.5.2.5. Reusability of celite-*At*HNL

Apart from giving enzyme stability, the other major advantage of immobilization is to use the biocatalyst repeatedly in biocatalysis. We have examined the reusability of celite-*At*HNL in the retro-Henry reaction catalysed preparation of (*S*)-NPE for four successive cycles. This study has shown decrease in *% ee* of (*S*)-NPE in second cycle onwards, although the % conversion remained unaffected. This is possibly due to enzyme leakage, as enzyme adsorption on the celite surface involves weak physical interactions such as H-bonding, van der Waals forces, and ionic interactions. Pectinase immobilized on celite545 on recyclability test has showed only 57% activity in 3rd cycle.[45] *Candida rugosa* lipase immobilized in vesicular silica through physical adsorption has showed enzyme leakage during reusability experiments.[46] There exist a number of examples of such enzyme leakage in case of enzyme immobilization involving weak physical interaction. To minimize the leakage, a further step of immobilization such as cross-linking or entrapment in a matrix is required, which can be made as another study. Okrob et al. however, in their celite R-633-*At*HNL catalysed (*R*)-mandelonitrile synthesis, could reuse the enzyme for five cycles with high conversion of product.[28]

3.5.2.6. Preparation of (*S*)-β-nitroalcohols

A dozen of chiral (*S*)-β-nitroalcohols were synthesized using the optimised retro-Henry reaction (**Scheme 3.3, Table 3.3**). Racemic aromatic β-nitroalcohols with substitutions at different positions of the ring were used in the celite-*At*HNL biocatalysis. Racemic NPE was converted into (*S*)-NPE in >97% *ee* and 48% conversion. High preference for benzaldehyde was observed in the *At*HNL catalysed cyanohydrin (99% *ee*) and NPE (91% *ee*) synthesis.[25,47] Racemic aromatic β-nitroalcohols carrying electron donating groups i.e. methyl and methoxy substituted at the *meta* position of the aromatic ring had undergone retro-Henry reaction to produce the corresponding (*S*)-β-nitroalcohols in high *% ee* (97-98) and 44-46% conversion. In our earlier study of free enzyme catalysed retro-Henry reaction, the *At*HNL has shown similar high enantioselectivity for substrates with methyl and methoxy at the *meta* position of the aromatic ring.[17] Presence of chloro functionality at the same position has reduced the *% ee* to moderate i.e., 72.7 with 54% conversion. *Meta* substituted nitro aromatic β-nitroalcohols had produced poor *% ee* of product (32.7%). These two substrates were not tested earlier in the *At*HNL catalysed retro-nitroaldol reaction. This could be attributed due to negative inductive effect of the chloro and nitro functionality. However *meta* chlorobenzaldehyde is a well-accepted substrate for *At*HNL in the synthesis of corresponding (*R*)-cyanohydrin or (*R*)-β-nitroalcohol where 91-99% *ee* were reported.[25,47] Racemic NPE with chloro at *ortho* position has showed only 32.8% *ee* and 48.6% conversion of product in the retro-Henry reaction. The poor *% ee* of the product cannot be predicted whether it is due to the position of the substitution or electronic effect, because this is the only *ortho* substituted substrate investigated in this study. This substrate was not studied earlier in the retro-Henry reaction, however in case of the reverse reaction, *ortho* chlorobenzaldehyde has showed only 68% *ee* of corresponding (*R*)-β-nitroalcohol.[25] In the cyanohydrin synthesis, *At*HNL has showed high preference for *ortho* chlorobenzaldehyde.[47] Aromatic β-nitroalcohols having *para* substitutions i.e. fluoro, methyl, chloro and allyloxy were when tested for the retro-Henry reaction, they showed 74.9, 67.6,

16.4, 16.3% *ee* and 44.2, 47.8, 47 and 53.9% conversion of corresponding (*S*)-β-nitroalcohols respectively. Among them *para* fluoro, chloro and allyloxy substrates were investigated for the first time in the retro-Henry reaction. This result shows that *para* substituted substrates are not preferred by the enzyme for the retro-Henry approach. However, the high difference in *% ee* of product between the *para* fluoro and chloro substrates is not understood. *At*HNL could efficiently convert *para* chloro and *para* fluoro benzaldehydes to their corresponding cyanohydrin in >99*% ee*.[47] The poor *% ee* by the *para* allyloxy substrate could be due to the possible stearic effect. Neither *para* allyloxy-NPE in *At*HNL catalysed retro-Henry reaction nor *para* allyloxy benzaldehyde in corresponding Henry reaction has been investigated earlier. The racemic β-nitroalcohol of cinnamaldehyde is yet another new substrate in the *At*HNL catalysed retro-Henry reaction. This substrate has showed only 5*% ee* and 47% conversion of the corresponding product indicating very poor selectivity. The substrate with methoxy at both the *meta* positions has showed only 48*% ee* and 56.3% conversion in the retro-Henry reaction. Although this substrate has not been tested in the retro-Henry reaction earlier, but aromatic substrates with di and tri-substitutions had showed poor *% ee* in case of free *At*HNL catalysed retro-Henry reaction.[17] Comparison of the substrate preference of *At*HNL between the retro-Henry reaction and the nitroaldol and cyanohydrin synthesis catalysed by the same enzyme, could not be efficiently done due to the partial overlap of the substrates used in these different reactions. The difference in enantioselectivity observed for similar substrates in these different reactions could be partly attributed due to the diverse reaction conditions used.

3.6. Conclusions

Immobilized *At*HNL was prepared by physical adsorption of the enzyme in celite®545, an inexpensive adsorbent. Retro-Henry reaction was investigated using celite-*At*HNL in the preparation of (*S*)-β-nitroalcohols. After optimisation of the biocatalysis conditions, the TTN

of the celite-*At*HNL was increased 2.3-fold. The celite-*At*HNL has showed good retro-Henry activity at low pH e.g., 4.5 and 5.0 with 62 - 97% *ee* and 41 - 42% conversion of (*S*)-NPE, compared to inactivation of the free enzyme at pH <5.0. This increased catalytic efficiency and pH stability could be possibly due to increased stability of *At*HNL by immobilization. A dozen of racemic β-nitroalcohols were converted into their corresponding (*S*)-β-nitroalcohols using this reaction; among them eight were not tested earlier. The immobilized enzyme has showed broad substrate selectivity in the retro-Henry reaction and products were obtained up to 98.5% *ee*. The celite-*At*HNL however was leaching due to physical adsorption, a poor interaction, which needs a further study such as cross-linking or sol-gel to increase the rigidity of the enzyme. The celite-*At*HNL being active at low pH 4.5 to 5.0 in the retro-Henry reaction, can be exploited in related cascade, and chemo-enzymatic syntheses that may require acidic pH.

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Chapter 4

Preparation of (*S*)-β-nitroalcohols by retro-Henry reaction using engineered *At*HNL

4.1. Introduction

Practical application of enzymes in organic synthesis is often hampered by their poor selectivity towards non-natural substrates. It is because active site of natural enzymes are not evolved to accommodate non-natural organic molecules for catalysis. Apparently, there is a need to alter the enzyme to accommodate new substrates. Protein engineering has brought a revolution in the area of biocatalysis. It has become an inevitable tool to improve several important properties of enzymes such as catalytic activity, substrate selectivity, stereoselectivity, thermostability, etc. While we aimed to prepare (*S*)-β-nitroalcohols using *At*HNL catalysed retro-Henry reaction, we observed that substrate selectivity of the wild type enzyme is a major bottleneck in this biotransformation.

The first retro-Henry reaction was reported using *Hb*HNL. It catalysed the cleavage of (*S*)-enantiomer of the racemic substrate 2-nitro-1-phenylethanol (NPE) and produced (*R*)-NPE. The major constraint of this method was, it prevented to achieve high conversion and % *ee* of the unreacted (*R*)-NPE. This is due to the formation of the benzaldehyde, which is a strong inhibitor of the enzyme. To overcome the benzaldehyde inhibition limitation, hydrogen cyanide (HCN) was added *in-situ* to the reaction mixture, which reacts with benzaldehyde and converts it to the less-inhibitive (probably) (*S*)-mandelonitrile. By following this strategy, it was possible to conduct the retro-Henry reaction practically to achieve 95% *ee* (enantiomeric excess), 49% conversion of (*R*)-NPE. However, substrate scope of this method was not studied.[1] After *Hb*HNL, *Arabidopsis thaliana* hydroxynitrile lyase (*At*HNL), an (*R*)-selective enzyme was reported to catalyse nitroaldol reaction with good enantioselectivity, but the maximum yield

was 30%.[2] We have demonstrated retro-Henry reaction by wild type *At*HNL, for the first time and illustrated the application of this method using ten different racemic β-nitroalcohols.[3] However, only in the case of six substrates, corresponding (*S*)-β-nitroalcohols were obtained with high enantioselectivity, i.e., 81–99% *ee* and 19–47% conversion (see chapter 2 of this thesis), while the remaining four substrates showed poor enantioselectivity i.e., 1–44% *ee* of product. We have also reported retro-Henry reaction catalysed by celite immobilised wild type *At*HNL. In this method, we used twelve different racemic β-nitroalcohols as substrates. However, only in the case of six substrates, the corresponding (*S*)-β-nitroalcohols could be observed in good to high enantioselectivity i.e., 67–98% *ee* and 44–54% conversion. In the case of the remaining six substrates, the corresponding products were found with poor enantioselectivity 5–48% *ee* (see chapter 3 of this thesis). Analysis of the above two cases suggests that, *At*HNL catalysed retro-Henry reaction using the wild type enzyme, either in its purified form or immobilized, could not produce high % *ee* of the (*S*)-enantiomeric product in the case of 2-Cl, 3-Cl, 4-Cl, 3-OH, 4-Me and 4-MeO derivatives of NPE as substrates. It clearly indicates the limited substrate scope of the wild type *At*HNL in catalysing the retro-Henry reaction.

Earlier studies shows that HNLs have been engineered to improve their substrate scope, $k_{cat,}$ and stability. The following are relevant examples where wild type HNLs have been engineered to improve their activity. *Acidobacterium capsulatum* ATCC 51196 hydroxynitrile lyase (*Ac*HNL) and *Granulicella tundricula* hydroxynitrile lyase (*Gt*HNL) have been reported to catalyse stereoselective synthesis of (*R*)-β-nitroalcohols.[4] Wild type *Ac*HNL and *Gt*HNL have shown moderate to poor enantioselectivity, 46–79% *ee* and poor conversion of 15–38% in the synthesis of (*R*)-NPE in 24 hours. However, when engineered, *Ac*HNL and *Gt*HNL variants, i.e., A40H, A40R, and A40H/V42T/Q110H have shown 93–98% *ee* and high conversion of 15–75% of (*R*)-NPE in 24 h. Improved enantioselectivity was also observed in the case of

stereoselective nitroaldol synthesis of three other aldehydes, 2-chloro benzaldehyde, cyclohexanecarboxaldehyde and hexanal. *Ac*HNL and *Gt*HNL variants have shown high enantioselectivity, i.e., 80–99% *ee* and high conversion 80–95% in the synthesis of the corresponding (*R*)-β-nitroalcohols. Kazlauskas and co-authors found improved retro-nitroaldolase activity with *Hevea brasiliensis* HNL (*Hb*HNL) variants compared to the wild type enzyme.[5] The best *Hb*HNL variant with the triple substitution, i.e., L121Y-F125T-L146M has shown a specific activity of 0.71 U.mg^{-1} towards the cleavage of racemic NPE, which is ~5.5 folds higher than that of the wild type activity (0.13 U mg^{-1}). Further, the k_{cat} for this variant was 3.3 times higher than that of the wild type. *At*HNL cannot be used for stereoselective cyanohydrin synthesis in aqueous media at pH < 5 due to its limited stability in such low pH. However, low pH reaction condition is required to suppress the spontaneous (uncatalysed) racemic cyanohydrin formation.[6] Therefore, *At*HNL was engineered to increase its stability at low pH, which produced a surface-modified (Surf-mod) variant having eleven amino acid changes on the protein surface. The surf-mod *At*HNL variant showed an increased stability below pH 5.8 and a 14-fold higher stability at pH 5.0 compared to the half-life of the wild type. It not only enhanced the stability, but also demonstrated high enantioselectivity in cyanohydrin synthesis. The surf-mod *At*HNL variant catalysed hydrocyanation of benzaldehyde, 3-fluorobenzaldehyde and 2-furfural performed at pH 4.5, have produced the corresponding (*R*)-cyanohydrins in 99, 92 and 95% *ee* and 70, 74 and 87% conversions respectively. Note that in the case of the wild type no enantioenriched products were formed most likely due to the rapid deactivation of the enzyme at such a low pH. Zheng et al., engineered *Prunus communis* hydroxynitrile lyase (*Pc*HNL5) to enable hydrocyanation of rigid benzo-ketal aldehydes with high enantioselectivity.[7] Specific activity of *Pc*HNL5 variant L331A towards 1,3-oxane ring-fused benzaldehyde was found to be 2.4 U/mg, which is 545 folds higher as compared to 4.4×10^{-3} U/mg by the wild type enzyme. *Pc*HNL5 engineering

has also improved the substrate scope of the enzyme. For fifteen different structurally diverse aldehydes, the turnover frequencies (TOF or k_{cat}) of the *Pc*HNL5 variants in the synthesis of their corresponding (*R*)-cyanohydrins were found to be 1.3–1249 s^{-1} with 41–97% yield and 95–99% *ee*. The TOF of wild type *Pc*HNL5 for the same set of fifteen aldehydes for hydrocyanation was in the range of 0.022–276 s^{-1} with 32–99% *ee*. Thus, compared to the wild type, TOF of the variants improved by 1.6 to 792-fold. It is clear from the aforementioned reports that HNL engineering could improve the substrate scope, $k_{cat,}$ and stability. Therefore, in the current study we envisioned to study the substrate scope of engineered *At*HNLs towards the retro-Henry reaction.

4.2. Objectives

1) To screen *At*HNL variant library towards retro-Henry reaction
2) To investigate the substrate scope with selected *At*HNL variants towards retro-Henry reaction

4.3. Materials and Methods

4.3.1. Materials

*At*HNL has been engineered in our laboratory by others with the intention of improving its substrate scope for nitroaldol reaction. In this study, we envisioned to use the variants towards retro-Henry reaction. Materials used in this chapter were mentioned in section **2.3.1** of the **Chapter 2**.

4.3.2. *At*HNL variant library

The library of *At*HNL variants used for screening in the current study consists of forty-eight mutants. It was created by site saturation mutagenesis at Tyr14, Phe179, together 20+19=39

mutants. The library also consisted of a semi-saturation library at position Phe82, where the Phe was substituted with polar amino acids, and other miscellaneous *At*HNL mutants present in our laboratory. This list includes variants F82A, F82K, F82P, F82Q, F82R, F82S, F82T, F80A, and H15A. The details about the preparation of these variants are described elsewhere.[8]

4.3.3. Preparation of crude enzyme extract

The recombinant *At*HNL genes (wild type and engineered variant genes) in pET28a plasmid were transformed into *Escherichia coli* BL21(DE3) 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_{600} 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 13751 *g* for 15 min at 4 °C and the cell pellet was suspended in 20 mM potassium phosphate buffer (KPB). From this step onwards, all the steps of crude enzyme preparation were done at 4 °C. The cell suspension was disrupted by sonication. Disrupted cells were centrifuged at 13751 *g* for 45 min. The supernatant was analysed by mandelonitrile cleavage assay to confirm HNL activity in the soluble fraction. The protein concentration of the supernatant was measured by using a Nanodrop. The resultant supernatant was used as crude enzyme in the screening of *At*HNL variants library in the retro-Henry reaction.

4.3.4. Expression and purification of wild type *At*HNL and its variants

Expression and purification of wild type *At*HNL and its variants were performed as mentioned in materials and methods section **2.3.2** of **Chapter 2**. The resultant purified wild type *At*HNL and its variants were used in the biotransformation.

4.3.5. HNL assay

HNL activity of either crude or purified enzymes of wild type or engineered, were measured using the method described in section **2.3.3** of **Chapter 2**.

4.3.6. Synthesis of racemic β-nitroalcohols

Racemic β-nitroalcohols were synthesised using the method as mentioned in section **2.3.5** of **Chapter 2.** Racemic β-nitroalcohols synthesized were characterized by ^{1}H and ^{13}C NMR spectroscopy (BRUKER 400 MHz NMR). They were used as analytical HPLC standards during chiral HPLC analysis.

4.3.7. Screening of *At*HNL variants library in the enantioselective cleavage of multiple racemic β-nitroalcohols

Screening of *At*HNL variants library in the enantioselective cleavage of multiple racemic β-nitroalcohols was performed as follows. The reaction mixture contained 2 µmol of racemic β-nitroalcohol (1 µmol of each substrate, two different substrates were added), 10 mg of crude lysate of *At*HNL mutant (25% v/v), 50 mM citrate phosphate buffer (CPB) (25% v/v) pH 5.5, and *n*-butyl acetate (50% v/v). The reaction mixture was shaken at 1200 rpm at 30 ℃ in an incubator shaker for 9 h. A 100 µL of aliquot from the organic layer was taken and added to 200 µL of hexane/2-propanol = 9:1, vortexed followed by centrifuged at 15000 *g* at 4 ℃ for 5 min. A 20 µL of the organic layer was analysed in a HPLC using chiral column.

4.3.8. Investigation of substrate scope of selected purified *At*HNL variants towards retro-Henry reaction

Investigation of substrate scope of selected purified *At*HNL variants towards retro-Henry reaction was performed as follows. The reaction mixture contained 1.33 mM racemic β-

nitroalcohol, 2 mg of purified *At*HNL variant in KPB pH 7.0, KPB pH 7.0 (enzyme and KPB together 17.5% v/v), 50 mM CPB pH 5.5 (17.5% v/v), and toluene (65% v/v). Total reaction volume was 1 mL. The reaction mixture was shaken at 1200 rpm, 30 ℃ in an incubator shaker for 7 h. Aliquots were taken at 3, 5 and 7 hours. A 100 µL of aliquot from the organic layer was taken and added to 200 µL of hexane/2-propanol = 9:1, vortexed, centrifuged at 15000*g* at 4 ℃ for 5 min. A 20 µL of the organic layer was analysed in a HPLC using chiral column.

4.4. Results

4.4.1. ^{1}H NMR and ^{13}C NMR characterization of racemic β-nitroalcohols

^{1}H NMR and ^{13}C NMR (Nuclear Magnetic Resonance) characterisation spectral data were shown (**Figures 4.3.1 to 4.3.16)**

1-(3,5-Dimethoxyphenyl)-2-nitroethanol[9]

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.80 (6H, s), 4.48-4.61 (2H, m), 5.37-5.40 (1H, dd, *J* = 2.5, 9.5 Hz), 6.43-6.44 (1H, t, *J* = 2.5), 6.54-6.55 (2H, d, *J* = 2.0 Hz) ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.4, 71.0, 81.2, 100.6, 103.8, 140.6, 161.2.

1-(3-Hydroxyphenyl)-2-nitroethanol[10]

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.82 (1H, d, *J* = 4 Hz), 4.55 (1H, dd, *J* = 3, 13.5 Hz), 4.59-4.63 (1H, m), 4.96 (1H, s), 5.43-5.44 (1H, m), 6.83-6.85 (1H, m), 6.93-6.98 (2H, m), 7.24 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 70.7, 80.8, 112.6, 116.1, 118.0, 130.1, 139.6, 156.0.

1-(4-Methoxylphenyl)-2-nitroethanol[2]

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.99-2.0 (1H, m), 3.77-3.78 (3H, m), 4.41-4.45 (1H, m), 4.52-4.58 (1H, m), 5.31-5.35 (1H, m), 6.86-6.90 (2H, m), 7.26-7.28 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.3, 70.6, 81.2, 114.3(X2), 127.3(X2), 130.5, 159.8.

1-(4-nitrophenyl)-2-nitroethanol[11]

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.51 (1H, d, *J* = 7, 14 Hz), 4.56-4.64 (2H, m), 5.63 (1H, dd, *J* = 5, 10 Hz), 7.65 (2H, d, *J* = 10 Hz), 8.29 (2H, d, *J* = 9 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 69.6, 80.1, 124.3 (2X), 126.6 (2X), 145.1, 148.1.

1-(4-Methylphenyl)-2-nitroethanol[2]

^{1}H NMR (500 MHz, $CDCl_3$) δ 2.38 (3H, s), 3.05 (1H, d, *J* = 3.5 Hz), 4.50 (1H, dd, *J* = 3.0, 13.0 Hz), 4.61 (1H, dd, *J* = 9.5, 13.0 Hz), 5.42 (1H, ddd, *J* = 3.0, 3.5, 9.5 Hz), 7.23 (2H, d, *J* = 8 Hz), 7.29 (2H, d, *J* = 8.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.1, 70.8, 81.2, 125.9 (X2), 129.7(X2), 135.2,138.9.

1-(2-Chlorophenyl)-2-nitroethanol[12]

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.44-4.49 (1H, m), 4.66-4.70 (1H, dd, *J* = 2.5, 14 Hz), 5.84-5.87 (1H, m), 7.28-7.40 (3H, m), 7.66-7.68 (1H, dd, *J* = 1.5, 7.5 Hz) ^{13}C NMR (125 MHz, $CDCl_3$) δ 67.8, 79.3, 127.5, 127.6, 129.7, 129.2, 131.4,135.5

1-(3-Chlorophenyl)-2-nitroethanol[9]

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.17 (1H, brs), 4.50-4.60 (2H, m), 5.43-5.46 (1H, dd, *J* = 3.5, 9.5 Hz), 7.27-7.29 (1H, m), 7.34-7.35 (2H, m), 7.430-7.437(1H, m); ^{13}C NMR (120 MHz, $CDCl_3$) δ 70.2, 80.9, 124, 126.2, 129 (X2),130.3, 135, 140.1.

1-(4-Chlorophenyl)-2-nitroethanol[9]

^{1}H NMR (400 MHz, $CDCl_3$): δ 3.04 (1H, brs), 4.48-4.61 (2H, m), 5.44-5.47 (1H, dd, *J* = 3.2, 9.2 Hz), 7.34-7.41 (4H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.3, 80.9, 127.3, 129.2, 134.8, 136.5

4.4.2. Chiral resolution of racemic β-nitroalcohols

Table 4.1: Details of chiral resolution of the racemic β-nitroalcohols used in this chapter using chiral HPLC

S. No	Substrate (β-nitroalcohol)	Retention time of aldehyde, corresponding (*R*)- and (*S*)- β-nitroalcohols (minutes)	Column Chiralpak	Mobile phase Hex/ IPA	Flow rate (mL/ min)
1[a]	1-(3,5-dimethoxyphenyl)-2-nitroethanol	3,5-Dimethoxybenzaldehyde = 6.63, RT_R = 25.02, RT_S = 19.78	IA	90:10	1
	1-(3-Hydroxyphenyl)-2-nitroethanol	3-Hydroxybenzaldehyde = 8.58, RT_R = 32.36, RT_S = 28.08			
2[a]	1-(4-Methoxyphenyl)-2-nitroethanol	4-Methoxybenzaldehyde = 3.94, RT_R = 10.16, RT_S = 12.16	IB	90:10	1
	1-(4-Nitrophenyl)-2-nitroethanol	4-Nitrobenzaldehyde = 6.92, RT_R = 16.56, RT_S = 19.46			
3	1-(4-Methoxyphenyl)-2-nitroethanol	4-Methoxybenzaldehyde = 7.46, RT_R = 14.44, RT_S = 16.86	IB	90:10	1
4	1-(4-Methylphenyl)-2-nitroethanol	4-Methylbenzaldehyde = 5.27, RT_R = 10.10, RT_S = 11.63	IB	90:10	1
5	1-(3-Hydroxyphenyl)-2-nitroethanol	3-Hydroxybenzaldehyde = 7.98, RT_R = 25.37, RT_S = 22.92	IA	90:10	1
6	1-(2-Chlorophenyl)-2-nitroethanol	2-Chlorobenzaldehyde = 7.42, RT_R = 21.04, RT_S = 21.88	IB	97.5:2.5	0.8
7	1-(3-Chlorophenyl)-2-nitroethanol	3-Chlorobenzaldehyde = 5.22, RT_R = 10.43, RT_S = 11.76	IB	90:10	1
8	1-(4-Chlorophenyl)-2-nitroethanol	4-Chlorobenzaldehyde = 5.18, RT_R = 10.83, RT_S = 12.58	IB	90:10	1

Absorbance at 210 nm, Hex/IPA = *n*-hexane: 2-propanol, RT_R = retention time of corresponding (*R*)-β-nitroalcohol, and RT_S = retention time of corresponding (*S*)-β-nitroalcohol. a: Two substrates were injected as a mixture and resolved together, four substrates of S. No. 1 and 2 were used in screening of *At*HNL variants library in the retro-Henry reaction.

4.4.3. Screening of *At*HNL variants library in the enantioselective cleavage of multiple racemic β-nitroalcohols

Forty-eight *At*HNL variants were screened towards the enantioselective cleavage of four racemic β-nitroalcohols (**Scheme 4.1**), i.e., 1-(3,5-dimethoxyphenyl)-2-nitroethanol, 1-(3-hydroxyphenyl)-2-nitroethanol, 1-(4-methoxyphenyl)-2-nitroethanol and 1-(4-nitrophenyl)-2-nitroethanol. To minimize the number of reactions during screening, the two substrates of S. No 1 of **Table 4.1.** were mixed and added. Similarly, the other two substrates of S. No 2 of **Table 4.1.** were mixed and added during screening. The wild type *At*HNL has shown only 8, 10, 8 and –1% *ee*, of the (*S*)-enantiomers and 50, 39, 50 and 29% conversions for the above four substrates respectively (**Table 4.2**). The screening has uncovered mutants with higher enantioselectivity in the case of the enantioselective cleavage of 1-(3,5-dimethoxyphenyl)-2-nitroethanol, 1-(3-hydroxyphenyl)-2-nitroethanol, and 1-(4-methoxyphenyl)-2-nitroethanol, while no better mutants were found for 1-(4-nitrophenyl)-2-nitroethanol. Better enantioselectivity against 1-(3,5-dimethoxyphenyl)-2-nitroethano was shown by F179H, F179M, and F179C. Similarly, against 1-(3-hydroxyphenyl)-2-nitroethanol, F179C, F179H, F179I, F179K, F179L, and F179M have shown higher enantioselectivity. Against 1-(4-methoxyphenyl)-2-nitroethanol, we found F179C, F179I, F179K, F179L, and F179M having higher % *ee* than the wild type. Based on the above initial screening results, we have chosen the six variants, F179C, F179H, F179I, F179K, F179L, and F179M, and attempted to reproduce the results before moving on to purify the enzymes for biotransformation. However, reproducibility with respect to % *ee* and conversion in the enantioselective cleavage of the three substrates (except 4-NO_2 NPE), could be obtained only in the case of F179K, and F179M.

In our laboratory, the *At*HNL mutants were screened in parallel towards the cleavage of racemic NPE. From this study, F179T and Y14M were selected, which showed higher

enantioselectivity as compared to the wild type.[8] Overall, four *At*HNL mutants F179K, F179M, F179T and Y14M were selected to study their substrate scope towards enantioselective cleavage of a series of racemic β-nitroalcohols using their purified enzymes.

4.4.4. Characterization of purified *At*HNL and its variants

The four mutants purified and used in biotransformation were characterized by SDS-PAGE (**Figure 4.1.**). All four mutant proteins have shown a 28kDa band in the SDS-PAGE similar to the wild type enzyme. Also, in case of all the four mutants, the protein expression was found to be very good.

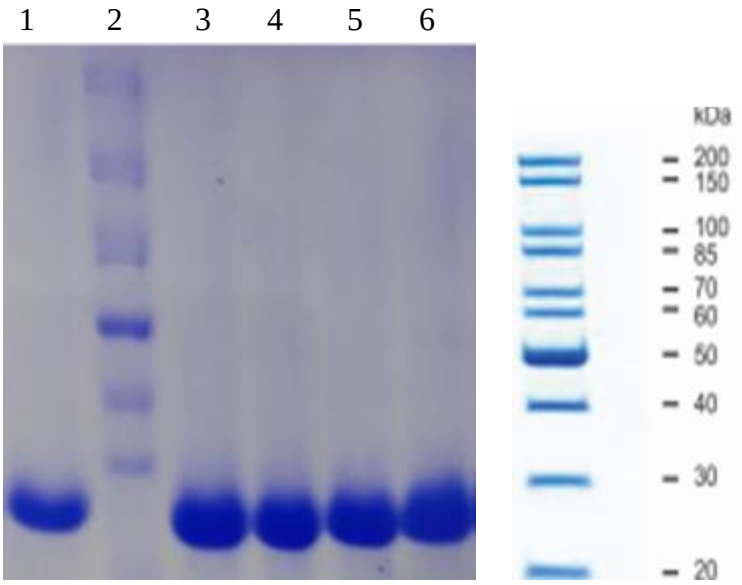
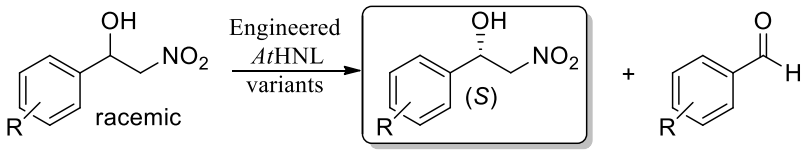


Figure 4.1: SDS PAGE image of purified *At*HNL and its variants. Lanes 1: wild type *At*HNL, 2: protein ladder, 3: F179K, 4: F179M, 5: F179T, and 6: Y14M



Scheme 4.1: Engineered *At*HNL variants catalysed retro-Henry reaction

Table 4.2: Screening of crude *At*HNL mutants in enantioselective cleavage of four different racemic β-nitroalcohols.

*At*HNL variants	1-(3,5-dimethoxyphenyl)-2-nitroethanol		1-(3-hydroxyphenyl)-2-nitroethanol		1-(4-methoxyphenyl)-2-nitroethanol		1-(4-nitrophenyl)-2-nitroethanol	
	% *ee*	% conv.	% *ee*	% conv.	% *ee*	% conv.	% *ee*	% conv.
wild type	8.16	49.97	9.51	38.95	7.88	49.92	–0.64	28.73
F179A	2.72	46.11	–4.16	43.65	0.21	48.26	–1.24	25.32
F179C	13.26	51.90	64.63	29.45	33.75	52.24	–0.55	26.42
F179D	–0.51	45.18	16.63	53.76	0.71	49.24	–0.96	24.43
F179E	–8.00	41.97	–5.65	44.26	1.95	49.88	–1.80	25.64
F179G	1.24	45.90	1.95	42.78	1.79	47.68	0.42	26.45
F179H	19.39	53.62	42.20	39.42	16.75	43.63	0.18	25.56
F179I	2.78	46.14	49.19	22.72	36.04	51.84	–1.72	24.58
F179K	6.31	48.16	71.22	36.15	26.35	41.69	1.99	26.49
F179L	8.20	49.02	75.64	30.45	33.38	41.94	1.94	27.11
F179M	10.73	49.24	68.97	29.19	25.39	44.18	0.54	24.35
F179N	1.58	45.73	15.75	50.53	1.28	48.55	–1.25	24.48
F179P	1.12	45.57	25.88	52.97	2.96	48.18	–3.01	23.61
F179Q	2.48	46.74	1.98	46.06	2.06	48.11	–1.06	24.45
F179R	3.55	46.94	2.94	46.83	0.46	48.24	0.85	25.99
F179S	6.00	47.62	6.96	49.99	1.65	48.46	–0.94	24.65
F179T	2.01	45.86	15.33	43.99	6.73	47.46	–0.27	25.48
F179V	7.50	48.49	32.22	45.07	8.89	46.02	–0.25	24.91
F179W	1.86	46.41	–1.15	44.70	1.09	48.50	–1.38	26.43
F179Y	2.73	46.30	17.35	47.06	3.39	47.92	–1.03	26.03
Y14A	3.70	48.00	0.35	45.73	–4.25	46.09	–1.04	29.03
Y14C	1.52	46.11	7.37	48.26	–0.85	47.41	–1.60	25.01

table continued in next page

	% *ee*	% conv.	% *ee*	% conv.	% *ee*	% conv.	% *ee*	% conv.
Y14D	3.52	47.18	–2.99	46.04	–1.16	47.70	–1.92	25.83
Y14E	3.11	47.20	–4.66	45.29	1.00	48.42	0.75	26.95
Y14F	4.30	47.54	31.75	46.75	9.14	46.70	–0.59	25.56
Y14G	–0.56	45.76	8.03	50.23	0.85	46.80	–1.53	23.97
Y14H	0.74	46.18	7.71	48.97	1.23	46.80	0.31	24.63
Y14I	–0.17	45.86	1.80	48.33	0.60	46.34	–0.27	25.80
Y14K	2.99	47.05	10.56	51.22	1.09	48.08	–2.05	24.26
Y14L	2.86	46.17	8.94	47.67	1.94	46.54	–1.02	24.38
Y14M	2.74	46.28	19.96	44.27	7.08	47.03	–0.27	23.83
Y14N	2.96	49.97	6.85	50.66	1.14	48.52	–0.61	39.17
Y14P	–1.88	44.27	13.51	53.40	0.22	48.05	–0.35	24.35
Y14Q	7.88	48.89	–12.42	40.29	0.83	48.33	–1.39	21.44
Y14R	1.77	46.53	–4.09	45.94	–0.11	48.11	–1.40	25.41
Y14S	3.56	47.35	3.20	47.55	0.75	47.98	0.27	25.98
Y14T	4.57	49.60	–0.63	46.95	0.64	48.18	–0.46	31.14
Y14V	2.27	46.89	0.16	47.37	0.71	48.51	–1.65	24.79
Y14W	0.67	46.08	–1.46	45.20	1.13	47.13	–1.24	26.15
A13G	-0.12	45.77	13.66	53.81	1.77	48.59	–1.81	19.68
F80A	0.00	45.40	5.40	47.29	0.53	47.70	–0.84	23.72
F82A	-0.01	45.94	–1.56	46.66	0.83	48.39	–0.43	26.16
F82K	4.78	47.93	6.55	50.62	0.86	47.43	–0.21	25.28
F82P	1.27	46.34	4.55	50.83	–0.98	47.17	–1.05	23.74
F82Q	3.15	47.27	0.98	47.54	1.53	47.59	–2.24	23.34
F82R	4.68	47.79	1.32	49.16	–0.54	47.72	0.19	25.73
F82S	4.81	48.26	1.26	47.78	0.38	47.52	–0.44	25.34
F82T	4.16	47.00	3.98	48.49	2.44	47.78	–1.49	21.37
H15A	2.82	46.69	–0.88	47.26	0.56	47.43	–2.05	22.31

4.4.5. Investigation of substrate scope of selected purified *At*HNL variants towards retro-Henry reaction

To study the substrate scope of the designated *At*HNL mutants, we have selected six different racemic β-nitroalcohols. Wild type *At*HNL catalysed enantioselective cleavage of racemic 2-chloro NPE, 3-chloro NPE, 4-chloro NPE, 3-hydroxy NPE, 4-methoxy NPE and 4-methyl NPE has produced corresponding (*S*)-β-nitroalcohols in 48, 85, 35, 67, 40 and 56% *ee* and 46, 51, 43, 34, 40 and 41% conversions respectively in 3 to 7 hours (**Table 4.3**). The low to medium range of % *ee* of products by the wild type *At*HNL indicates its poor enantioselectivity and narrow substrate scope towards the abovementioned six substrates. Of the variants tested, F179K has shown high activity towards enantioselective cleavage of 2-chloro NPE, 4-chloro NPE, 3-hydroxy NPE and 4-methoxy NPE compared to the wild type *At*HNL, and produced corresponding (*S*)-β-nitroalcohols in 98, 74, 99, 99 % *ee* and 43, 43, 29, 33% conversions respectively in 3 to 7 hours. (HPLC chromatograms: **Figures 4.4.1 to 4.4.6)** The F179T variant has shown high activity towards enantioselective cleavage of 3-chloro NPE and 4-methyl NPE than the wild type, and produced the corresponding (*S*)-β-nitroalcohols in 99, 98% *ee* and 54, 43% conversions respectively in 3 hours. The above data is represented in **Figure 4.2**.

Table 4.3: Investigation of substrate scope of purified *At*HNL variants in retro-Henry reaction

Enzyme	4 - Methoxy NPE		4 - Methyl NPE		3 - Hydroxy NPE		2 - Chloro NPE		3 - Chloro NPE		[a]4 - Chloro NPE	
	% *ee*	% conv	% *ee*	% conv	% *ee*	% conv	% *ee*	% conv	% *ee*	% conv	% *ee*	% conv
WT	39.94	39.95	56.07	41.10	66.72	34.07	48.10	45.78	85.46	50.86	34.82	43.33
F179K	98.87	33.20	93.43	46.96	99.16	29.34	98.06	43.30	94.74	52.26	74.71	42.77
F179M	62.40	37.07	64.09	40.38	99.22	24.98	74.03	42.99	96.15	51.10	32.02	42.98
F179T	96.94	40.33	97.58	42.57	99.41	27.63	95.18	42.59	98.59	54.32	57.90	41.89
Y14M	61.09	33.51	79.64	42.66	98.41	32.15	63.67	47.63	84.58	52.47	33.95	43.32

a: reaction time of WT *At*HNL and four variants towards 4-Choro NPE cleavage is 7 h and towards other five substrates cleavage the reaction time is 3 h.

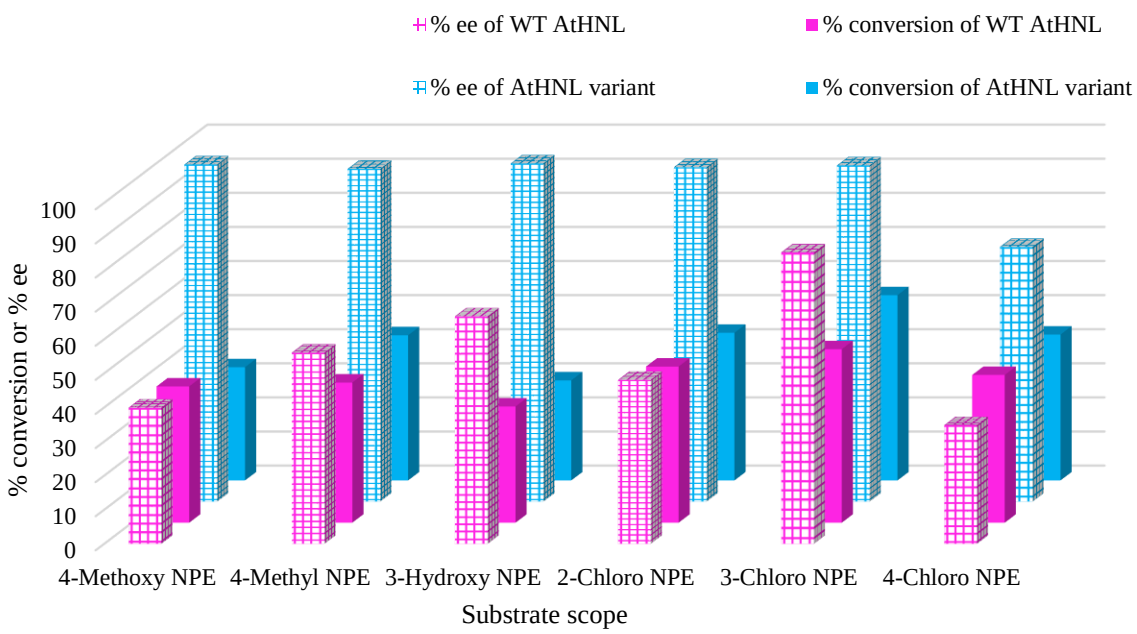


Figure 4.2: Substrate scope of *At*HNL variants in the retro-Henry reaction. The variants compared with wild type for the set of substrates are: 4-methoxy NPE-F179K, 4-methyl NPE- F179T, 3-hydroxy NPE- F179K, 2-chloro NPE- F179K, 3-chloro NPE-F179T, and 4-chloro NPE- F179K.

4.5. Discussion

Improved substrate scope by protein engineering has been reported in the case of *Ac*HNL and *Gt*HNL catalysed stereoselective synthesis of (*R*)-β-nitroalcohols.[4] The *Gt*HNL and *Ac*HNL variants, e.g., A40H, A40R, and A40H-V42T-Q110H, were tested in the synthesis of (*R*)-β-nitroalcohols. Wild type *Ac*HNL has shown moderate enantioselectivity i.e., 79% and poor yield of 38% in the synthesis of (*R*)-NPE in 24 hours. Similarly, wild type *Gt*HNL also showed poor enantioselectivity and yield i.e., 46% *ee* and 15% yield in the synthesis of (*R*)-NPE. However, *Ac*HNL variants have shown 66–74% conversion and 93–97% *ee* of (*R*)-NPE in 24 h reaction time. In 2 to 4 h, up to >99% *ee* of the product was achieved. Three *Gt*HNL muteins produced 15–75% conversion and 94–98% *ee* of the (*R*)-NPE in 24 h. When 2-chlorobenzaldehyde was used as substrate, *Ac*HNL-A40H and A40R variants showed high activity in the synthesis of corresponding

(*R*)-β-nitroalcohol i.e., 89–95% conversion with 80–83% *ee*, as compared to other *Ac*HNL and *Gt*HNL muteins. In the case of cyclohexanecarboxaldehyde as substrate, triple variants of both *Ac*HNL and *Gt*HNL have shown 80–86% conversion and 96.9–98% *ee* of its corresponding (*R*)-β-nitroalcohol. Similarly, in the case of hexanal, synthesis of its corresponding (*R*)-β-nitroalcohol by both the triple variants gave 93.7–95% conversion and >99% *ee*. Not only nitroaldol reaction, but also in case retro-nitroaldol (Henry) reaction, improved enzymatic activity was observed by *Hb*HNL variants compared to the wild type enzyme.[5] *Hb*HNL triple mutant, L121Y-F125T-L146M has shown ~5.5-fold higher specific activity, e.g., 0.71 U mg^{-1} vs. 0.13 U mg^{-1} and k_{cat} 3.3 times higher than the wild type towards the cleavage of racemic NPE.

A literature survey of different biocatalytic methods known in the synthesis of enantiopure β-nitroalcohols that are discussed in the current study are summarized below. *Hb*HNL catalysed Henry reaction was reported in the synthesis of (*S*)-1-(3-hydroxyphenyl)-2-nitroethanol in 18% *ee* and 46% yield in 48 h, at pH 7.0.[13] In our study *At*HNL F179K variant has produced (*S*)-1-(3-hydroxyphenyl)-2-nitroethanol in 99% *ee* and 29% conversion (**Table 4.3.).** This clearly shows the higher enantioselectivity of F179K variant compared to *Hb*HNL although the former has shown lesser conversion to (*S*)-1-(3-hydroxyphenyl)-2-nitroethanol than the latter. In a study, to increase the enantiomeric excess of (*S*)-β-nitroalcohols in the *Hb*HNL catalysed Henry reaction, it was performed at pH 5.5. This biotransformation has produced (*S*)-1-(2-chlorophenyl)-2-nitroethanol, (*S*)-1-(3-chlorophenyl)-2-nitroethanol, (*S*)-1-(4-chlorophenyl)-2-nitroethanol and (*S*)-1-(4-methoxyphenyl)-2-nitroethanol in 95, 98, 97 and 99% *ee* and 23, 36, 25, and 20% yield respectively.[14] In our current study, *At*HNL-F179K has produced (*S*)-1-(2-chlorophenyl)-2-nitroethanol, (*S*)-1-(4-chlorophenyl)-2-nitroethanol and (*S*)-1-(4-methoxyphenyl)-2-nitroethanol in 98, 75 and 99% *ee* and 43, 42 and 33% conversion respectively (**Table 4.3**). Comparison of the

results specifies better % *ee* and high yield by the *At*HNL-F179K than *Hb*HNL in the synthesis of 2-chloro and 4-methoxy substituted (*S*)-β-nitroalcohols, despite of the fact we used retro-Henry reaction as compared to Henry reaction by *Hb*HNL. However, in the production of (*S*)-1-(4-chlorophenyl)-2-nitroethanol, *At*HNL F179K variant showed low enantioselectivity than *Hb*HNL, although the conversion was higher in case of the former. In the case of (*S*)-1-(3-chlorophenyl)-2-nitroethanol production, *At*HNL-F179T showed better enantioselectivity (99% *ee*) and high conversion (54%) as compared to *Hb*HNL.

A two-step enzymatic method comprising of D-aminoacylase catalysed nitroaldol reaction and immobilized *Burkholderia cepacia* (PS-IM) lipase catalysed kinetic resolution has produced enantiopure (*R*)-acetates of β-nitroalcohols and (*S*)-β-nitroalcohols with excellent enantiomeric excess and conversion.[15] Reactions of β-nitroalcohols with *meta* or *para* substituents such as 3-Cl, 4-Cl, 4-MeO and 4-Me NPEs have produced corresponding unreacted (*S*)-β-nitroalcohols with 91, 97, 84, and 84% *ee* and 53, 54, 48 and 47% conversion (these % conversions were calculated as 100 minus the given % conversions to *R*-β-nitroalcohol acetates as found in the publication) respectively. However, in the case of β-nitroalcohol with *ortho* substituted substrates such as 2-Cl NPE, almost no product was observed (<1% yield and no *ee*). In our study, *At*HNL-F179K catalysed retro-Henry reaction of 4-Cl NPE has produced its corresponding (*S*)-β-nitroalcohol with less enantioselectivity and moderate conversion compared to PS-IM with 74% *ee* and 43% conversion. When 4-MeO NPE was used, *At*HNL-F179K has produced its corresponding (*S*)-β-nitroalcohol in 99% *ee* and 33% conversion. Here the % conversion was found to be moderate, while % *ee* of the product was very high as compared to PS-IM catalysed kinetic resolution. In the case of 2-Cl NPE as the substrate, *At*HNL-F179K has produced its corresponding (*S*)-β-nitroalcohol with 98% *ee* and 43% conversion, while PS-IM has failed to accept this substrate. In

the case of 3-Cl NPE and 4-Me NPE, our approach has found *At*HNL-F179T that produced their corresponding (*S*)-β-nitroalcohols in 99, 98% *ee* and 54, 43% conversion (**Table 4.3.**) respectively as compared to 91 and 84% *ee* by PS-IM.

*At*HNL catalysed nitroaldol reaction has been reported in the synthesis of a number of β-nitroalcohols, but with (*R*)- selectivity.[2] Thirteen different aromatic aldehydes were converted into the corresponding (*R*)-β-nitroalcohols by the wild type enzyme with 68–99.9% *ee* and 2–34% yield in 2 h. Reaction with aliphatic aldehydes proceeded slowly and traces of corresponding adduct was formed which indicates poor activity of *At*HNL towards aliphatic aldehydes. *Burkholderia cepacia* lipase (lipase PS, Amano IM) catalysed enantioselective transesterification of racemic aromatic β-nitroalcohols has produced (*R*)-β-nitroalcohols and the corresponding (*S*)-acetylated products.[16] The optimized reaction conditions used here are, 5: 1 (w/w) enzyme to substrate ratio in hexane, while the reaction time was 12–24 h. This kinetic resolution has used *meta* and *para* substituted substrates such as 3-Cl, 4-Cl and 4-Me NPEs and produced corresponding (*S*)-β-nitroalcohol acetates with 98, 99, and 98% *ee* and 52, 49, and 51% conversions respectively. We found similar results by *At*HNL-F179T towards 3-Cl and 4-Me NPE where corresponding (*S*)-β-nitroalcohols were produced in 99 and 98% *ee* and 54 and 43% conversion (**Table 4.3.**). In the case of 4-Cl NPE, our *At*HNL-F179K has gave 74% *ee* and 43% conversion of its (*S*)-β-nitroalcohol. The advantage of our method is it produced (*S*)-β-nitroalcohols, while the lipase PS catalysed kinetic resolution produced its acetate derivatives. 2-Cl NPE was not catalysed by the lipase PS, while we achieved high % *ee* of product *At*HNL-F179K as explained earlier.

Human serum albumin (HSA) catalysed asymmetric nitroaldol (Henry) reaction in the synthesis of (*R*)-β-nitroalcohols was reported in water at neutral pH.[17] Eight different aromatic aldehydes were converted into the corresponding enantioenriched β-nitroalcohols with 33–79% *ee* and 44–

93% yield in 168 h at 0 °C. Acyl peptide releasing enzyme from *Sulfolobus tokodaii* (ST0779) was reported to catalyse asymmetric nitroaldol reaction in the synthesis of enantioenriched β-nitroalcohols.[18] Here too, eight aromatic enantioenriched β-nitroalcohols were synthesized in 34-92% yield, and 17-99% *ee* in 18-90 h reaction time, however the absolute configuration of the products was not clearly defined. Two products, 4-Cl NPE and 4-MeO NPE found in this report can be compared with our study. In the case of 4-Cl NPE, and 4-MeO NPE, the ST0779 has produced the corresponding products with 78 and 86% *ee* and 45, and 32% yield respectively, in 60-72 h. Our *At*HNL-F179K has shown similar activity compared to ST0779 with 74 and 99% *ee* and 43 and 33% conversions of (*S*)-4-Cl NPE and (*S*)-4-MeO NPE respectively in only 3-7 hours (**Table 4.3.**).

We have prepared ten aromatic (*S*)-β-nitroalcohols using wild type *At*HNL catalysed retro-Henry reaction under optimized biocatalytic reaction conditions.[3] At least half a dozen aromatic (*S*)-β-nitroalcohols were prepared with up to 99% *ee* and 47% conversion and *E* values up to 84. Racemic β-nitroalcohols such as 3-OH NPE, 4-MeO NPE, and 4-Me NPE were converted into their corresponding (*S*)-β-nitroalcohols with 81, 44 and 85% *ee* and 19, 41 and 45% conversions respectively. In the current study, *At*HNL F179K variant has shown very high enantioselectivity of 99% *ee* each towards the preparation of (*S*)-3-OH NPE, and (*S*)-4-MeO NPE in 29 and 33% conversions respectively (**Table 4.3.**). Similarly, in the case of 4-Me NPE corresponding (*S*)-product was formed by *At*HNL-F179T in high *ee* (98%) and 43% conversion compared to the wild type enzyme. Recently we have shown celite immobilized *At*HNL catalysed retro-Henry reaction and produced a dozen (*S*)-β-nitroalcohols with 5–98% *ee* and 44–61% conversions in 9–30 h reaction time.[19] Racemic β-nitroalcohols such as 2-Cl NPE, 3-Cl NPE, 4-Cl NPE and 4-Me NPE were converted into their corresponding (*S*)-β-nitroalcohols with 33, 73, 16 and 68% *ee* and 49,

55, 47 and 48% conversions respectively. In our current study, *At*HNL-F179K has shown high enantioselectivity compared to celite immobilized *At*HNL towards the production of (*S*)-2-Cl NPE, and (*S*)-4-Cl NPE with 98 and 75% *ee* and 43 and 43% conversion respectively (**Table 4.3**). Similarly, *At*HNL F179T variant showed high enantioselectivity compared to celite immobilized *At*HNL towards the production of (*S*)-3-Cl NPE, and (*S*)-4-Me NPE with 99 and 98% *ee* and 54 and 43% conversion respectively.

Ketoreductases (KREDs) were reported to catalyse asymmetric reduction of class I (1-aryl-2-nitro-1-ethanone) and class II α-nitro ketones (1-aryloxy-3-nitro-2-propanone).[12] YGL039w from *Saccharomyces cerevisiae* was used in the reduction of 23 different class I (1-aryl-2-nitro-1-ethanone) α-nitro ketones and produced the corresponding enantiomers of β-nitroalcohols with 18–99% conversion and 6–99% *ee*. YGL039w showed (*S*)-selectivity for all tested substrates except for 1-naphthyl, benzyl, and phenethyl derivatives. Class I α-nitro ketones having 2-Cl, 3-Cl, 4-Cl, 4-OMe and 4-Me substituents on the phenyl ring were converted into their corresponding (*S*)-NPEs with 90, 29, 98, 99 and 96% *ee* and 63, 73, 99, 96 and 96% conversions respectively. In the present study *At*HNL-F179K showed comparable enantioselectivity to YGL039w and produced 2-Cl, 4-Cl, and 4-OMe NPEs in 98, 75, and 99% *ee*, however with 43, 43, and 33% conversions respectively (**Table 4.3.**). Towards the preparation of (*S*)-3-Cl and (*S*)-4-Me NPEs, *At*HNL-F179T has shown excellent enantioselectivity similar to YGL039w (99 and 98% *ee*) but with 54 and 43% conversion respectively. Undoubtedly, the biocatalytic asymmetric reduction has the advantage of achieving higher conversion than the retro-Henry reaction, however, the earlier process requires additional chemical synthesis of the α-nitro ketones which are not readily available and cofactor dependent enzymes, while racemic β-nitroalcohols used in our method can be easily prepared from the commercially available aldehydes.

4.6. Conclusions

Screening of *At*HNL variants library resulted from alteration at three positions, Phe82, Phe179 and Tyr14, was carried out using crude enzyme extracts towards the enantioselective cleavage of multiple racemic β-nitroalcohols. The screening has identified F179K and F179M as the mutants with best activity. Towards cleavage of 1-(3,5-dimethoxyphenyl)-2-nitroethanol, 1-(3-hydroxyphenyl)-2-nitroethanol, 1-(4-methoxyphenyl)-2-nitroethanol and 1-(4-nitrophenyl)-2-nitroethanol wild type *At*HNL has shown poor enantioselectivity of –1 to 10% *ee*, and 29–50% conversion. Pleasantly, the *At*HNL-F179K has shown higher enantioselectivity towards the cleavage of 1-(3-hydroxyphenyl)-2-nitroethanol and 1-(4-methoxyphenyl)-2-nitroethanol with 71 and 26% *ee* and 36 and 42% conversion respectively. The *At*HNL-F179M has shown higher enantioselectivity in case of the retro-Henry reaction of 1-(3,5-dimethoxyphenyl)-2-nitroethanol, 1-(3-hydroxyphenyl)-2-nitroethanol and 1-(4-methoxyphenyl)-2-nitroethanol and produced the corresponding (*S*)-NPEs with 11, 69 and 25% *ee* and 49, 29 and 44% conversions respectively. F179T and Y14M were selected from another relevant study where the same library was screened against enantioselective cleavage of racemic NPE. Overall, four *At*HNL mutants F179K, F179M, F179T and Y14M have been selected to find out their substrate scope towards enantioselective cleavage of a six different racemic β-nitroalcohols. Substrate scope of F179K, F179M, F179T and Y14M was investigated towards the retro-Henry reaction. Racemic 2-Cl NPE, 3-Cl NPE, 4-Cl NPE, 3-OH NPE, 4-MeO NPE and 4-Me NPE were used in the wild type *At*HNL catalysed enantioselective cleavage study. These biotranformations have produced corresponding (*S*)-β-nitroalcohols in 48, 85, 35, 67, 40 and 56% *ee* and 46, 51, 43, 34, 40 and 41 % conversions respectively in 3 to 7 hours. Of the variants tested, F179K has shown higher enantioselectivity towards enantioselective cleavage of 2-Cl NPE, 4-Cl NPE, 3-OH NPE and 4-MeO NPE compared

to wild type *At*HNL. It produced corresponding (*S*)-β-nitroalcohols in 98, 74, 99, 99 % *ee* and 43, 43, 29, 33% conversions respectively. We also found F179T that showed higher enantioselectivity towards the enantioselective cleavage of 3-Cl NPE and 4-Me NPE compared to the wild type. It produced corresponding (*S*)-β-nitroalcohols in 99, 98% *ee* and 54, 43% conversions respectively in 3 hours. The % *ee* of various (*S*)-β-nitroalcohols obtained by retro-Henry reaction are comparable with that of obtained lipase catalysed kinetic resolution or asymmetric reduction of corresponding α-nitro ketones. The yields of (*S*)-β-nitroalcohols prepared by the current method are in par with the kinetic resolution method, and in some cases even better (2-Cl NPE by PS-IM), but are less than that of the asymmetric reduction approach. Nonetheless, the asymmetric reduction process requires cofactor dependent enzymes, and addition chemical synthesis of the substrate α-nitro ketones which are not readily available, while our approach uses a cofactorless enzyme and racemic β-nitroalcohols as substrates which can be easily prepared from the commercially available aldehydes. For the first time, *At*HNL engineered mutants have been used in retro-Henry reaction. By using these variants, we have successfully produced six different (*S*)-β-nitroalcohols with high enantiomeric excess and conversions.

[1]H NMR and [13]C NMR Characterization of racemic β-nitroalcohols

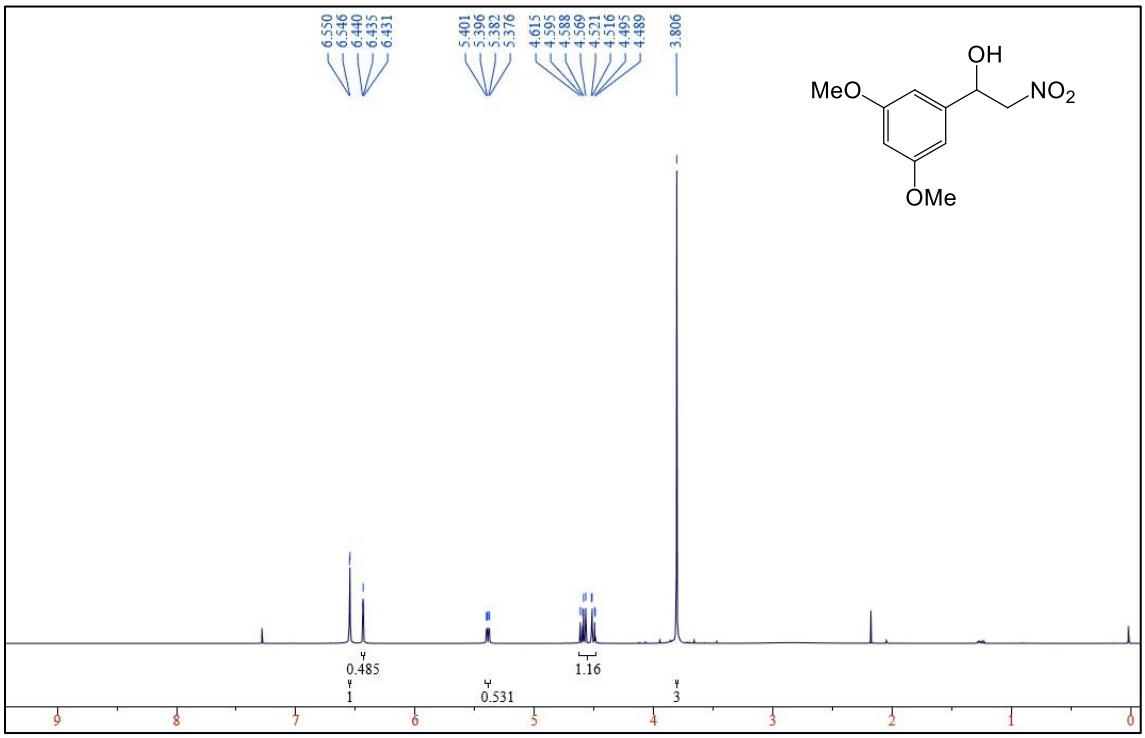


Figure 4.3.1: [1]H NMR spectrum of 1-(3,5-dimethoxyphenyl)-2-nitroethanol

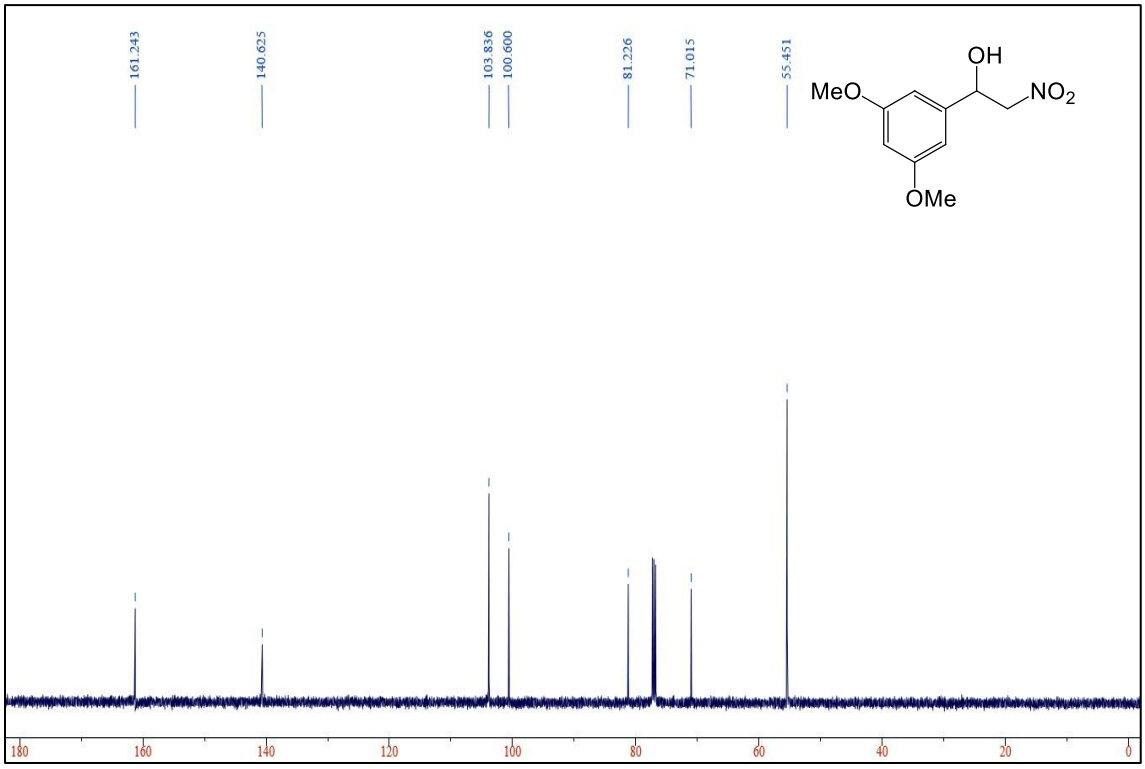


Figure 4.3.2: [13]C NMR spectrum of 1-(3,5-dimethoxyphenyl)-2-nitroethanol

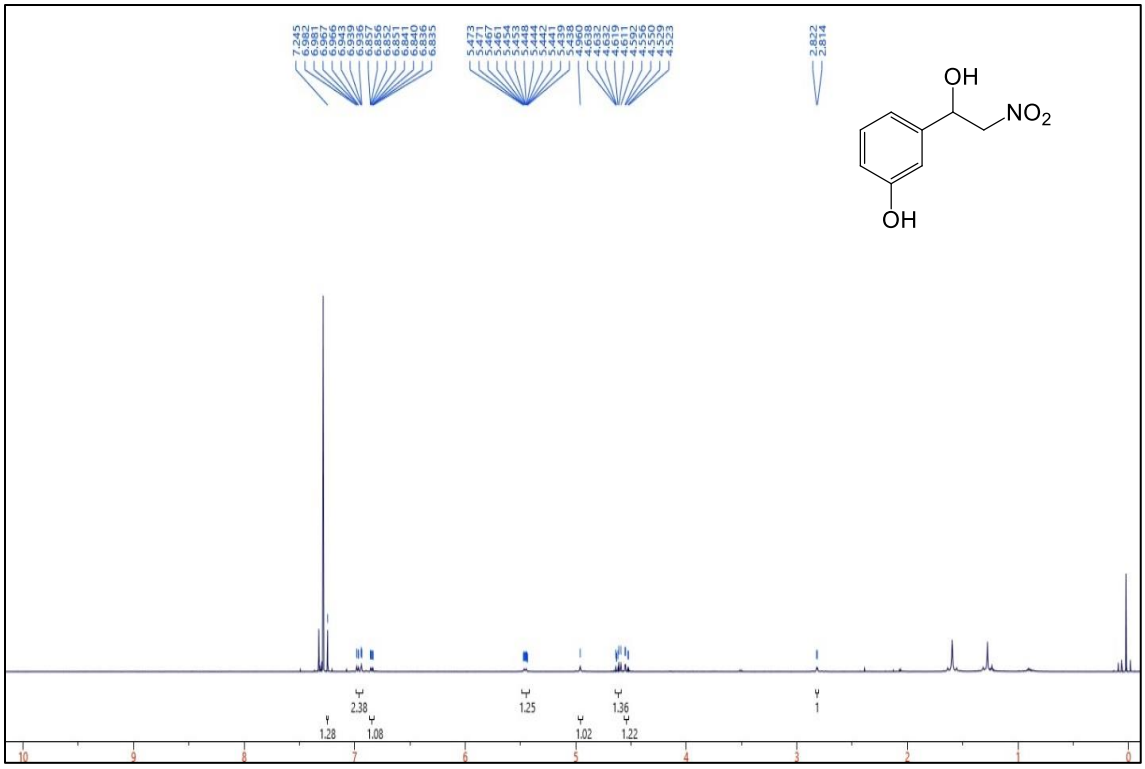


Figure 4.3.3: ^{1}H NMR spectrum of 1-(3-Hydroxyphenyl)-2-nitroethanol

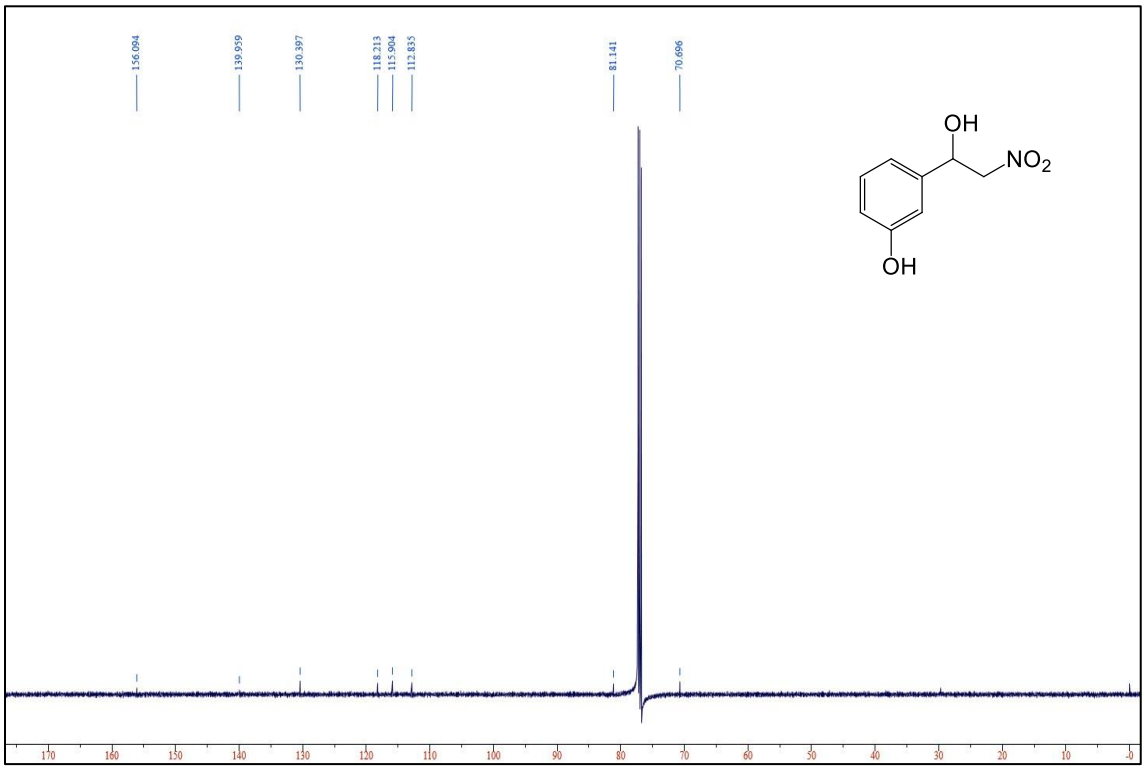


Figure 4.3.4: ^{13}C NMR spectrum of 1-(3-Hydroxyphenyl)-2-nitroethanol

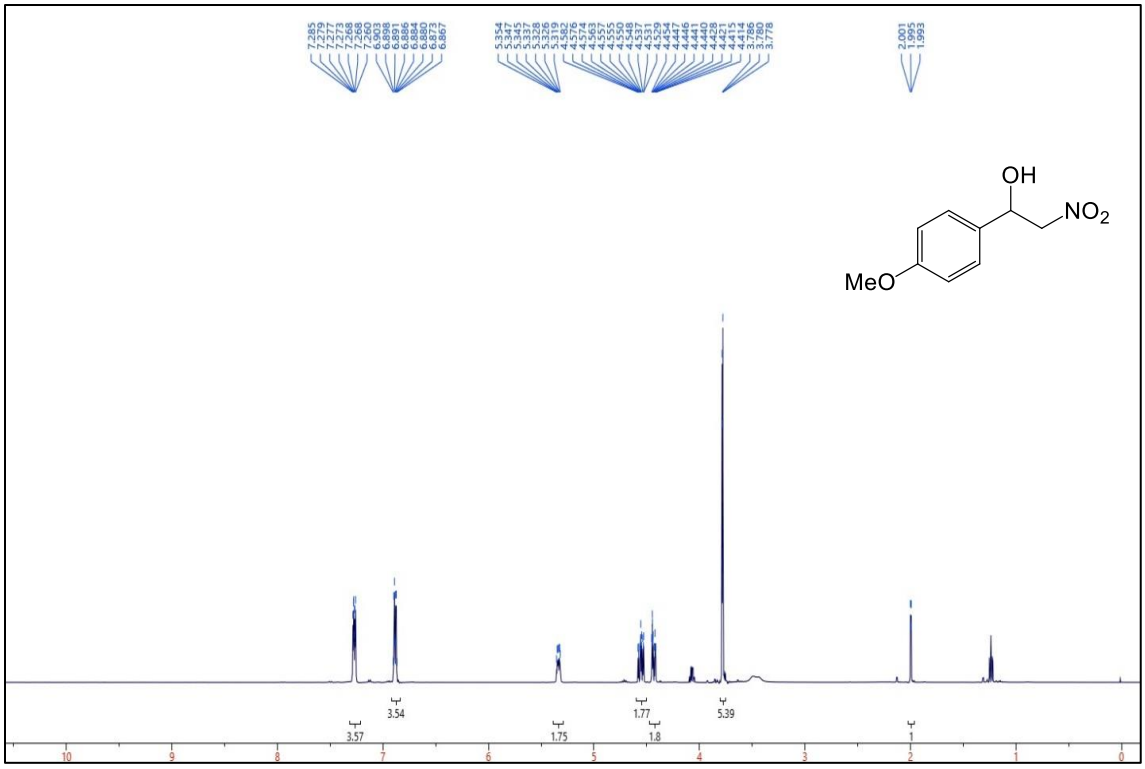


Figure 4.3.5: ^{1}H NMR spectrum of 1-(4-Methoxyphenyl)-2-nitroethanol

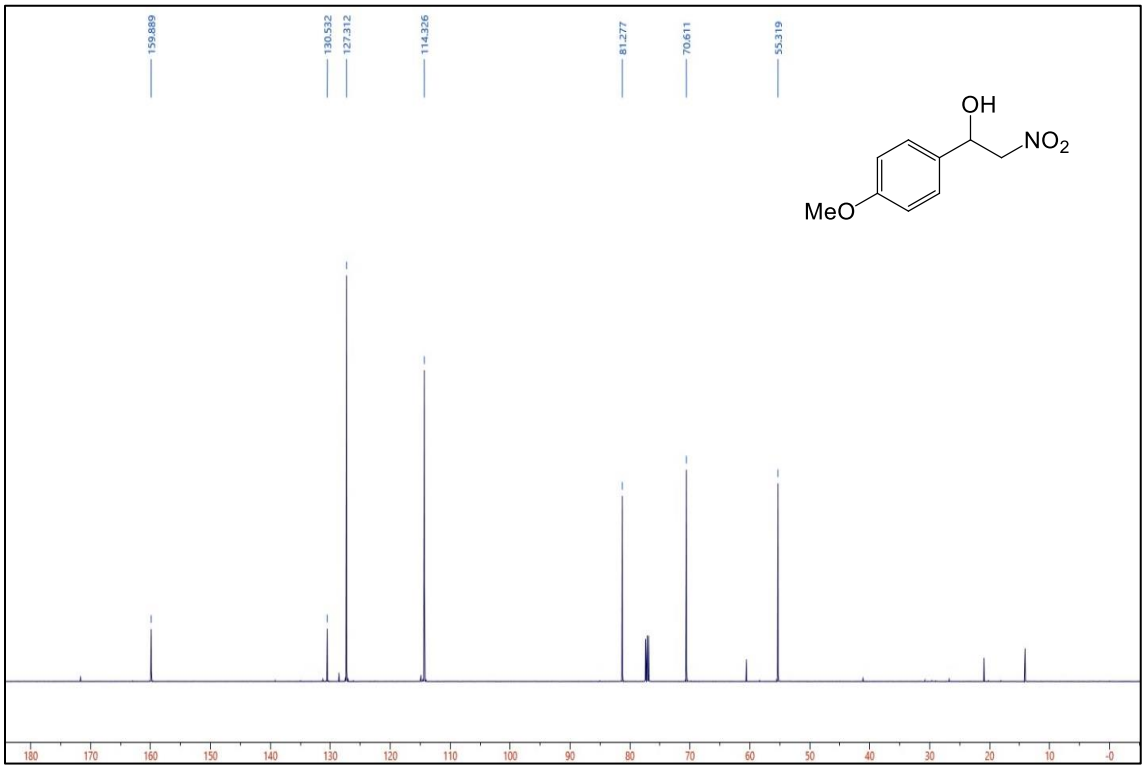


Figure 4.3.6: ^{13}C NMR spectrum of 1-(4-Methoxyphenyl)-2-nitroethanol

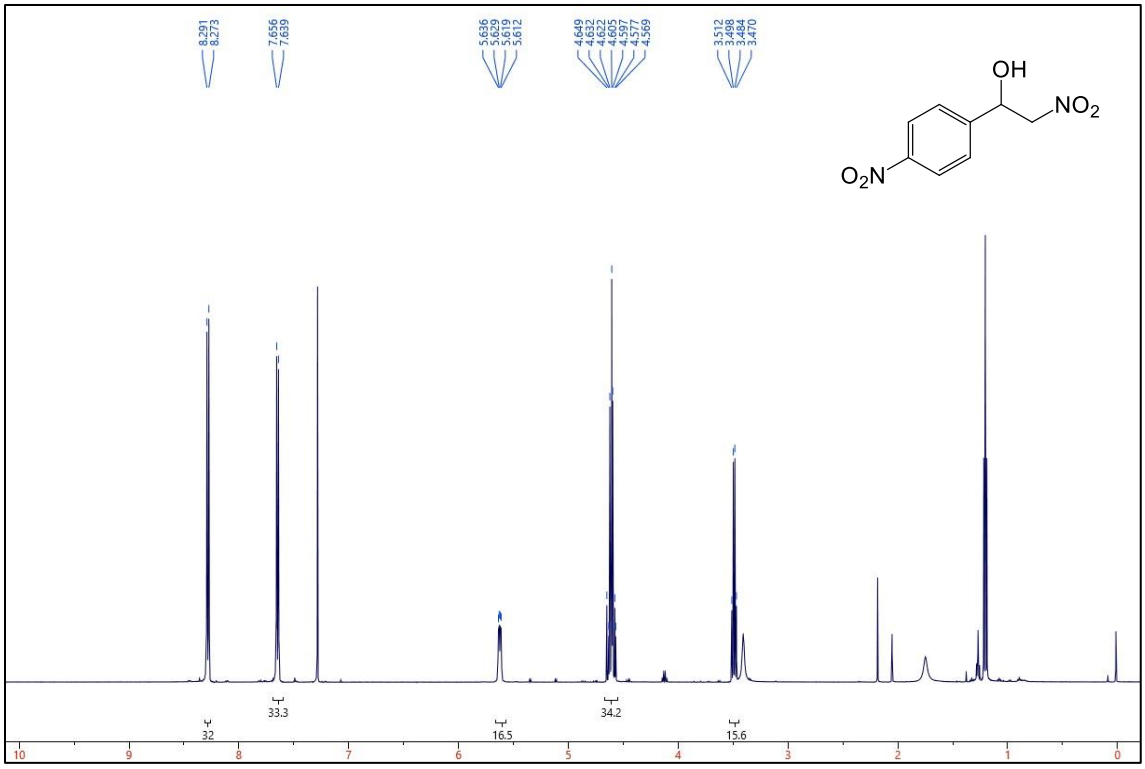


Figure 4.3.7: ^{1}H NMR spectrum of 1-(4-Nitrophenyl)-2-nitroethanol

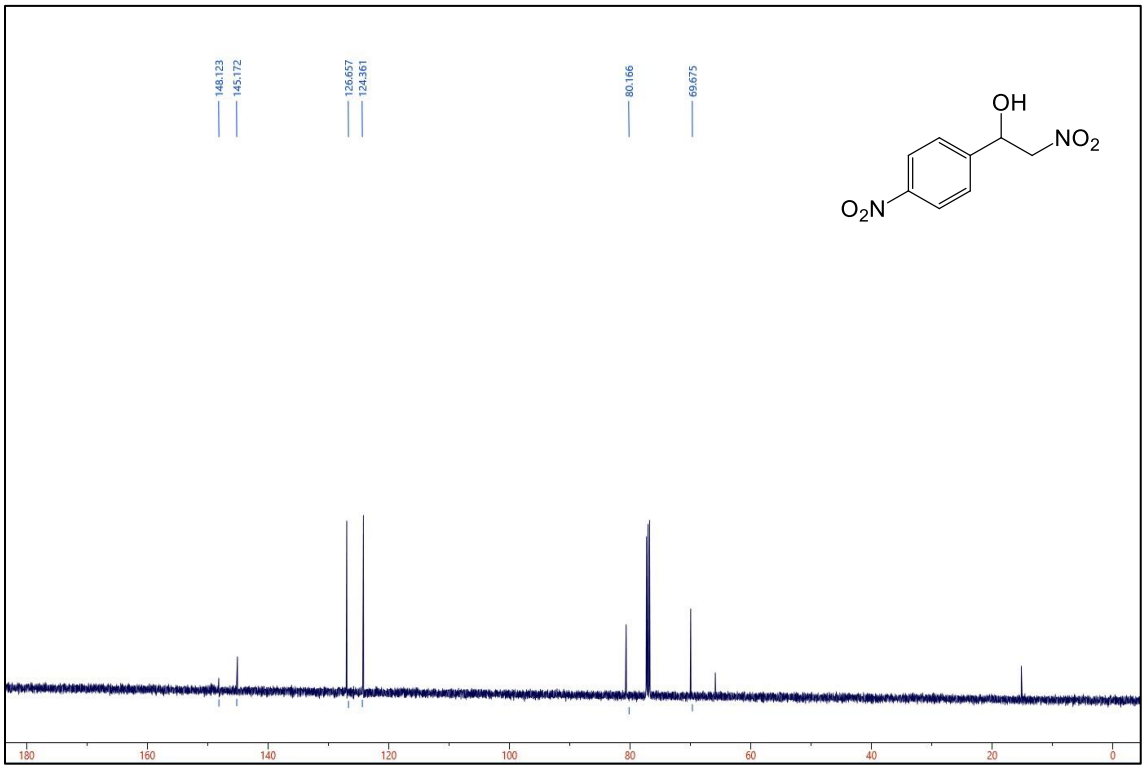


Figure 4.3.8: ^{13}C NMR spectrum of 1-(4-Nitrophenyl)-2-nitroethanol

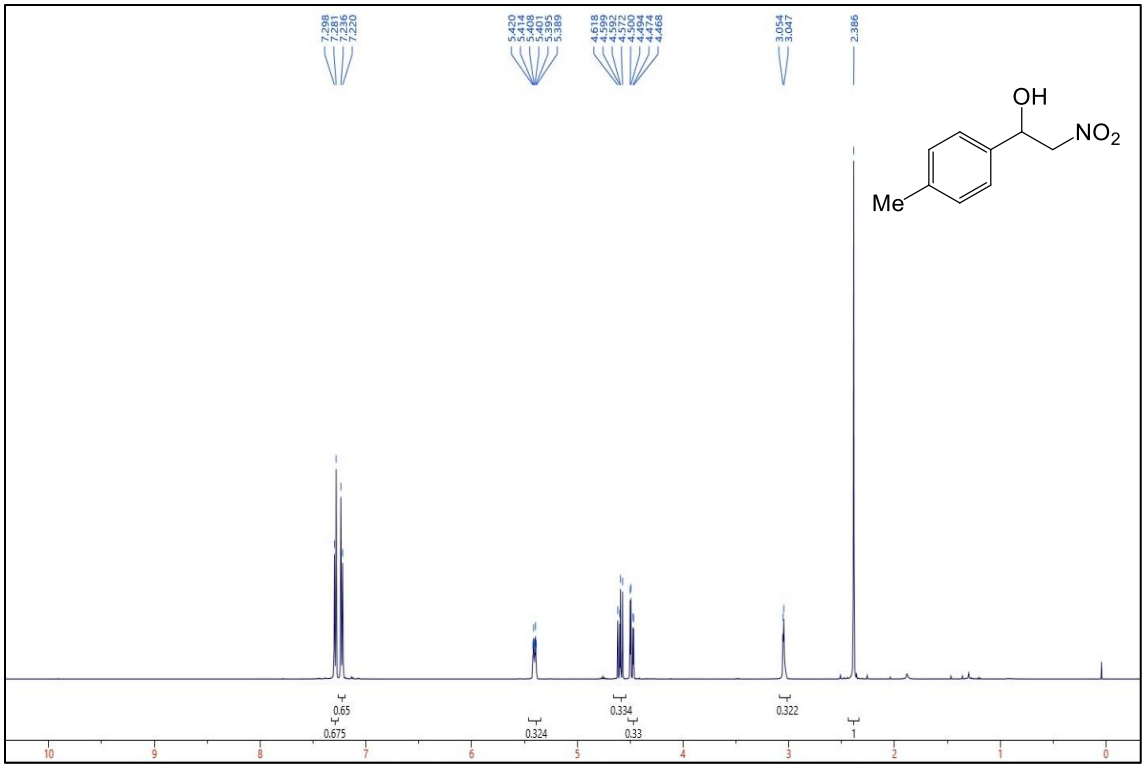


Figure 4.3.9: ^{1}H NMR spectrum of 1-(4-Methylphenyl)-2-nitroethanol

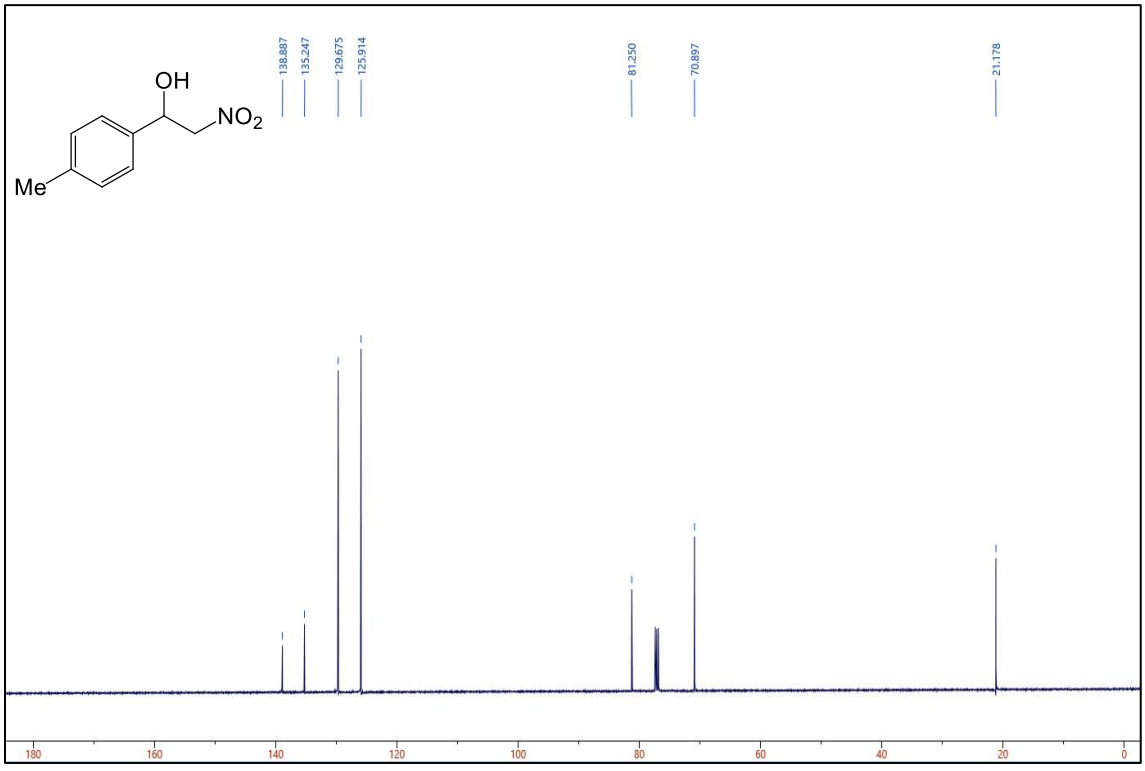


Figure 4.3.10: ^{13}C NMR spectrum of 1-(4-Methylphenyl)-2-nitroethanol

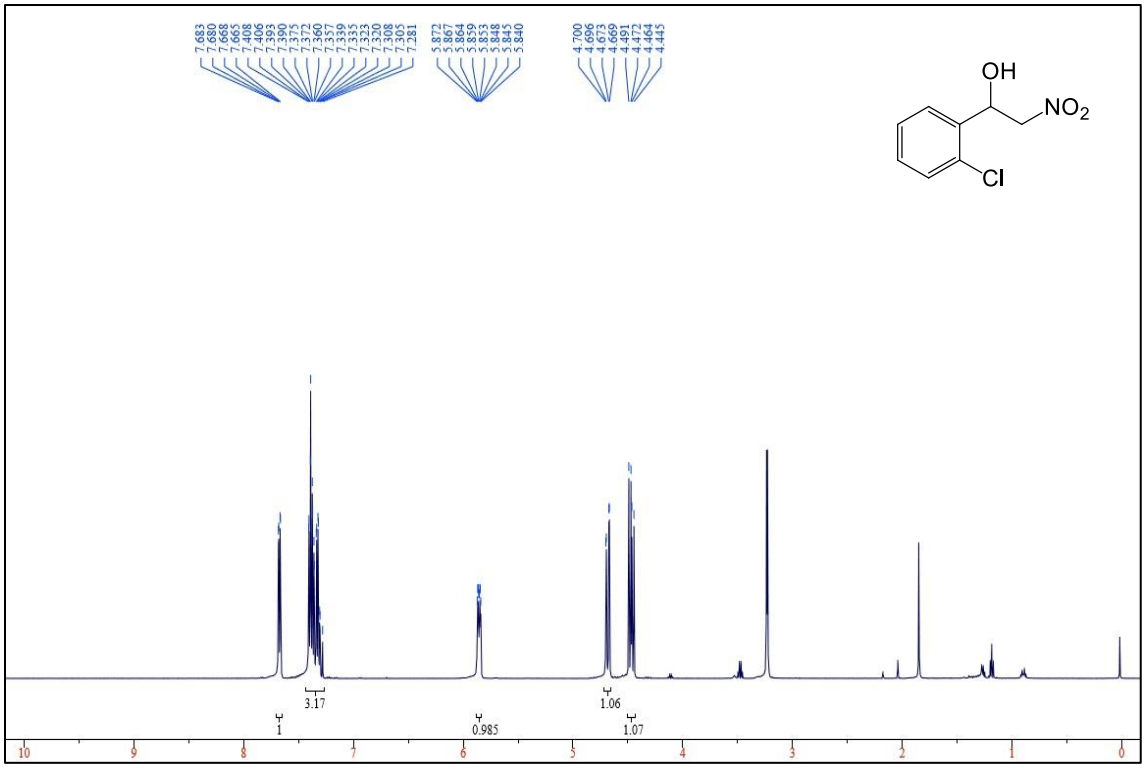


Figure 4.3.11: ^{1}H NMR spectrum of 1-(2-Chlorophenyl)-2-nitroethanol

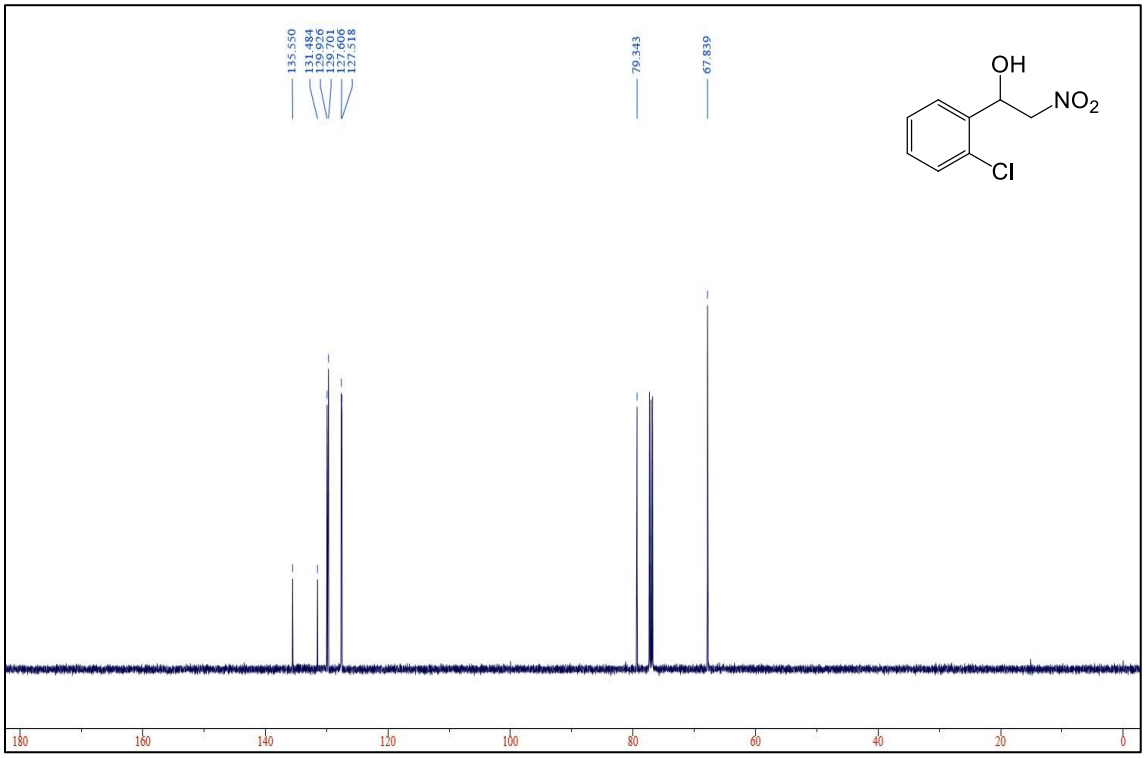


Figure 4.3.12: ^{13}C NMR spectrum of 1-(2-Chlorophenyl)-2-nitroethanol

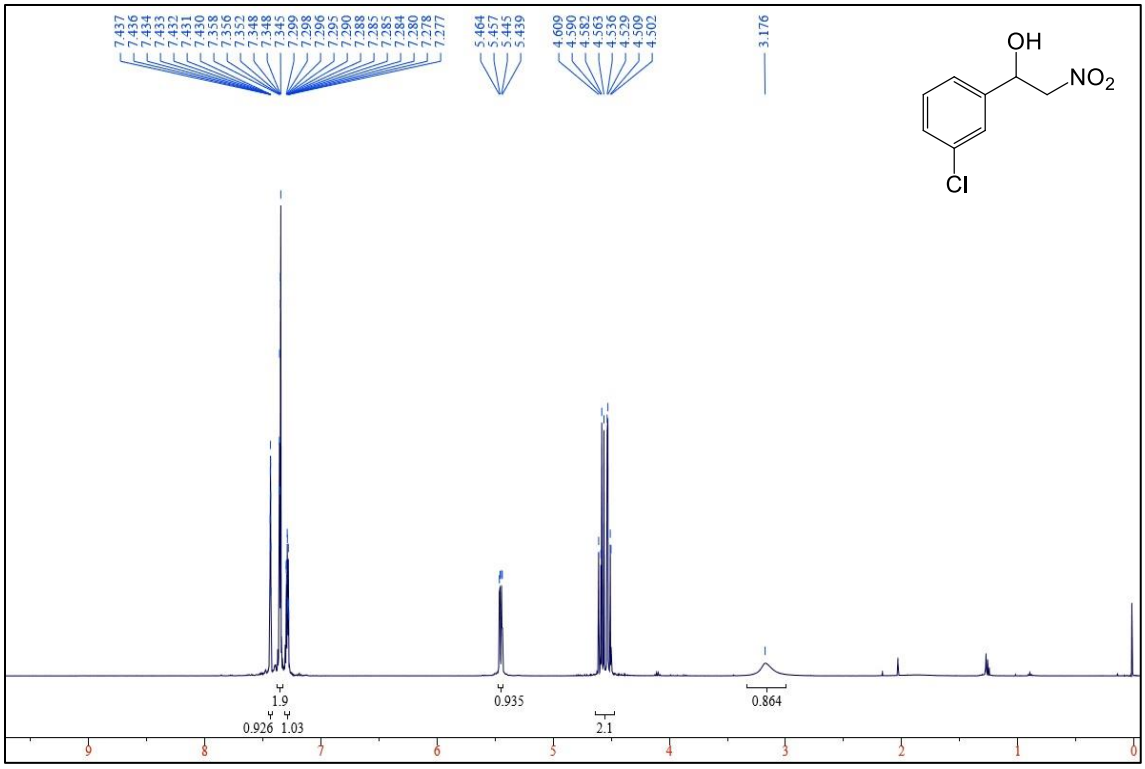


Figure 4.3.13: ^{1}H NMR spectrum of 1-(3-Chlorophenyl)-2-nitroethanol

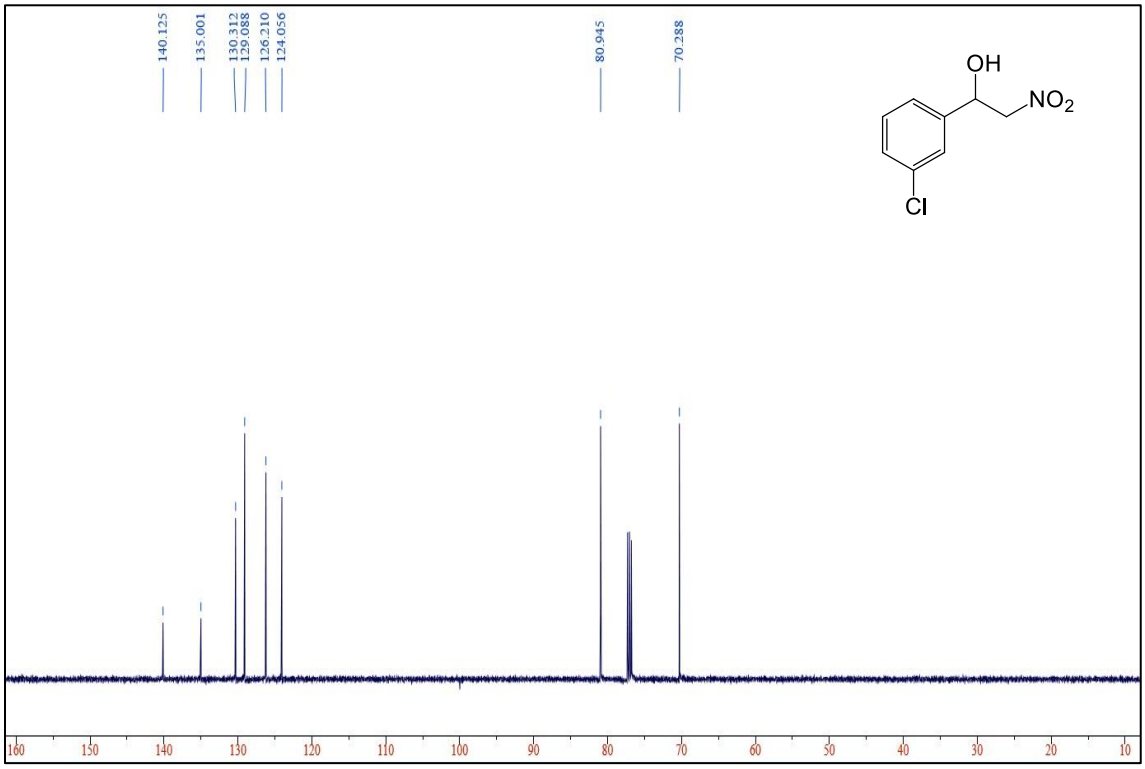


Figure 4.3.14: ^{13}C NMR spectrum of 1-(3-Chlorophenyl)-2-nitroethanol

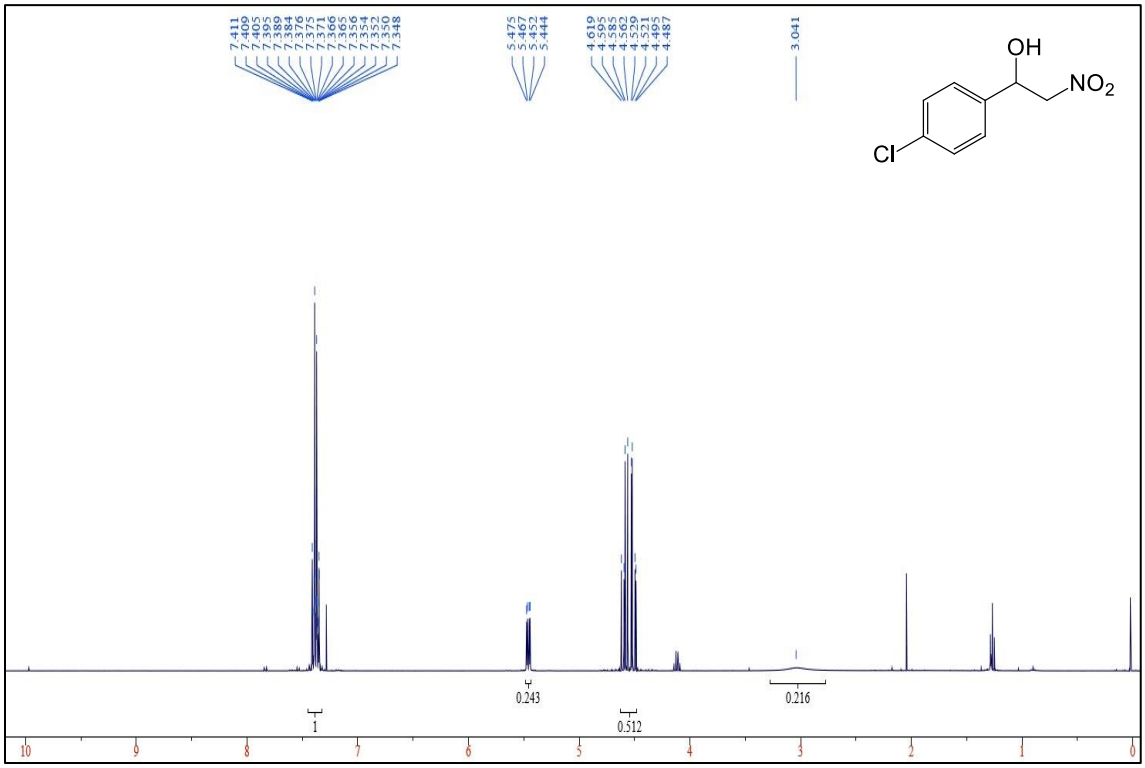


Figure 4.3.15: ^{1}H NMR spectrum of 1-(4-Chlorophenyl)-2-nitroethanol

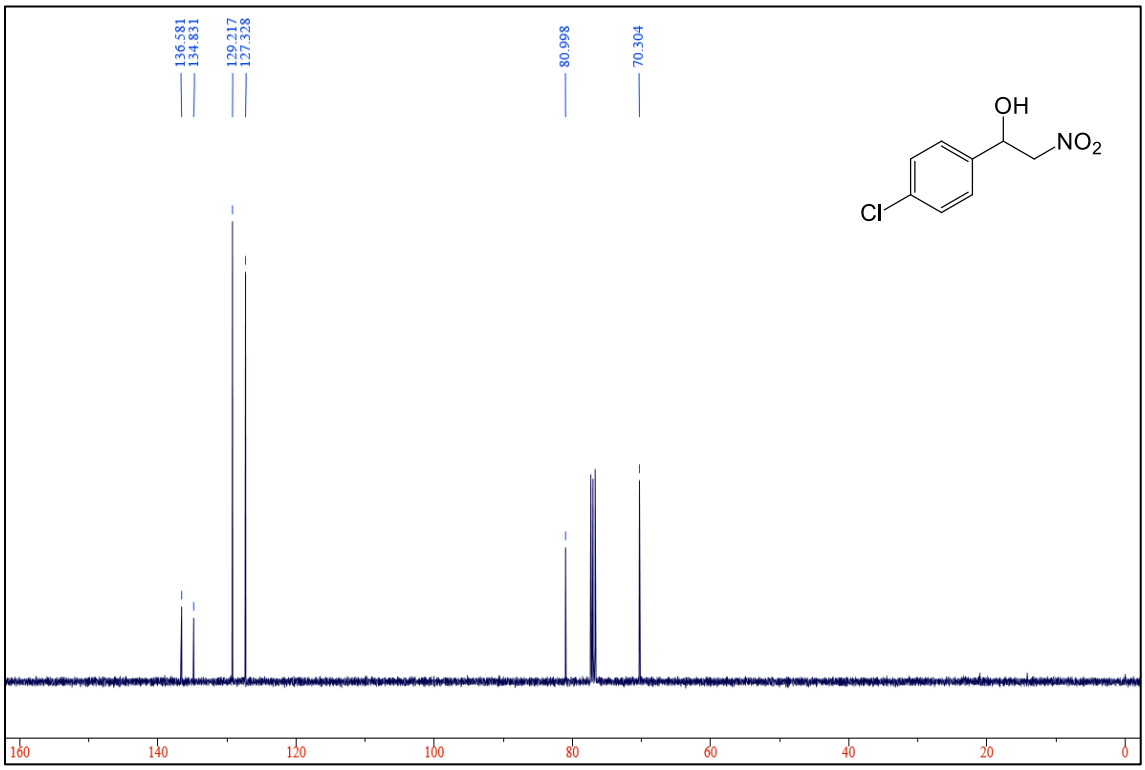
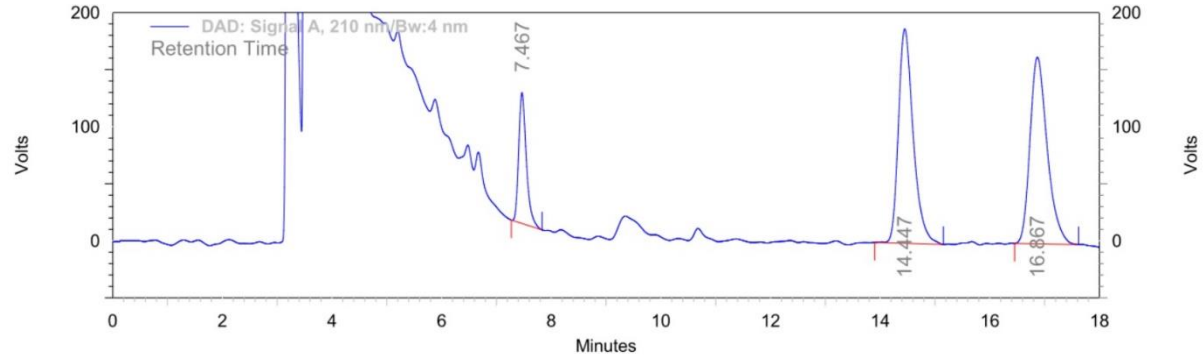
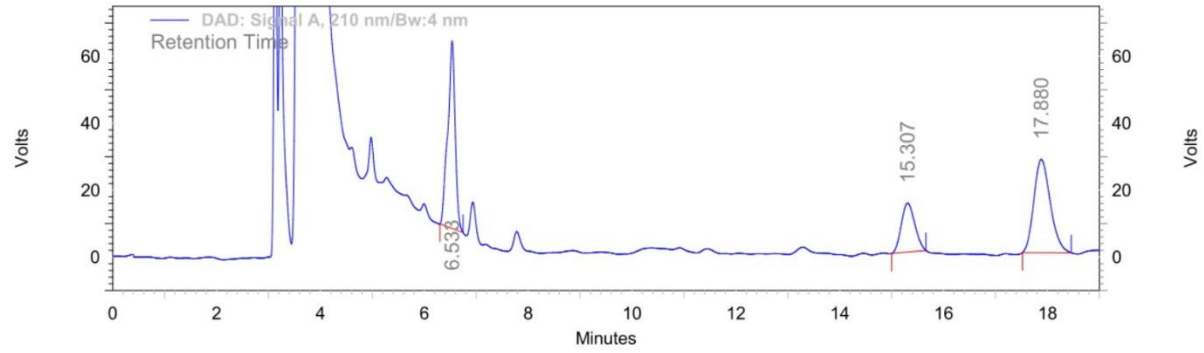


Figure 4.3.16: ^{13}C NMR spectrum of 1-(4-Chlorophenyl)-2-nitroethanol



(a)



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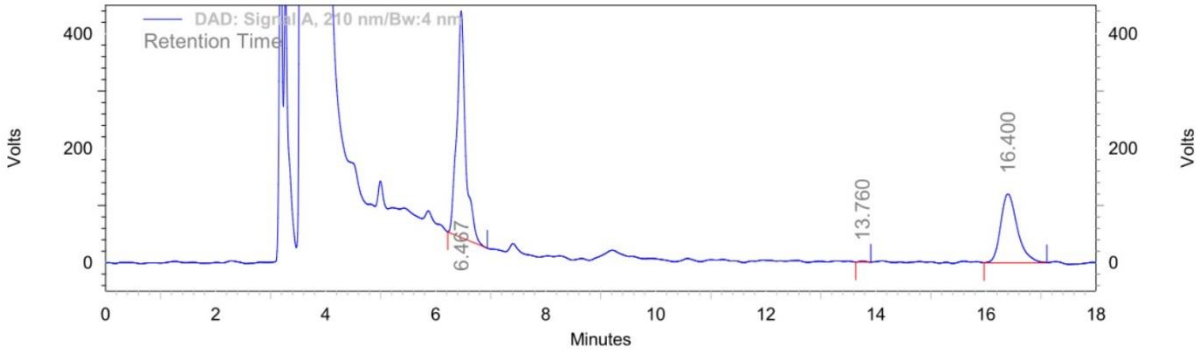


(c)

Figure 4.4.1: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of racemic 1-(4-Methoxyphenyl)-2-nitroethanol in the preparation of (*S*)-1-(4-Methoxyphenyl)-2-nitroethanol. (a), (b) and (c) represents control, WT *At*HNL and *At*HNL F179K variant catalysed reactions respectively.

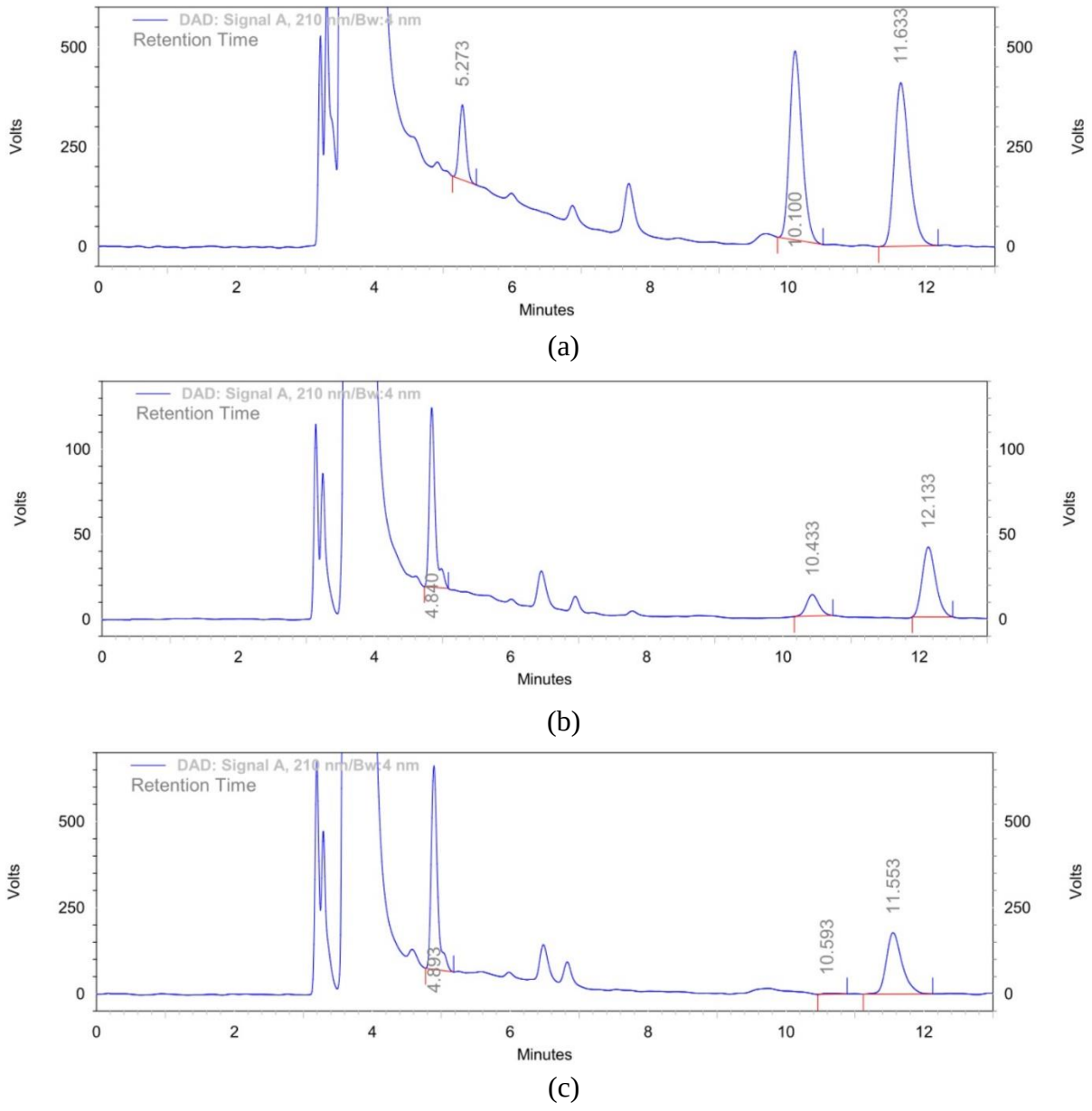
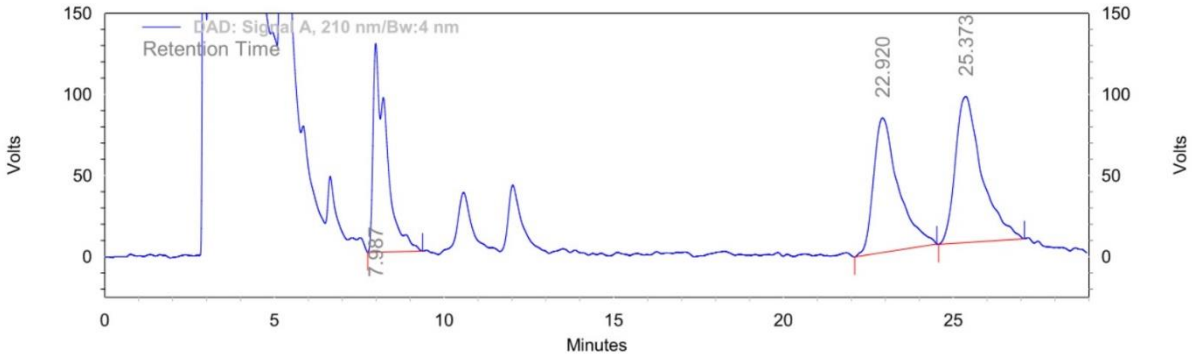


Figure 4.4.2: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of racemic 1-(4-Methylphenyl)-2-nitroethanol in the preparation of (*S*)-1-(4-Methylphenyl)-2-nitroethanol. (a), (b) and (c) represents control, WT *At*HNL and *At*HNL F179T variant catalysed reactions respectively.



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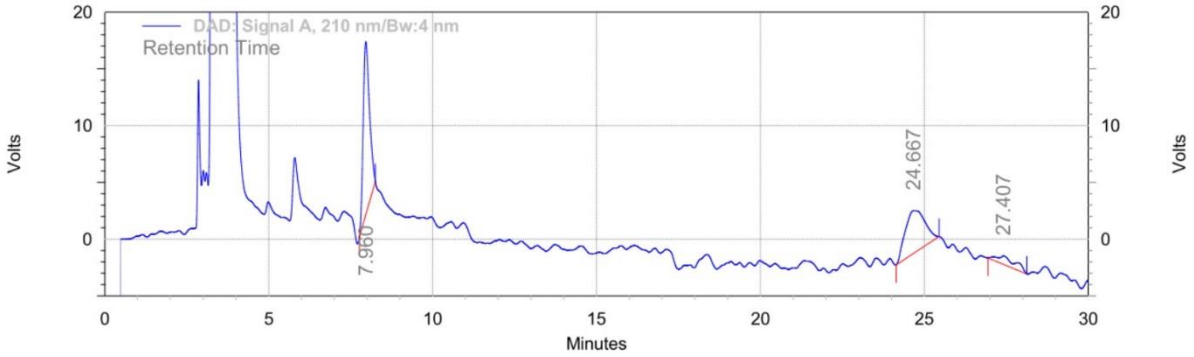

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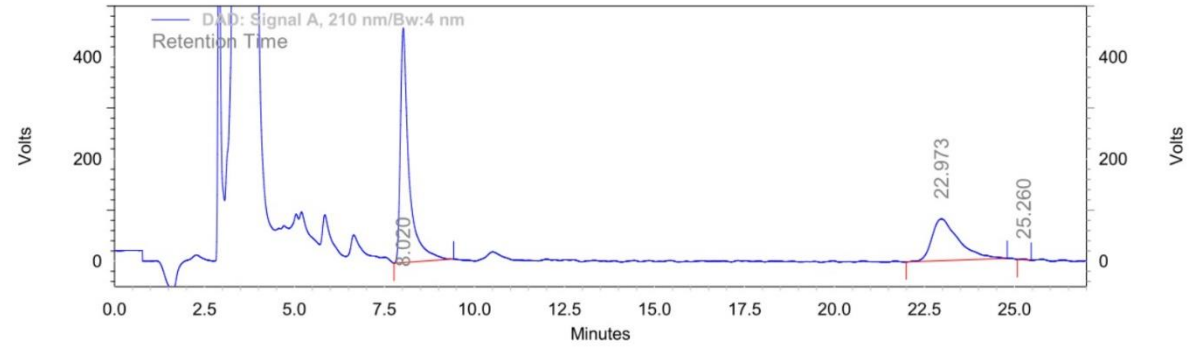


(c)

Figure 4.4.3: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of racemic 1-(3-Hydroxyphenyl)-2-nitroethanol in the preparation of (*S*)-1-(3-Hydroxyphenyl)-2-nitroethanol. (a), (b) and (c) represents control, WT *At*HNL and *At*HNL F179K variant catalysed reactions respectively.

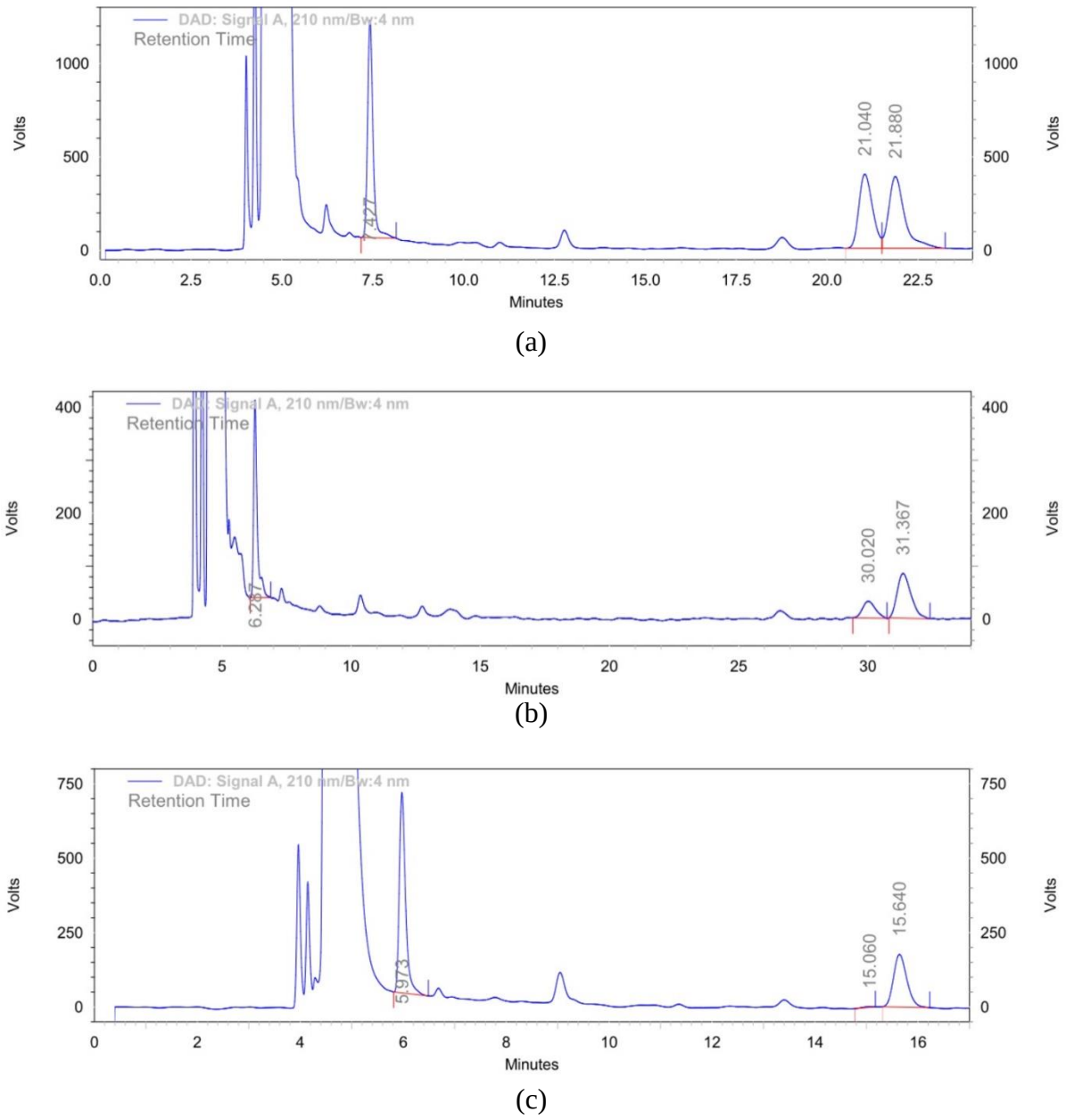
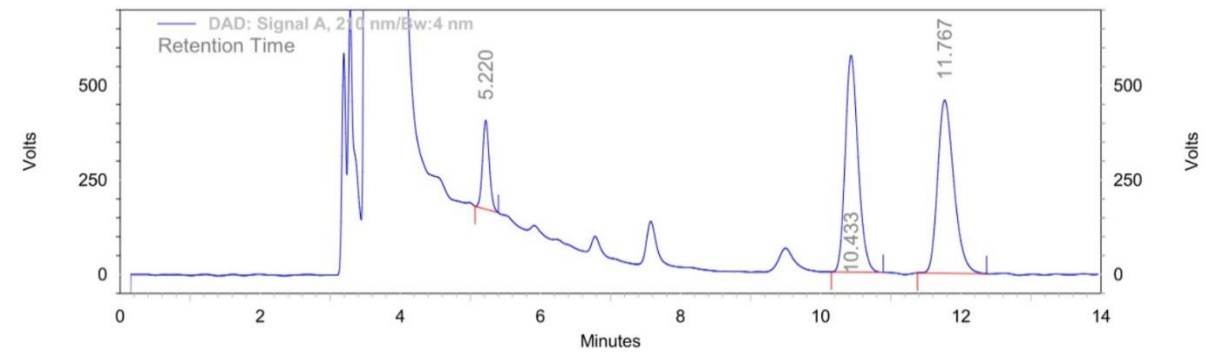
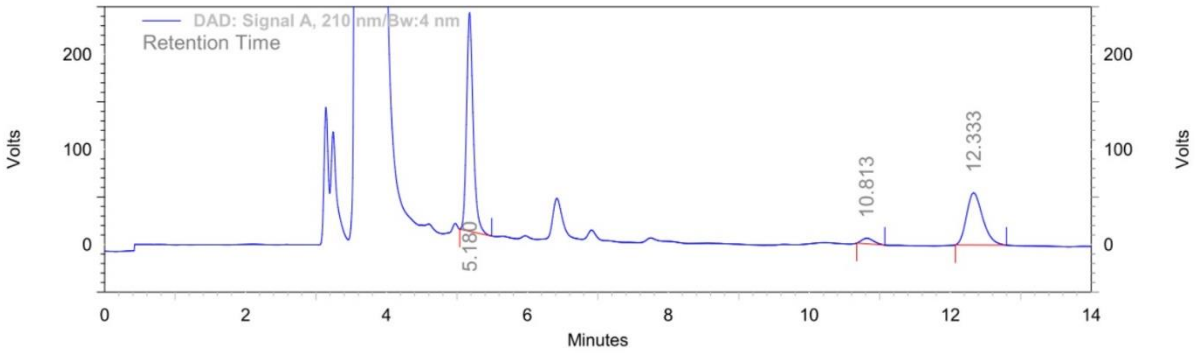


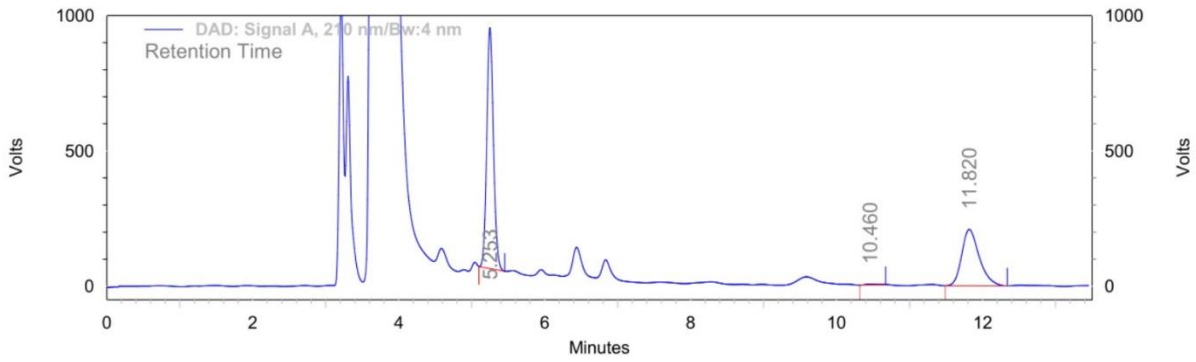
Figure 4.4.4: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of racemic 1-(2-Chlorophenyl)-2-nitroethanol in the preparation of (*S*)-1-(2-Chlorophenyl)-2-nitroethanol. (a), (b) and (c) represents control, WT *At*HNL and *At*HNL F179K variant catalysed reactions respectively.



(a)

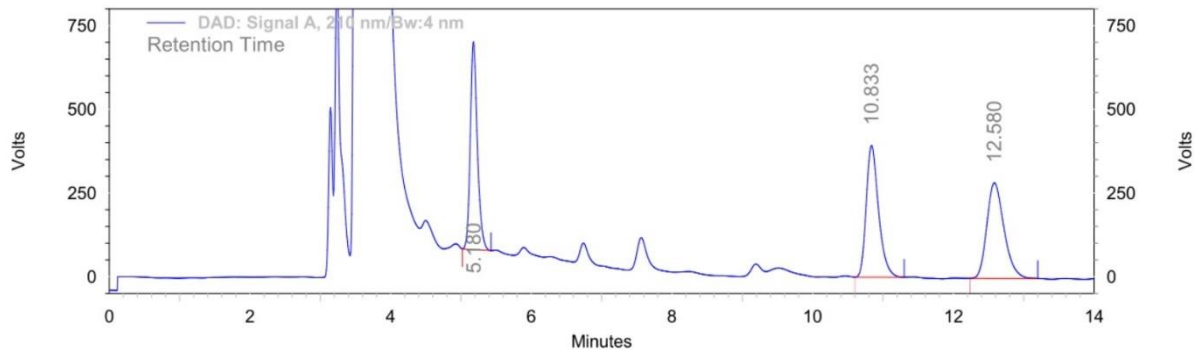


(b)

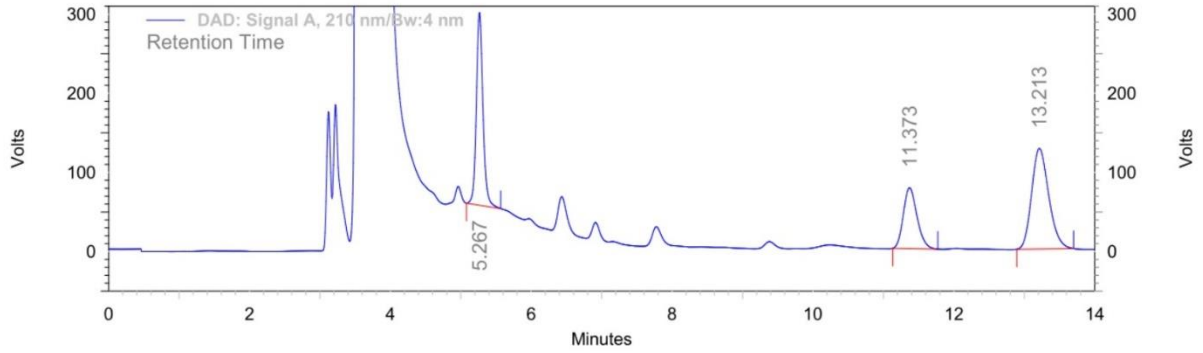


(c)

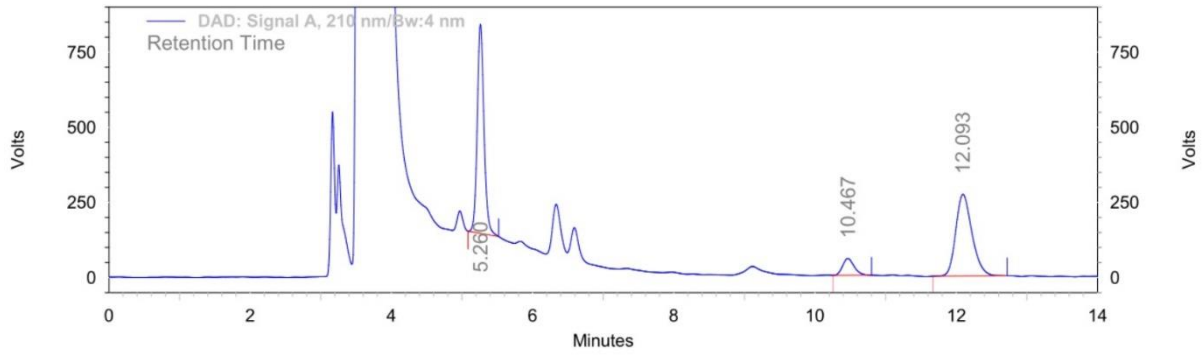
Figure 4.4.5: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of racemic 1-(3-Chlorophenyl)-2-nitroethanol in the preparation of (*S*)-1-(3-Chlorophenyl)-2-nitroethanol. (a), (b) and (c) represents control, WT *At*HNL and *At*HNL F179T variant catalysed reactions respectively.



(a)



(b)



(c)

Figure 4.4.6: HPLC chromatogram of *At*HNL catalysed enantioselective cleavage of racemic 1-(4-Chlorophenyl)-2-nitroethanol in the preparation of (*S*)-1-(4-Chlorophenyl)-2-nitroethanol. (a), (b) and (c) represents control, WT *At*HNL and *At*HNL F179K variant catalysed reactions respectively.

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Chapter 5

Engineered *Arabidopsis thaliana* Hydroxynitrile lyase for Dynamic Kinetic Asymmetric Nitroaldol Reaction based diastereocomplementary synthesis of β-aryl-α-hydroxynitroalkanes

5.1. Introduction

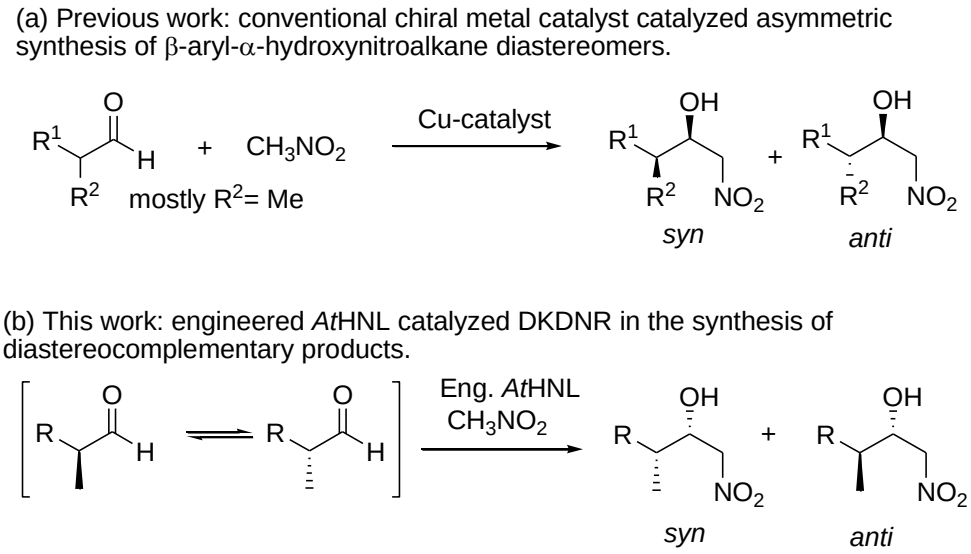
Nature evolved enzymes are increasingly used in sustainable biocatalysis for the production of chiral molecules. Their high degree of selectivity, rate acceleration, and clean reaction conditions make them favorable catalysts in asymmetric synthesis. Enzymes are tailored to gain one or more critical catalytic properties, e.g., broad substrate scope, high catalytic efficiency, and greater stereoselectivity that lead to the expansion of their versatile synthetic applications. In the past two decades significant advancements in protein engineering led to the development of new and efficient biocatalytic processes for a repertoire of asymmetric transformations.[1-6] Despite of advances in enzyme engineering, development of biocatalysts for non-natural reactions and synthesis of unconventional stereoisomers remains a major challenge.

Stereoselective nitroaldol or Henry reaction is a powerful atom-economy transformation in organic synthesis. In a single step, it makes a C–C bond formation between an electrophilic carbonyl center and a nucleophilic nitroalkane to produce an optically pure β-nitroalcohol; a longer carbon framework of organic molecules with diverse functional groups. They are precursors of pharmaceuticals, agrochemicals, and several important chemical intermediates. A lot of effort has been made to prepare diasteroselective nitroaldol products. Significant contributions toward this include transition metal catalysts, organcatalysts, and double helix. Conventional diastereoselective nitroaldol reaction using α-branched aldehydes uses chiral Cu complexes (**Scheme 5.1a**).[7-9]

Biocatalytic synthesis of enantiopure β-nitroalcohols by C-C bond formation is a promiscuous enzymatic activity.[10-15] Diastereoselective nitroaldol reaction produces two contiguous stereogenic centers and hence its biocatalytic synthesis remains challenging. So far, three hydroxynitrile lyases (HNLs) have been confirmed in the biocatalytic diastereoselective nitroaldol synthesis. They are *Hb*HNL (*Hevea brasiliensis*), an (*S*)-selective, and *Gt*HNL (*Granulicella tundricula*), and *Ac*HNL (*Acidobacterium capsulatum*), both (*R*)-selective HNLs.[10,13] However, two major limitations were noticed in these diastereoselective transformations. First, limited substrates have been exploited in the stereoselective synthesis, and second, all the HNLs show only *anti* enriched diastereomers. This confines the synthetic applicability of the enzymes, and demands to create enzymes for broad substrate scope as well as access to the unconventional diastereomers. To address these challenges, we have selected *Arabidopsis thaliana* HNL (*At*HNL), a (*R*)-selective metal-independent α/β hydrolase fold HNL that has not even tested in diastereoselective nitroaldol reaction.

Dynamic kinetic resolution (DKR) exemplifies as one of the most important strategies in asymmetric synthesis to prepare enantiopure molecules efficiently. Several enzymes, e.g., lipases,[16] dehydrogenases,[17] ω-transaminases,[18] and imine reductases[19] have been used in chemoenzymatic DKR in the synthesis of a diverse range of chiral molecules. Chemoenzymatic enantioselective syntheses of β-nitroalcohols have been reported using lipase catalysed kinetic resolution in combination with racemization of the unfavored enantiomer by a chemical catalyst.[20-22] However, the DKR based β-nitroalcohol syntheses have been limited to a single chiral center. In case of α-branched aldehydes as substrates, the nitroaldol reaction produces four stereoisomers of β-nitroalcohols, each having two stereogenic centers. To synthesize such β-nitroalcohols in a stereocontrolled manner, we envisaged the process of Dynamic Kinetic Diastereoselective Nitroaldol Reaction (DKDNR), a combination of DKR with HNL catalysed diastereoselective

nitroaldol reaction (**Scheme 5.1b**). We investigated the stereoselective addition of nitromethane to 2-phenylpropionaldehyde in the presence of WT *At*HNL. Herein, we report for the first time, an HNL in the DKR process to prepare diastereoselective nitroaldol products. WT *At*HNL has shown poor diastereoselectivity of the products. We have prepared tailor made enzymes to access the unusual diastereoisomeric products. Engineered *At*HNL variants catalysed C-C bond formation between an α-branched aldehyde and nitromethane, to produce diastereocomplementary nitroaldol products in a diastereoconvergent process.



Scheme 5.1: Nitroaldol reaction in the diastereoselective synthesis of β-aryl-α-hydroxynitroalkanes

5.2. Objectives

a) Optimization of reaction conditions of wild type *At*HNL catalysed DKDNR in the stereoselective synthesis of 1-nitro-3-phenylbutan-2-ol (NPB)

b) Screening of *At*HNL variant library for DKDNR in the stereoselective synthesis of NPB

c) Determination of kinetic parameters of *At*HNL wild type and variant(s) for DKDNR in the stereoselective synthesis of NPB

5.3. Materials and Methods

5.3.1. Materials

Materials used in this chapter were mentioned in section **2.3.1.** of **Chapter 2**.

5.3.2. Synthesis of racemic 1-nitro-3-phenylbutanol

Racemic NPB was synthesized from 2-phenylpropionaldehyde (PPA) by using $Ba(OH)_2$ as catalyst, the protocol of synthesis was as mentioned in section **2.3.5.** of **Chapter 2.** Racemic NPB synthesized was characterized by ^{1}H and ^{13}C NMR spectroscopy. They were used as analytical HPLC standards.

5.3.3. *At*HNL variant library

The library of *At*HNL variants used for screening in the current study consists of forty-eight mutants and were generated as described in the section **4.3.2.** of **Chapter 4**.

5.3.4. Preparation of crude enzyme extract

WT *At*HNL and its variants crude extract was prepared as per section **4.3.3.** of the **Chapter 4**. The resultant supernatant was used as crude enzyme in the screening of *At*HNL variant library in the stereoselective synthesis of diastereomers of NPB.

5.3.5. Expression and purification of WT *At*HNL and its variants

Expression and purification of WT *At*HNL and its variants was performed as mentioned in materials and methods section **2.3.2.** of the **Chapter 2.** The resultant purified WT *At*HNL and its

variants were used in optimization of reaction conditions of wild type *At*HNL catalysed DKDNR and in determination of kinetic parameters of WT *At*HNL and *At*HNL F179L variant in the synthesis of four diastereomers of NPB.

5.3.6. HNL assay

HNL activity was measured using the method described in section **2.3.3.** of **Chapter 2**.

5.3.7. Optimization of reaction conditions of wild type *At*HNL catalysed DKDNR in the stereoselective synthesis of 1-nitro-3-phenylbutanol

The ratio between the two substrates, i.e., nitromethane, and PPA, in WT *At*HNL catalysed DKDNR was optimized. The reaction mixture contained 8 mM of PPA, the molar ratio of PPA to the nitromethane was varied from 1:1 to 1:62.5, 30 units of purified *At*HNL (85 µL), 50% v/v *n*-butyl acetate (250 µL), and 165 µL of 100 mM phosphate buffer saline (PBS) pH 6.0. The total reaction volume was 0.5 mL. Reaction mixture was shaken at 1200 rpm at 30 ℃ in an incubator shaker. The reaction was monitored at different time intervals. A 50 µL of aliquot from the organic layer was added to 50 µL of hexane/2-propanol = 9:1, dried over anhydrous sodium sulphate and centrifuged at 15000 *g* at 4 ℃ for 5 min. A 20 µL of the organic layer was analyzed in a HPLC using chiral column. The pH of *At*HNL catalysed DKDNR was optimized with similar reaction conditions as above, except that the 1:62.5 molar ratio of PPA to nitromethane was taken and the pH of 100 mM PBS was varied, i.e., 5.8, 7.0 and 7.4. Extraction and analysis remained same as in the case of nitromethane optimization.

% Conversion, diastereomeric ratio (*de*) and % *ee* were calculated using the following formulae

$$\%\ conversion$$

$$= \frac{[(2S,\ 3S) + (2R,\ 3R) + (2S,\ 3R) + (2R,\ 3S)]}{[(\text{Aldehyde} \times \text{conversion factor} + (2S,\ 3S) + (2R,\ 3R) + (2S,\ 3R) + (2R,\ 3S)]} \times 100$$

$$diastereomeric\ ratio\ (de) = anti\ /syn\ = \frac{[(2S, 3S) + (2R, 3R)]}{[(2S, 3R) + (2R, 3S)]} \times 100$$

$$isomeric\ content = \frac{(\text{Area of one diastereomer peak})}{(\text{Area of sum total of four diastereomers})} \times 100$$

$$\%\ ee\ of\ (2S, 3S) = \frac{[(2S,\ 3S) - (2R,\ 3R)]}{[(2S,\ 3S) + (2R,\ 3R)]} \times 100$$

$$\%\ ee\ of\ (2S, 3R) = \frac{[(2S,\ 3R) - (2R,\ 3S)]}{[(2S,\ 3R) + (2R,\ 3S)]} \times 100$$

5.3.8. Screening of *At*HNL variant library for DKDNR catalysed synthesis of NPB

Screening of *At*HNL variant library in the diastereoselective synthesis of NPB was performed as follows. The reaction mixture of 0.6 mL contained 6.6 mM of 2-phenylpropionaldehyde, 416 mM of nitromethane (molar ratio of 2-phenylpropionaldehyde to nitromethane was 1:62.5), 9 mg of crude *At*HNL lysate in 20 mM potassium phosphate buffer (KPB) pH 7.0, 300 µL of *n*-butyl acetate, 100 mM PBS pH 7.0. The mixture was shaken at 1200 rpm at 30 °C in an incubator shaker. The reaction was monitored at different time intervals. Aliquot extraction and analysis were done as mentioned in section **5.3.7.** above.

5.3.9. Determination of kinetic parameters of WT *At*HNL and the varaint F179L for DKDNR catalysed synthesis of NPB stereoisomers

Kinetic parameters WT *At*HNL were determined using the diastereoselective synthesis of NPB. The kinetic parameters were calculated against the synthesis of each of the four diastereomers of NPB. The details of experiment were as follows. Multiple reactions were kept with different PPA

concentrations varied from 2 to 50 mM dissolved in *n*-butyl acetate, corresponding concentrations of nitromethane with 1:62.5 of PPA: nitromethane, 30 units of purified *At*HNL in KPB pH 7.0 (1.58 mg, 107 μL, concentration of enzyme is 14.8 mg/mL, specific activity = 19 U/mg based on mandelonitrile cleavage), 143 μL of 100 mM PBS pH 7.0, 250 μL of *n*-butyl acetate. In case of each reaction, the total volume was 0.5 mL. The control experiment had all the reaction components except that the enzyme was replaced by its corresponding buffer. The reaction mixture was shaken at 1200 rpm at 30 ℃ in an incubator shaker for 60 minutes. The reaction mixture containing 50% v/v of each of organic and aqueous phase was centrifuged at 15000 *g* for 5 min at 4 °C. Total product and substrate commonly found in the organic phase. From 250 μL of the organic phase, 10 μL was taken and added into 190 μL of Hex/IPA 90:10 and 20 μL of this mixture was injected into HPLC for chiral analysis. Control reaction was subtracted from that of the enzymatic reaction. One unit of HNL activity is defined as the amount of enzyme that produces 1 μmol of NPB from PPA per minute. The best fit of the data to the Michaelis–Menten equation was achieved by using the Solver function of Microsoft Excel. Kinetic of *At*HNL-F179L towards the synthesis of four diastereomers of NPB was performed using similar methodology used above except that F179L mutant was used instead of WT *At*HNL and the reaction time was 30 minutes. Substrate concentration ranging from 2 to 70 mM was taken. The kinetic parameters were determined from the MM plot.

5.4. Results

5.4.1. ^{1}H NMR characterization of racemic NPB[8]

Racemic NPB synthesized was characterized by ^{1}H NMR, spectral data was shown (**Figure 5.13**). ^{1}H-NMR (400 MHz, CDCl3) δ 7.21-7.38 (m, 5H), 4.38-4.52 (m, 1H), 4.22-4.34 (m, 2H), 2.92-2.97 (qw, 1H, J=7.2 Hz), 2.81-2.90 (m, 1H), 1.38-1.44 (dd, 3H, J= 6.8, 15.6 Hz) ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.4, 43.7, 73.2, 79.5, 127.4, 129.1, 142.0.

5.4.2. Chiral resolution of racemic NPB

The details of HPLC based chiral resolution of the racemic NPB is given in **Table 5.1**. Corresponding HPLC chromatogram is present in **Figure 5.14**

Table 5.1.: HPLC chiral resolution of the racemic NPB.

S. No	Product	Retention time of PPA and NPB diastereomers (minutes)	Column Chiralpak	Mobile phase Hex/ IPA	Flow rate (mL/ min)
1	NPB	PPA = 4.7 min, (2*S*,3*S*)- NPB = 16.9 min, (2*R*,3*R*)-NPB = 17.9 min, (2*R*,3*S*)-NPB = 18.7 min and (2*S*,3*R*)- NPB = 19.4 min	IB	Gradient; 96:04 (*v*/*v*), 0 - 12 min and 97:03 from 12 - 25 min	1

Absorbance at 210 nm, Hex/IPA = *n*-hexane: 2-propanol

5.4.3. Characterization of purified *At*HNL and its variant

Prior to use the purified enzymes in biotransformation, both the wild type and F179L were characterized by SDS-PAGE (**Figure 5.1**). Presence of ~28kDa band in the SDS-PAGE confirms the proteins. A good protein expression was seen in the case of *At*HNL-F179L.

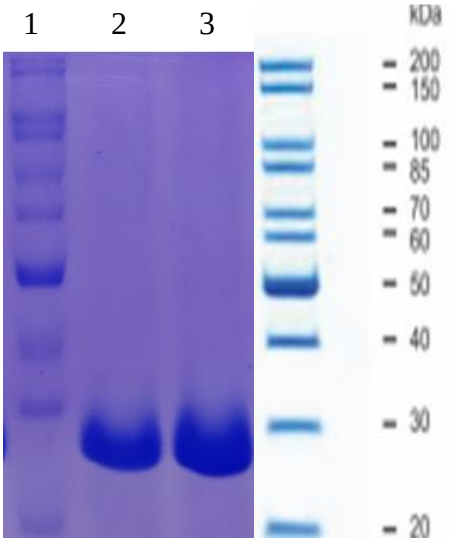
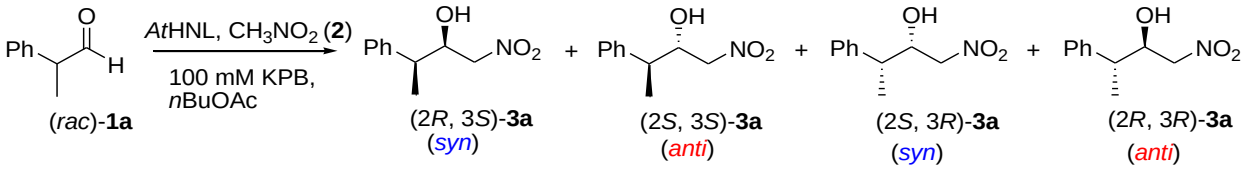


Figure 5.1: SDS PAGE image of purified *At*HNL wild type and *At*HNL-F179L. Lanes 1: protein ladder, 2: *At*HNL wild type and 3: *At*HNL F179L

5.4.4. Optimization of reaction conditions of wild type *At*HNL catalysed DKDNR in the synthesis of NPB stereoisomers

The key process of DKDNR is dynamic kinetic resolution. To observe dynamic kinetic resolution during the nitroaldol reaction, we focused to optimize two important biocatalytic parameters. They are (i) mole equivalents of nitromethane, and (ii) pH of the buffer. The DKDNR was performed using purified wild type *At*HNL, nitromethane (**2**) and racemic PPA (**1a**) (**Scheme 5.2**). To shift the equilibrium of the reaction forward, we need excess nitroalkane. Hence, a number of transformations were carried out by varying the molar ratio of PPA to nitromethane from 1:1 to 1:62.5. In case of 1:1 of PPA to nitromethane, the *At*HNL has shown only 2% conversion, 1:1 *anti* / *syn* ratio, 7% *ee* of (2*S*, 3*S*)-NPB, **3a** and 99% *ee* of (2*S*, 3*R*)-**3a** at 6 h with buffer pH 6.0 (**Table 5.2**). When the ratio of PPA to nitromethane was increased, *At*HNL's activity also gradually increased. The highest activity of 41% conversion, 3:1 *anti* / *syn* ratio, 5% *ee* of (2*S*, 3*S*)-**3a** and

98% *ee* of (2*S*, 3*R*)-**3a** was found in the case of 1:62.5 ratio, at 6 h, and buffer pH 6.0. Even when the reaction time was extended to 24 h, we observed a similar trend of highest activity in the case of 1:62.5 ratio of PPA to nitromethane. Here, the *At*HNL has shown 70% conversion, 3:1 *anti* / *syn* ratio, –1% *ee* of (2*S*, 3*S*)-**3a** and 93% *ee* of (2*S*, 3*R*)-**3a** at 24 h. This clearly shows that with 1:62.5 of PPA to nitromethane, the enzyme provides highest DKDNT activity. Next we studied the effect of pH of buffer in the DKDNR process. The wild type *At*HNL catalysed DKDNR was carried out in phosphate buffer of pH ranging from 5.8 to 7.4. The biotransformation at the lowest pH 5.8, has resulted in 76% total conversion of **3a**, 4:1 ratio of *anti* : *syn*, 3% *ee* of (2*S*, 3*S*)-**3a** and 92% *ee* of (2*S*, 3*R*)-**3a** in 24 h. At pH 7.0, we found 91% total conversion of **3a**, 4:1 diastereomeric ratio (*anti* / *syn*), 14% *ee* of (2*S*, 3*S*)-**3a** and 90% *ee* of (2*S*, 3*R*)-**3a** at 24 h (**Table 5.2**). The total conversion was increased marginally and % *ee* of (2*S*, 3*R*)-**3a** decreased slightly when the pH was increased from 5.8 to pH 7.0. At the highest pH 7.4, the wild type enzyme has produced 95% total conversion of **3a**, 4:1 diastereomeric ratio (*anti* / *syn*) ratio, 13% *ee* of (2*S*, 3*S*)-**3a** and 86% *ee* of (2*S*, 3*R*)-**3a** at 24 h. At 12 h, 84% conversion was obtained with 3:1 diastereomeric ratio (*anti* / *syn*) ratio, 9% *ee* of (2*S*, 3*S*)-**3a** and 93% *ee* of (2*S*, 3*R*)-**3a**.



Scheme 5.2: *At*HNL catalysed DKDNR in the synthesis of NPB diastereomers

Table 5.2: Optimization of reaction conditions of wild type *At*HNL catalysed DKDNR in the synthesis of NPB stereoisomers

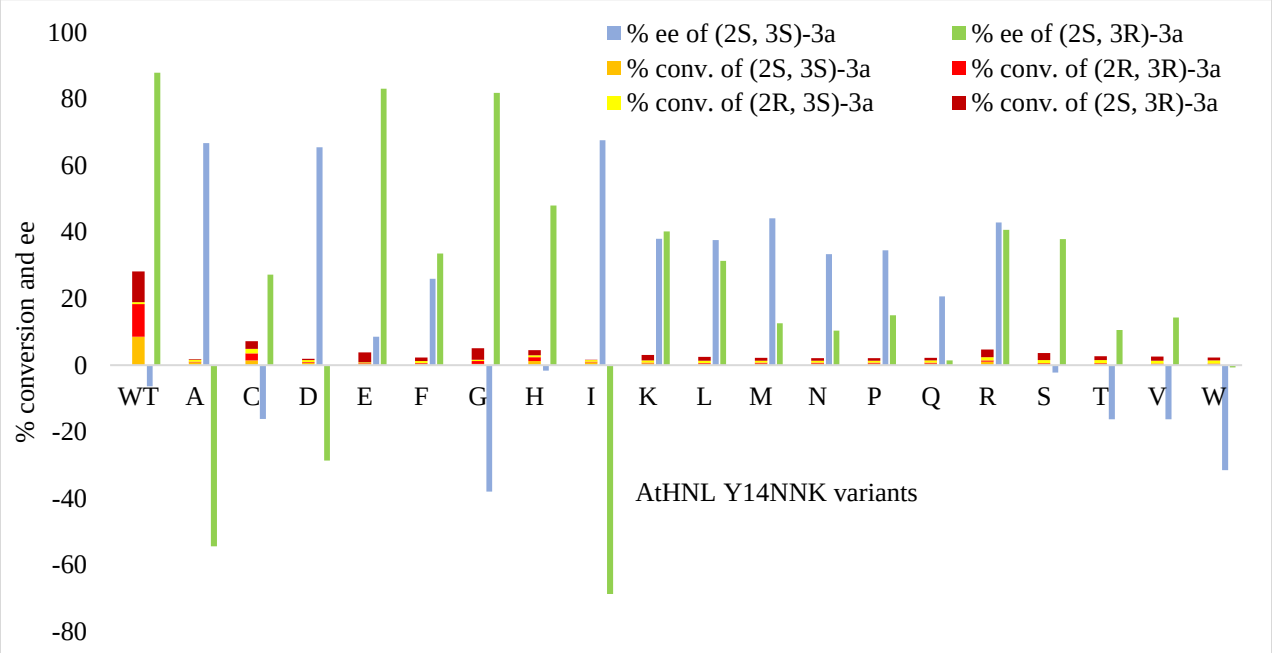
Entry	pH	**1a**:**2**[a]	t (h)	Conv. [%][b] **3a**	*anti* / *syn*[c]	*ee* [%][c] (2*S*, 3*S*)-**3a**	*ee* [%][c] (2*S*, 3*R*)-**3a**
1	5.8	1:62.5	12	63	3:1	–5	92
2	5.8	1:62.5	24	76	4:1	3	92
3	6.0	1:1	6	2	1:1	7	99
4	6.0	1:5	6	6	2:1	21	97
5	6.0	1:10	6	11	2:1	10	97
6	6.0	1:31.25	6	26	3:1	7	85
7	6.0	1:62.5	6	41	3:1	5	98
8	6.0	1:1	24	5	3:1	26	95
9	6.0	1:5	24	18	3:1	14	93
10	6.0	1:10	24	31	3:1	6	93
11	6.0	1:31.25	24	61	3:1	1	94
12	6.0	1:62.5	24	70	3:1	–1	93
13	7.0	1:62.5	12	80	3:1	10	88
14	7.0	1:62.5	24	91	4:1	14	90
15	7.4	1:62.5	12	84	3:1	9	93
16	7.4	1:62.5	24	95	4:1	13	86

[a] Molar ratio of PPA:NM, [b] The total % conversion to **3a** and [c] diastereomeric ratio, and enantiomeric excess were determined by HPLC on a chiral stationary phase.

5.4.5. Screening of *At*HNL variant library for DKDNR catalysed synthesis of NPB stereoisomers

The library of *At*HNL variants used for screening has a total of forty-eight of them. It consisted of three single mutants (A13G, H15A, and F80A), one partially saturated library at Phe82 (Ala, Lys, Pro, Gln, Arg, Ser, and Thr), and two saturation libraries at Tyr 14 and Phe179. Screening of the library towards DKDNR was performed using crude lysates of the variants. The products were analyzed by chiral HPLC. From the chromatograms, % conversion of each diastereomer was calculated as described in **5.3.7.** above. The % *de* and % *ee* of the major stereoisomers were also calculated. In case of wild type *At*HNL catalysed DKDNR, only 28% conversion to **3a** was found, with 6% *ee* of (2*R*,3*R*)-**3a** and 88% *ee* of (2*S*,3*R*)-**3a** (**Figure 5.2 top**). In the Tyr14 library, Y14E and Y14G has provided 83, and 82% *ee* of (2*S*,3*R*)-**3a** respectively. In case of Y14A and Y14I,

(2*R*,3*S*)-**3a** was produced in 54 and 69% *ee*. Similarly, Y14A, Y14D, and Y14I resulted in the synthesis of (2*S*,3*S*)-**3a** in 66-67% *ee*. However, in all the cases of Y14 variants the total conversion was less than 5%. In the second set of variants screened (**Figure 5.2 middle**), A13G, F80A, F82K, F82P, F82Q, F82S, and F82T resulted in 66-90% *ee* of (2*S*,3*R*)-**3a**. Surprisingly, the F80A variant gave 65% *ee* of (2*R*,3*R*)-**3a**. All the successful variants of this set however had <10% conversion. The major outcome of screening of F179 library was F179D and F179L, both gave (2*S*,3*R*)-**3a** in 85 and 98% *ee* respectively. The % conversion to NPB by F179D was <5%. However, significantly high activity was found in the case of F179L. This variant has catalysed the DKDNR and produced a total of 82% conversion to **3a**, with 94% *ee* of (2*S*, 3*S*)-**3a** and 98% *ee* of (2*S*, 3*R*)-**3a** (**Figure 5.2 bottom**).



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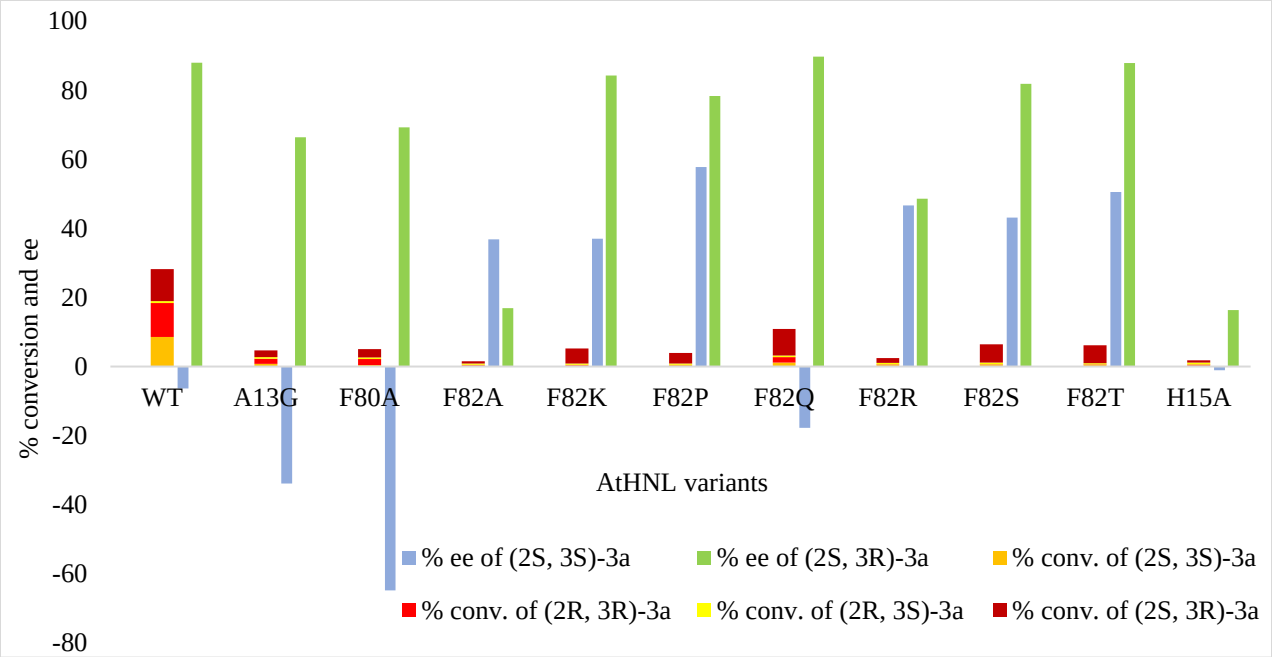

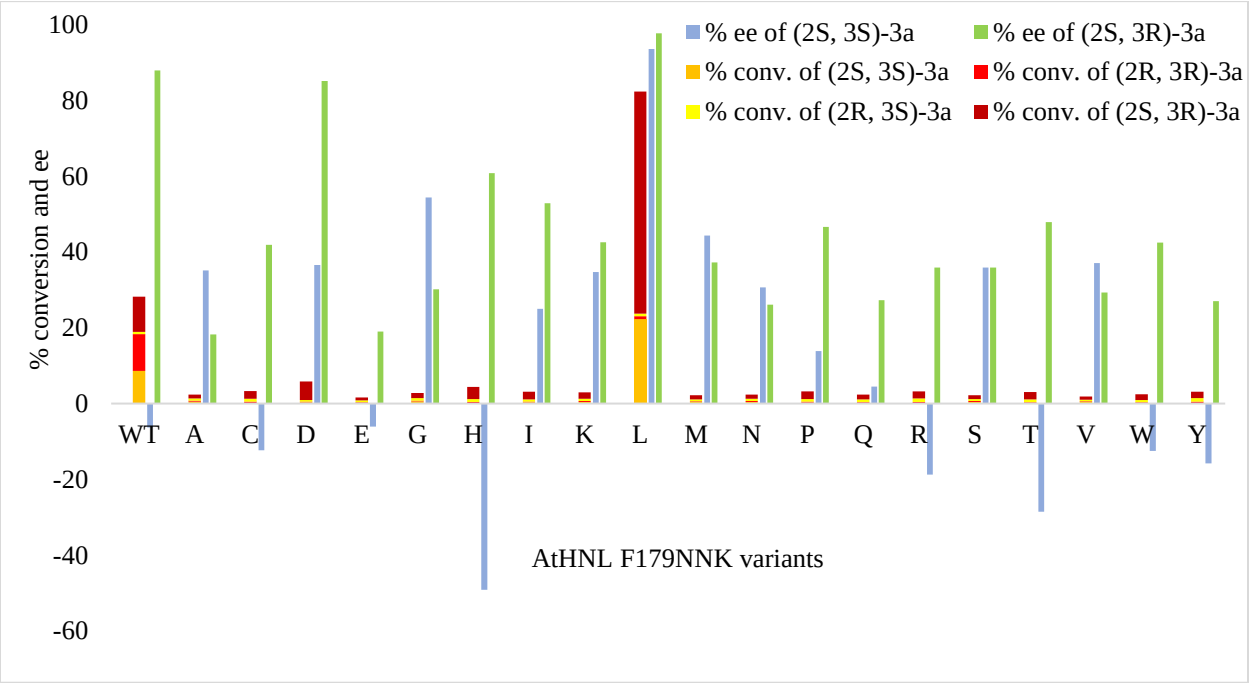

Figure 5.2: *At*HNL variants library screening for DKDNR catalysed synthesis of NPB stereoisomers. Top graph shows results from saturation library at Tyr14. Middle one has miscellaneous mutations, while the bottom one represents results of Phe179 saturation library. The % conversion of each diastereomer of **3a**, enantiomeric excess of (2*S*,3*S*)-**3a**, and (2*S*,3*R*)-**3a** were determined by HPLC on a chiral stationary phase.

5.4.6. Determination of kinetic parameters of *At*HNL wild type and F179L in the DKDNR catalysed synthesis of NPB stereoisomers

Having found *At*HNL-F179L as the best mutant with high activity for DKDNR from the screening, we then aimed to know the catalytic efficiency of this variant. Therefore, we determined the kinetic parameters of *At*HNL wild type and the variant F179L using DKDNR catalysed synthesis of NPB stereoisomers. The kinetic parameters of both the enzymes were found towards synthesis of NPB. From the data, we have calculated the parameters based on the formation of individual NPB diastereomers, as well as total formation of NPB. Wild type *At*HNL has shown K_M = 21.09 mM, k_{cat} = 0.62 s^{-1}, and k_{cat}/K_M = 0.03 M^{-1} s^{-1} (**Figure 5.3, Table 5.3**) towards the synthesis of total NPB (all the four diastereomers). *At*HNL-F179L has shown K_M = 11.35 mM, k_{cat} = 1.62 s^{-1}, and k_{cat}/K_M = 0.143 M^{-1} s^{-1} towards the synthesis of total NPB (**Figure 5.8**). It is evident from this data that *At*HNL F179L has shown lower K_M, 2.6-folds of higher k_{cat} and 4.76-fold higher k_{cat}/K_M compared to the wild type *At*HNL in the synthesis of total NPB, i.e., all the four diastereomers.

When the data was analysed based on the synthesis of individual diastereomers, wild type *At*HNL showed K_M = 16.203 mM, k_{cat} = 0.082 s^{-1} and k_{cat}/K_M = 0.005 M^{-1} s^{-1} towards the synthesis of (2*S*, 3*S*)-NPB (**Table 5.4, Figure 5.4**) and towards the synthesis of same diastereomer *At*HNL F179L showed K_M = 9.847 mM, k_{cat} = 0.142 s^{-1} and k_{cat}/K_M = 0.014 M^{-1} s^{-1} (**Table 5.4, Figure 5.9**). Compared to the wild type, *At*HNL F179L showed 2.8-fold higher k_{cat}/K_M in the synthesis of (2*S*,3*S*)-NPB. In the case of (2*S*,3*R*)-NPB synthesis, wild type showed K_M = 0.005 mM, k_{cat} = 0.128 s^{-1} and k_{cat}/K_M = 0.008 M^{-1} s^{-1} (**Table 5.4, Figure 5.7**), while *At*HNL-F179L showed K_M = 12.006 mM, k_{cat} = 1.354 s^{-1} and k_{cat}/K_M = 0.112 M^{-1} s^{-1} (**Table 5.4, Figure 5.12**). Especially, in the synthesis of (2*S*,3*R*)-NPB, *At*HNL-F179L has shown 14-fold higher k_{cat}/K_M than the wild type.

Towards the synthesis of (2*R*,3*R*)-NPB, wild type *At*HNL has shown K_M = 24.56 mM, k_{cat} = 0.30 s^{-1} and k_{cat}/K_M = 0.01 M^{-1} s^{-1} (**Table 5.4, Figure 5.5)** and *At*HNL-F179L has shown K_M = 13.38 mM, k_{cat} = 0.00 s^{-1} and k_{cat}/K_M = 0.00 M^{-1} s^{-1} (**Table 5.4, Figure 5.10**). Compared to the wild type, *At*HNL-F179L has shown negligible value of k_{cat}/K_M in the synthesis of (2*R*,3*R*)-NPB. In case of synthesis of (2*R*,3*S*)-NPB, we observed K_M = 10 mM, k_{cat} = 0.00024 s^{-1} and k_{cat}/K_M = 0.0002 M^{-1} s^{-1} by the wild type (**Table 5.4, Figure 5.6**), while *At*HNL-F179L has shown K_M = 2.0 mM, k_{cat} = 0.011 s^{-1} and k_{cat}/K_M = 0.006 M^{-1} s^{-1} (**Table 5.4, Figure 5.11**). Here too, *At*HNL-F179L has shown 30-fold improved catalytic efficiency than the wild type in the synthesis of (2*R*,3*S*)-NPB.

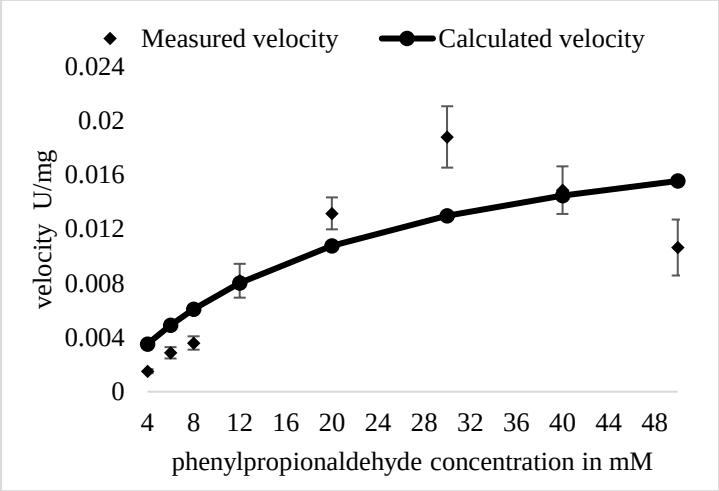

Figure 5.3: Michaelis–Menten kinetics plot of wild type *At*HNL catalysed DKDNR in the synthesis of NPB [(2*S*,3*S*) + (2*R*,3*R*) + (2*R*,3*S*) + (2*S*,3*R*)]

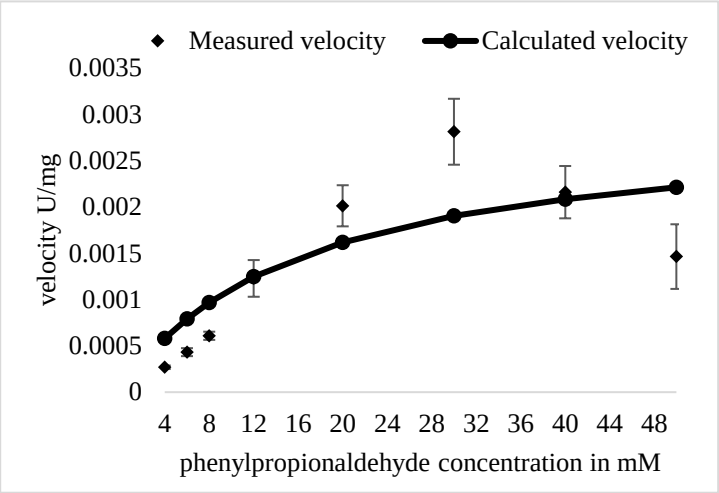

Figure 5.4: Michaelis–Menten kinetics plot of wild type *At*HNL catalysed DKDNR in the synthesis (2*S*,3*S*)-NPB

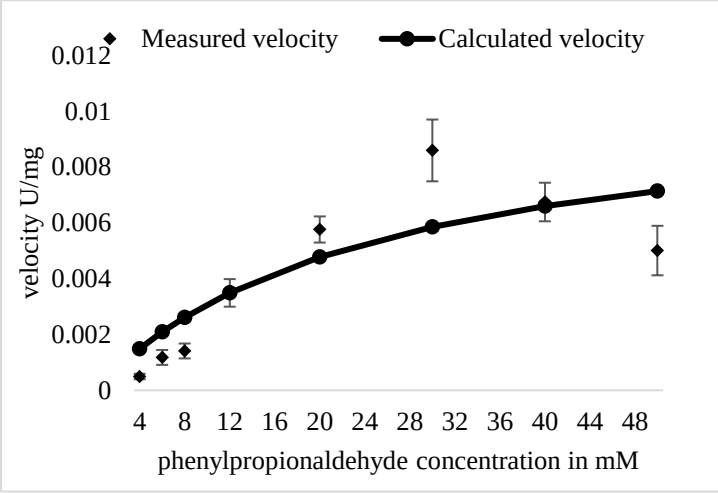

Figure 5.5: Michaelis–Menten kinetics plot of wild type *At*HNL catalysed DKDNR in the synthesis (2*R*,3*R*)-NPB

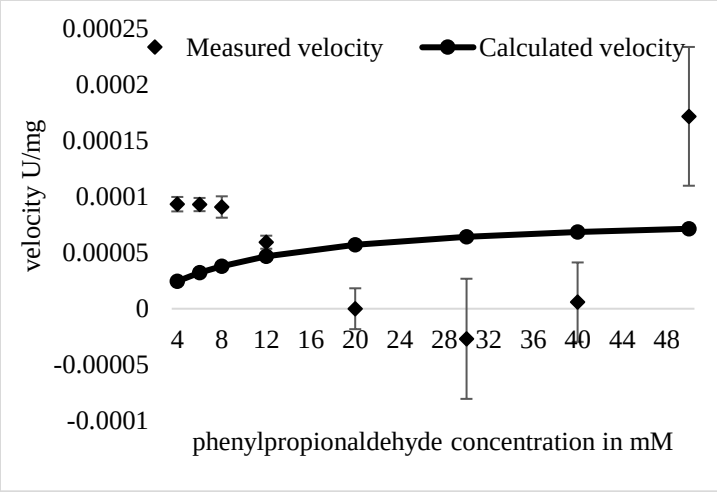

Figure 5.6: Michaelis–Menten kinetics plot of wild type *At*HNL catalysed DKDNR in the synthesis (2*R*,3*S*)-NPB

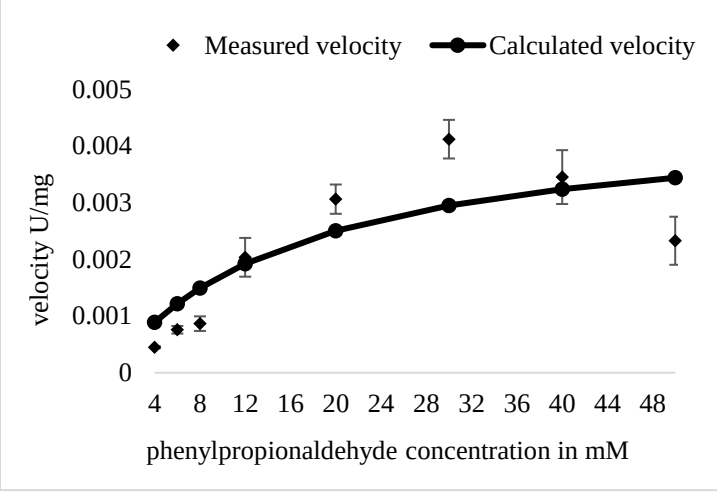

Figure 5.7: Michaelis–Menten kinetics plot of wild type *At*HNL catalysed DKDNR in the synthesis (2*S*,3*R*)-NPB

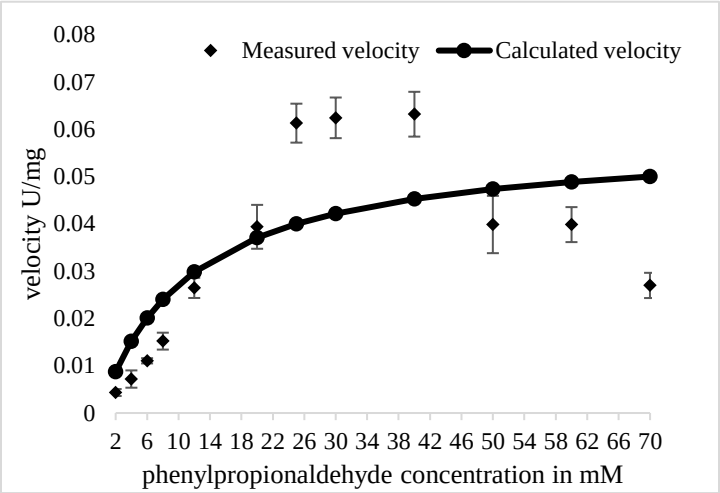

Figure 5.8: Michaelis–Menten kinetics plot of *At*HNL-F179L catalysed DKDNR in the synthesis of NPB [(2*S*,3*S*) + (2*R*,3*R*) + (2*R*,3*S*) + (2*S*,3*R*)]

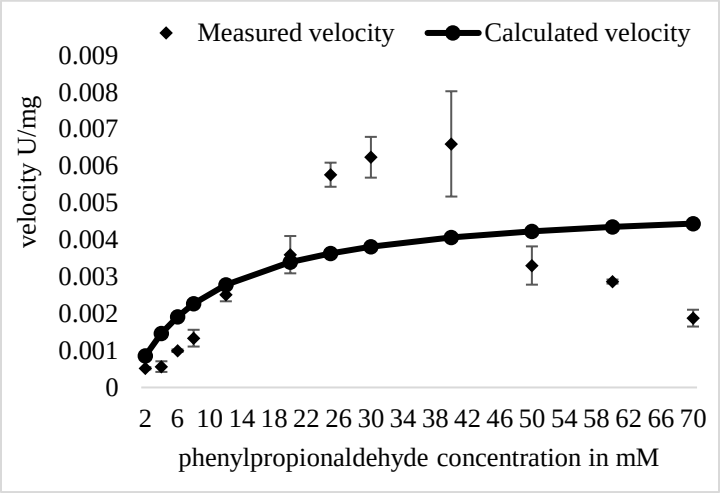

Figure 5.9: Michaelis–Menten kinetics plot of *At*HNL-F179L catalysed DKDNR in the synthesis (2*S*,3*S*)-NPB

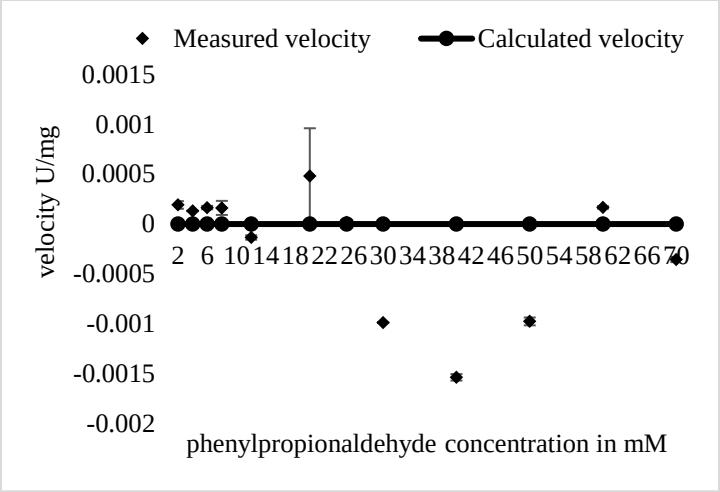

Figure 5.10: Michaelis–Menten kinetics plot of *At*HNL-F179L catalysed DKDNR in the synthesis (2*R*,3*R*)-NPB

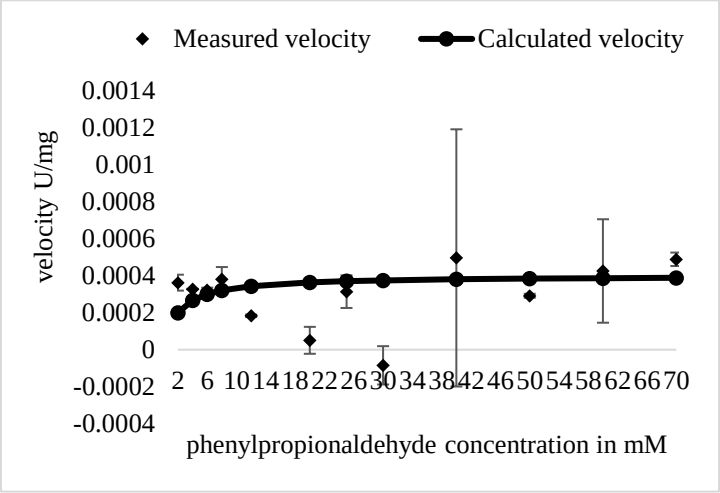

Figure 5.11: Michaelis–Menten kinetics plot of *At*HNL-F179L catalysed DKDNR in the synthesis (2*R*,3*S*)-NPB

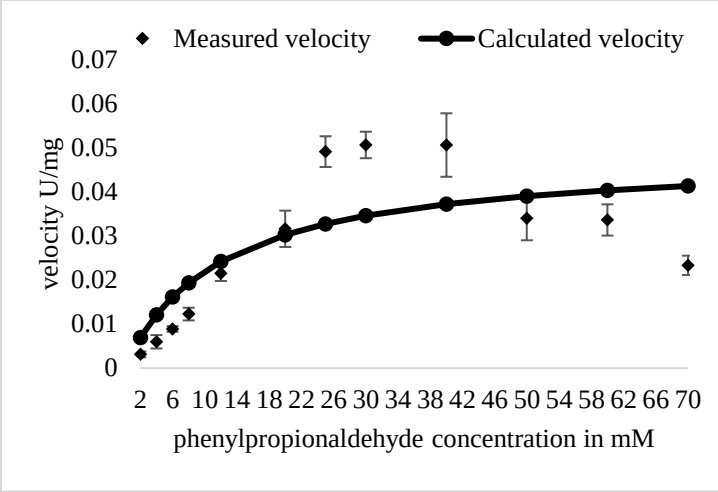

Figure 5.12: Michaelis–Menten kinetics plot of *At*HNL-F179L catalysed DKDNR in the synthesis (2*S*,3*R*)-NPB

Table 5.3: Kinetic parameters of *At*HNL wild type and F179L variant in the DKDNR catalysed synthesis of total NPB (four diastereomers)

Enzyme	**[(2*S*,3*S*) + (2*R*,3*R*) + (2*R*,3*S*) + (2*S*,3*R*)]-NPB**		
	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}mM^{-1}$)
WT	21.09	0.62	0.03
F179L	11.359	1.628	0.143

Table 5.4: Kinetic parameters of *At*HNL wild type and F179L variant in the DKDNR catalysed synthesis of four different NPB diastereomers.

Enzyme	**(2*S*,3*S*)-NPB**			**(2*R*,3*R*)-NPB**			**(2*R*,3*S*)-NPB**			**(2*S*,3*R*)-NPB**		
	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})
WT	16.203	0.082	0.005	24.56	0.30	0.01	10.00	0.0024	0.0002	0.005	0.128	0.008
F179L	9.847	0.142	0.014	13.380	0.000	0.000	2.000	0.011	0.006	12.006	1.354	0.112

5.5. Discussion

5.5.1. Optimization of reaction conditions of wild type *At*HNL catalysed DKDNR in the synthesis of NPB stereoisomers

To attain dynamic kinetic asymmetric nitroaldol reaction, we emphasized on two critical biocatalysis parameters, mole equivalents of nitromethane and pH of the buffer. We commenced our study by nitromethane (**2**) addition to racemic PPA (**1a**) in the presence of purified WT *At*HNL (**Scheme 5.2**). It is expected that nitromethane addition to the fast enantiomer of the aldehyde would provide a nitroaldol product via *At*HNL catalysed kinetic resolution. The molar ratio of PPA to nitromethane was varied from 1:1 to 1:62.5. Higher nitromethane concentration is assumed to drive the reaction equilibrium forward and hence favor the DKDNR. At 1:1 PPA to nitromethane ratio, modest activity of *At*HNL was observed, i.e., 2% conversion, 1:1 *anti* / *syn* ratio, 7% *ee* of (2*S*, 3*S*)-NPB, **3a** and 99% *ee* of (2*S*, 3*R*)-**3a** at 6 h with buffer pH 6.0 (**Table 5.2**). With increase in PPA to nitromethane ratio, activity of *At*HNL has been gradually increased and highest was observed in the case of 1:62.5 ratio, i.e., 41% conversion, 3:1 *anti* / *syn* ratio, 5% *ee* of (2*S*, 3*S*)-**3a** and 98% *ee* of (2*S*, 3*R*)-**3a** at 6 h, buffer pH 6.0. A similar trend of result has been observed at 24 h reaction time with KPB pH 6.0 buffer. Highest activity of *At*HNL was observed in the case of 1:62.5 ratio, i.e., 70% conversion, 3:1 *anti* / *syn* ratio, –1% *ee* of (2*S*, 3*S*)-**3a** and 93% *ee* of (2*S*, 3*R*)-**3a** at 24 h. Therefore 1:62.5 ratio of aldehyde to nitromethane was chosen as optimum ratio. Phosphate buffer pH 7.0 or 7.5 have been reported for spontaneous racemization of the α-branched aldehydes.[17,18,23] However, the challenge was to carry out the DKDNR at a lower pH. At pH> 7.0, *At*HNL shows decreased enantioselectivity in the nitroaldol reaction due to predominate non-enzymatic reaction.[12] We thus investigated the DKDNR in phosphate buffer of pH ranging from 5.8 to 7.4. At pH 5.8, 76% total conversion of **3a**, 4:1 ratio of *anti : syn*, 3% *ee* of (2*S*, 3*S*)-**3a** and

92% *ee* of (2*S*, 3*R*)-**3a** were observed at 24 h. At pH 7.0, 91% total conversion of **3a**, 4:1 diastereomeric ratio (*anti* / *syn*), 14% *ee* of (2*S*, 3*S*)-**3a** and 90% *ee* of (2*S*, 3*R*)-**3a** were observed at 24 h (**Table 5.2**). Compared to pH 5.8, at pH 7.0, total conversion was increased marginally and % *ee* of (2*S*, 3*R*)-**3a** decreased slightly. At pH 7.4, 95% total conversion of **3a**, 4:1 diastereomeric ratio (*anti* / *syn*) ratio, 13% *ee* of (2*S*, 3*S*)-**3a** and 86% *ee* of (2*S*, 3*R*)-**3a** were observed at 24 h**.** Compared to pH 7.0, at pH 7.4 the % conversion of **3a** was increased but % *ee* of (2*S*, 3*R*)-**3a** was decreased**.** Therefore pH 7.0 was chosen as the optimum pH for DKDNR.

5.5.2. Screening of *At*HNL variant library for DKDNR catalysed synthesis of NPB stereoisomers

With the modest stereoselectivity achieved by the wild type enzyme in DKDNR, we began screening a panel of forty-eight *At*HNL variants prepared by altering six different binding site residues (Ala13, Tyr14, His15, Phe80, Phe82, and Phe179). These residues were chosen based on their close proximity and orientation to the substrate. DKDNR by wild type *At*HNL has produced 28% conversion to **3a**, 6% *ee* of (2*R*, 3*R*)-**3a** and 88% *ee* of (2*S*,3*R*)-**3a** (**Figure 5.2**). Among the forty-eight *At*HNL variants evaluated for biocatalytic DKDNR, reasonable stereoselectivity in the synthesis of (2*S*,3*R*)-**3a** was obtained in the case of several variants, while a few also shown selectivity towards the synthesis of uncommon diastereomers such as (2*S*,3*S*)-**3a**, (2*R*,3*R*)-**3a**, and (2*R*,3*S*)-**3a** (**Figure 5.2**). Unfortunately, poor conversion (less than 10%) by most of the variants showing diverse diastereoselectivity. To our pleasure, one variant F179L has shown significantly high activity compared to the wild type. In case of *At*HNL- F179L, 82% conversion to **3a,** 94% *ee* of (2*S*,3*S*)-**3a** and 98% *ee* of (2*S*,3*R*)-**3a** was obtained. This result shows that not only high enantioselectivity towards the regular stereoisomer (2*S*,3*R*)-**3a** (produced by wild type), but also towards an uncommon stereoisomer (2*S*,3*S*)-**3a** was achieved by the F179L. It clearly proves our

accomplishment of diastereocomplementary synthesis of β-aryl-α-hydroxynitroalkanes by the engineered *At*HNL via DKDNR. Protein engineering to improve enantioselectivity is not so uncommon, however examples of gaining access to a new stereoisomer by a single mutation is rare. We are yet to understand the molecular basis of how F179L has been able to produce the (2*S*,3*S*)-**3a**. *At*HNL has been engineered earlier to improve its stability.[24] In the previous chapter we have found engineered *At*HNLs being able to prepare (*S*)-β-nitroalcohols by retro-Henry reaction, where increased substrate scope was explored. This is the first report not only with respect to *At*HNL, but among all the HNLs, where an engineered enzyme is used in DKDNR and a single mutation has successfully produced one *anti*, i.e., (2*S*,3*S*)-**3a** and one *syn*, i.e., (2*S*,3*R*)-**3a** diasteromers of NPB with high enantioselectivity.

5.5.3. Determination of kinetic parameters of *At*HNL wild type and F179L in the DKDNR catalysed synthesis of NPB stereoisomers

In order to explain the efficiency of an enzyme catalyzing a particular reaction, it is important to find out its kinetic parameters. As per the kinetic parameters of *At*HNL wild type and F179L determined in section **5.4.6** above, the latter has shown higher binding affinity (low K_M) to the substrate PPA, 2.6-fold of higher turnover number (k_{cat}), and 4.76-fold of higher k_{cat}/K_M compared to the wild type in the synthesis of total NPB (all the four diastereomers) (**Table 5.3**). Therefore, the engineered *At*HNL has proved to be efficient than the wild type. This variant was also found to be efficient in the synthesis (2*S*,3*S*)-NPB. Analysis of the kinetics data reveals that, in the synthesis of (2*S*,3*S*)-NPB, the variant has shown a lower K_M, 1.73-fold higher turnover number (k_{cat}), and 2.8-fold higher k_{cat}/K_M compared to the wild type (**Table 5.4**). In the case of synthesis of (2*S*,3*R*)-NPB, the variant showed 10.57-fold of higher k_{cat} and 14-fold of higher k_{cat}/K_M compared to the wild type (**Table 5.4**). A recent study on HNL engineering followed by

determination of kinetic parameters has revealed catalytically more efficient variants compared to the wild type enzyme. Zheng et al., engineered *Prunus communis* hydroxynitrile lyase (*Pc*HNL5) to enable hydrocyanation of rigid benzo-ketal aldehydes with high enantioselectivity.[25] Specific activity of *Pc*HNL5 variant L331A towards 1,3-oxane ring-fused benzaldehyde was found to be 2.4 U/mg, which is 545-folds higher as compared to the wild type enzyme. *Pc*HNL5 engineering has also improved the substrate scope of the enzyme. For fifteen different structurally diverse aldehydes, the turnover frequency (TOF or k_{cat}) of the *Pc*HNL5 variants in the synthesis of their corresponding (*R*)-cyanohydrins were found to be 1.3–1249 s^{-1} with 41–97% yield and 95–99% *ee*. The TOF of wild type *Pc*HNL5 for the same set of fifteen aldehydes for hydrocyanation was in the range of 0.022–276 s^{-1} with 32–99% *ee*. Thus, compared to the wild type, TOF of the variants improved by 1.6 to 792-folds. Kazlauskas and co-authors found improved retro-nitroaldolase activity with *Hevea brasiliensis* HNL (*Hb*HNL) variants than the wild type enzyme.[26] The best *Hb*HNL variant with the triple substitution, i.e., L121Y-F125T-L146M has shown a specific activity of 0.71 $U.mg^{-1}$ towards the cleavage of racemic NPE, which is ~5.5-folds higher than that of the wild type activity (0.13 U mg^{-1}). Further, the k_{cat} for this variant was 3.3 times higher than that of the wild type. Our kinetic study further proves the catalytic efficiency of the variant F179L towards the DKDNR compared to the wild type enzyme.

5.6. Conclusions

We have envisioned to synthesize β-aryl-α-hydroxynitroalkanes via DKDNR using engineered *At*HNL. Towards this, we optimized the aldehyde to nitromethane ratio of the DKDNR based biotransformation. The optimization in the case of 1:62.5 ratio of aldehyde to nitromethane ratio in the wild type *At*HNL catalysed DKDNR in the stereoselective synthesis of NPB has produced 70% conversion, 3:1 *anti* / *syn* ratio, −1% *ee* of (2*S*,3*S*)-NPB and 93% *ee* of (2*S*,3*R*)-NPB at 24 h.

Next, we have investigated the DKDNR in phosphate buffer of pH ranging from 5.8 to 7.4. At pH 7.0 91% total conversion of NPB, 4:1 diastereomeric ratio (*anti* / *syn*) ratio, 14% *ee* of (2*S*,3*S*)-NPB and 90% *ee* of (2*S*,3*R*)-NPB were observed at 24 h and is selected as the optimum pH. Forty-eight *At*HNL variants were evaluated for biocatalytic DKDNR using crude lysates of the variants. Wild type *At*HNL catalysed DKDNR has shown 28% conversion to NPB**,** 6% *ee* of (2*R*,3*R*)-NPB and 88% *ee* of (2*S*,3*R*)-NPB. The screening has identified F179L variant that showed significantly high activity compared to the wild type, 82% conversion to NPB**,** 94% *ee* of (2*S*,3*S*)-NPB and 98% *ee* of (2*S*,3*R*)-NPB. Kinetic study of the wild type and F179L was carried out to find out the efficiency of the engineered variant towards the synthesis of different diastereomers of NPB by DKDNR. *At*HNL-F179L has shown higher binding affinity (low K_M) to the substrate PPA, 2.6-fold higher turnover number (k_{cat}) and 4.76-fold higher k_{cat}/K_M than the wild type in the synthesis of total amount of NPB (all the four diastereomers), hence proved to be efficient than the wild type. Towards the synthesis of (2*S*,3*S*)-NPB, the variant has shown a lower K_M to the substrate PPA, 1.73-fold higher k_{cat} and 2.8-fold higher k_{cat}/K_M than the wild type. Similarly, F179L has shown higher efficiency towards the synthesis of (2*S*,3*R*)-NPB. It showed 10.57-fold higher k_{cat} and 14-fold higher k_{cat}/K_M compared to the wild type. Therefore, F179L has proven its higher catalytic efficiency not only in the synthesis of total amount of NPB, but also towards the synthesis of the regular stereoisomer (2*S*,3*R*)-NPB and the uncommon stereoisomer (2*S*,3*S*)-NPB. This study has shown that a single variant F179L in *At*HNL has efficiently performed the diastereocomplementary synthesis of β-aryl-α-hydroxynitroalkanes via DKDNR and produced two stereoisomers in high enantiopurity.

^{1}H NMR Characterization of 1-nitro-3-phenylbutanol

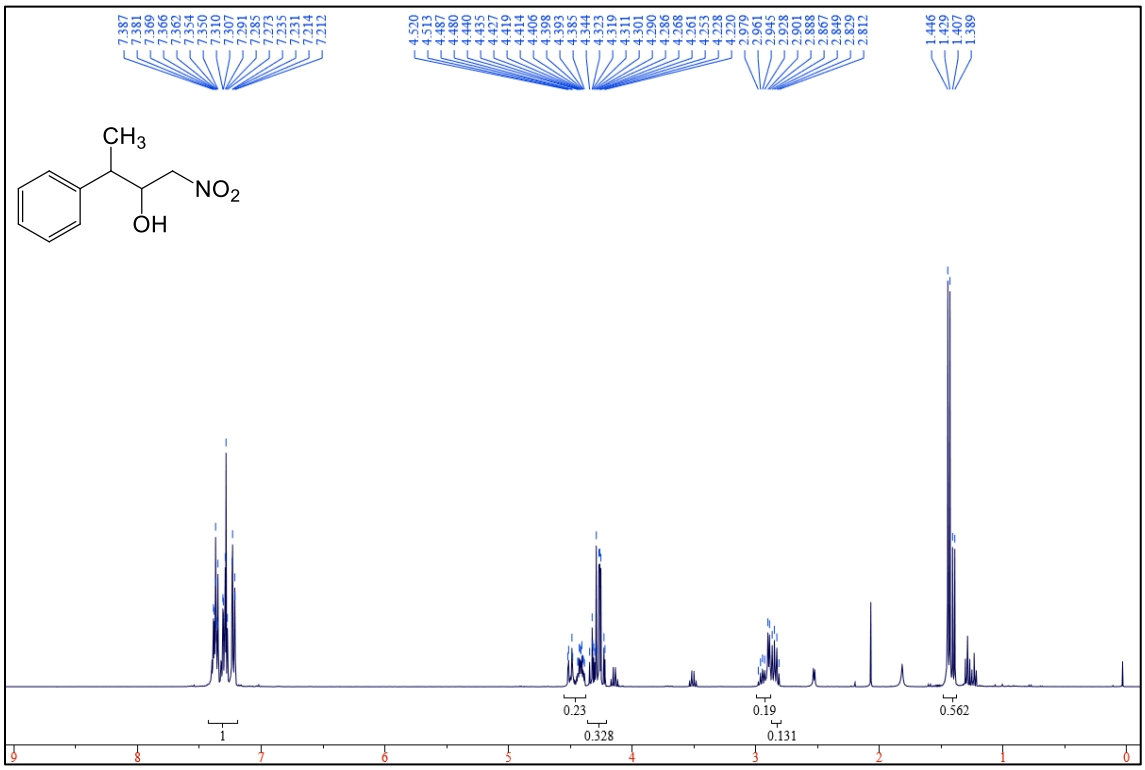

Figure 5.13.1: ^{1}H NMR spectrum of 1-nitro-3-phenylbutanol

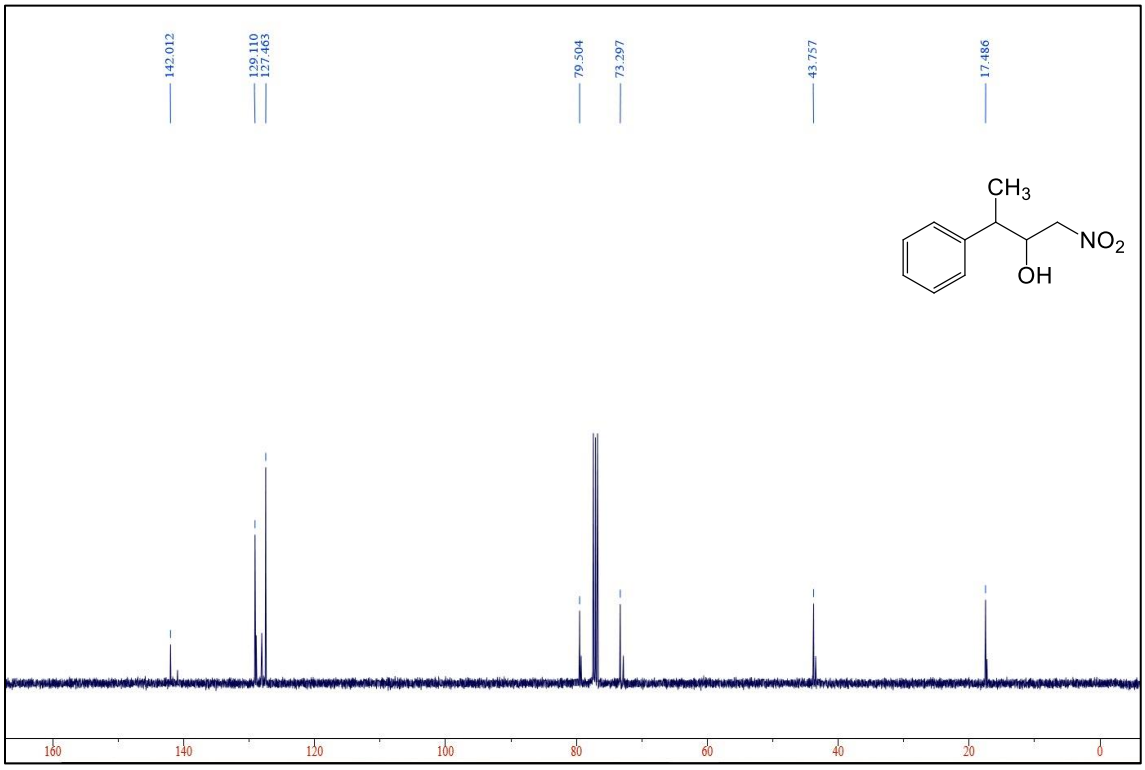

Figure 5.13.2: ^{13}C NMR spectrum of 1-nitro-3-phenylbutanol

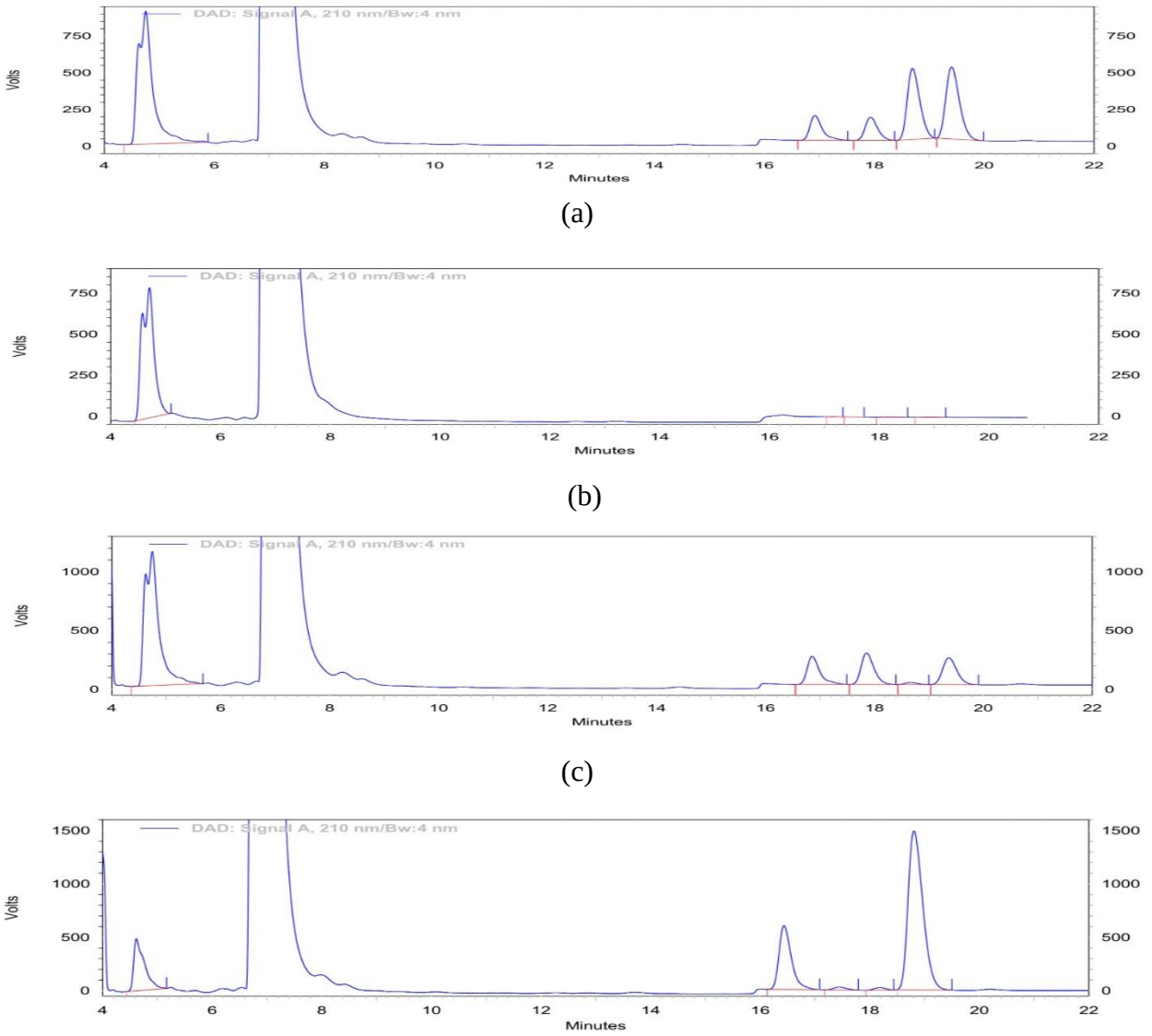

Figure 5.14: HPLC chromatograms of *At*HNL catalysed DKDNR in the synthesis of NPB stereoisomers. (a), (b), (c) and (d) represents standard, control, wild type and *At*HNL-F179L variant catalysed reactions respectively.

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Chapter 6

Conclusions and future prospects

Enantiopure β-nitroalcohols are structural motifs of several chiral drugs and biologically important molecules. With the recent demand for greener synthetic methods for industrially important fine chemicals and chiral intermediates, enzyme catalysis are increasingly studied. Among the existing biocatalytic approaches for synthesis of chiral β-nitroalcohols, hydroxynitrile lyase (HNL) catalyzed Henry reaction is considered to be one of the best because of its own advantages. The retro-Henry reaction appeared even more fascinating as it could be used to prepare enantioenriched β-nitroalcohols having absolute configuration opposite to that of the stereopreference of an HNL. Not only that, but also rate of enantioselective cleavage of a β-nitroalcohol is high as compared to the synthesis, which makes the retro-Henry reaction an efficient biocatalytic approach. The retro-Henry reaction despite of its potential significance, had remained as an underdeveloped method to prepare enantiopure β-nitroalcohols. In order to prepare (*S*)-β-nitroalcohols by this approach, we have envisioned to use *Arabidopsis thaliana* (*At*HNL), a (*R*)-selective HNL, known to catalyze the promiscuous stereoselective nitromethane addition to aldehydes to prepare (*R*)-β-nitroalcohols. The aim of the thesis was to develop the *At*HNL catalyzed retro-Henry reaction to prepare a diverse range of (*S*)-β-nitroalcohols, and also to prepare immobilized enzyme to improve the enzymatic stability, and activity, and use it further in the enantioselective C-C bond cleavage reaction. We also intended to exploit engineered *At*HNL variants to enhance the substrate scope of the enzyme in the enantioselective preparation of (*S*)-β-nitroalcohols. To improve the existing methods of diastereoselective synthesis of β-nitroalcohol, we aimed to develop a one-pot dynamic kinetic resolution cum nitroaldol reaction in the synthesis of β-nitroalcohol diastereomers.

To achieve one of the major objectives of the thesis, we have exploited the retro-Henry reaction by *At*HNL in the preparation of (*S*)-β-nitroalcohols starting from their racemic counterparts. Kinetic studies of *At*HNL using cleavage of racemic 2-nitro-1-phenyl ethanol (NPE) has revealed 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. 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-nitroalcohols having substituents at different positions of the aromatic ring were tested in the retro-Henry reaction, and their corresponding (*S*)-β-nitroalcohols were obtained with varied enantioselectivity. This proves the broad substrate selectivity of *At*HNL and also the efficacy of the method. To demonstrate the feasibility of the *At*HNL catalyzed retro-Henry reaction as a practical method, we have carried out a preparative scale biotransformation that produced (*S*)-NPE in 54% yield and 93% *ee*. We have proved that this is the fastest HNL catalyzed route known so far to synthesize a series of (*S*)-β-nitroalcohols.

In order to address the second objective *At*HNL was immobilized by physical adsorption on celite®545, an inexpensive adsorbent. The celite-*At*HNL catalyzed retro-Henry reaction was investigated in the preparation of (*S*)-β-nitroalcohols. After optimisation of the biocatalysis conditions, the total turnover number of the celite-*At*HNL was increased 2.3-fold. The celite-*At*HNL has shown good retro-Henry activity at low pH e.g., 4.5 and 5.0 with 62 - 97% *ee* and 41 - 42% conversion of (*S*)-NPE, compared to inactivation of the free enzyme at pH <5.0. This increased catalytic efficiency and pH stability could be possibly due to increased stability of

*At*HNL by immobilization. A dozen of racemic β-nitroalcohols were converted into their corresponding (*S*)-β-nitroalcohols using this reaction; among them eight were not tested earlier. The immobilized enzyme has shown broad substrate selectivity in the retro-Henry reaction and products were obtained up to 98.5% *ee*. However, during the recyclability study, the celite-*At*HNL showed decreased % *ee* of NPE in second cycle onwards. This could be probably due to leaching of the enzyme.

To further improve the *At*HNL catalyzed retro-Henry reaction to attain higher eanntioselectivity and broad substrate selectivity, a series of variants of this enzyme were studied. The *At*HNL variants library resulted from alteration at three positions Phe82, Phe179 and Tyr14 was screened towards retro-Henry reaction using crude enzyme extracts with multiple racemic β-nitroalcohols. Best activity was found in the case of F179K and F179M. Towards cleavage of 1-(3,5-dimethoxyphenyl)-2-nitroethanol, 1-(3-hydroxyphenyl)-2-nitroethanol, 1-(4-methoxyphenyl)-2-nitroethanol and 1-(4-nitrophenyl)-2-nitroethanol wild type *At*HNL has shown poor enantioselectivity of –1 to 10% *ee*, and 29–50% conversion. Pleasantly, the *At*HNL-F179K has shown higher enantioselectivity towards the cleavage of 1-(3-hydroxyphenyl)-2-nitroethanol and 1-(4-methoxyphenyl)-2-nitroethanol with 71 and 26% *ee* and 36 and 42% conversion respectively. The *At*HNL-F179M has shown higher enantioselectivity in case of the retro-Henry reaction of 1-(3,5-dimethoxyphenyl)-2-nitroethanol, 1-(3-hydroxyphenyl)-2-nitroethanol and 1-(4-methoxyphenyl)-2-nitroethanol and produced the corresponding (*S*)-NPEs with 11, 69 and 25% *ee* and 49, 29 and 44% conversions respectively. F179T and Y14M were selected from another relevant study where the same library was screened against enantioselective cleavage of racemic NPE. Overall, four *At*HNL mutants F179K, F179M, F179T and Y14M were selected to find out their substrate scope towards enantioselective cleavage of a six different racemic β-nitroalcohols.

Racemic 2-Cl NPE, 3-Cl NPE, 4-Cl NPE, 3-OH NPE, 4-MeO NPE and 4-Me NPE were used in the wild type *At*HNL catalysed enantioselective cleavage study. These biotranformations have produced corresponding (*S*)-β-nitroalcohols in 48, 85, 35, 67, 40 and 56% *ee* and 46, 51, 43, 34, 40 and 41 % conversions respectively in 3 to 7 hours. Among the variants tested, F179K has shown higher enantioselectivity towards enantioselective cleavage of 2-Cl NPE, 4-Cl NPE, 3-OH NPE and 4-MeO NPE compared to wild type *At*HNL. It produced corresponding (*S*)-β-nitroalcohols in 98, 74, 99, 99 % *ee* and 43, 43, 29, 33% conversions respectively. We also found F179T that showed higher enantioselectivity towards the enantioselective cleavage of 3-Cl NPE and 4-Me NPE compared to the wild type. It produced corresponding (*S*)-β-nitroalcohols in 99, 98% *ee* and 54, 43% conversions respectively in 3 hours. The % *ee* of various (*S*)-β-nitroalcohols obtained by retro-Henry reaction are comparable with that of obtained lipase catalysed kinetic resolution or asymmetric reduction of corresponding α-nitro ketones. The yields of (*S*)-β-nitroalcohols prepared by the current method are in par with the kinetic resolution method, and in some cases even better (2-Cl NPE by PS-IM), but are less than that of the asymmetric reduction approach. Nonetheless, the asymmetric reduction process requires cofactor dependent enzymes, and addition chemical synthesis of the substrate α-nitro ketones which are not readily available, while our approach uses a cofactor-less enzyme and racemic β-nitroalcohols as substrates which can be easily prepared from the commercially available aldehydes. For the first time, *At*HNL engineered mutants have been used in retro-Henry reaction. By using these variants, we have successfully produced six different (*S*)-β-nitroalcohols with high enantiomeric excess and conversions.

To synthesize two chiral centered β-nitroalcohols in a stereocontrolled manner using *At*HNL, we have used α-branched aldehydes as substrates. We envisaged the process of Dynamic Kinetic Diastereoselective Nitroaldol Reaction (DKDNR), a combination of DKR with HNL catalysed

diastereoselective nitroaldol reaction to synthesize such β-nitroalcohol stereoisomers. The DKDNR has synthesized chiral β-aryl-α-hydroxynitroalkane, in particular stereoisomers of 1-nitro-3-phenylbutan-2-ol (NPB). Towards this, the aldehyde to nitromethane ratio of the DKDNR based biotransformation was optimized. In the case of 1:62.5 ratio of aldehyde to nitromethane in the wild type *At*HNL catalysed stereoselective DKDNR, NPB was produced in 70% conversion, 3:1 *anti* / *syn* ratio, −1% *ee* of (2*S*,3*S*)-NPB and 93% *ee* of (2*S*,3*R*)-NPB at 24 h. Next, the DKDNR was investigated in phosphate buffer of pH ranging from 5.8 to 7.4. At pH 7.0, 91% total conversion of NPB, 4:1 diastereomeric ratio (*anti* / *syn*) ratio, 14% *ee* of (2*S*,3*S*)-NPB and 90% *ee* of (2*S*,3*R*)-NPB were observed at 24 h and is selected as the optimum pH. Forty-eight *At*HNL variants were evaluated for biocatalytic DKDNR using crude lysates of the variants. Wild type *At*HNL catalysed DKDNR has shown 28% conversion to NPB**,** 6% *ee* of (2*R*,3*R*)-NPB and 88% *ee* of (2*S*,3*R*)-NPB. The screening has identified F179L variant that showed significantly high activity compared to the wild type, 82% conversion to NPB**,** 94% *ee* of (2*S*,3*S*)-NPB and 98% *ee* of (2*S*,3*R*)-NPB. Kinetic study of the wild type and F179L was carried out to find out the efficiency of the engineered variant towards the synthesis of different diastereomers of NPB by DKDNR. *At*HNL-F179L has shown higher binding affinity (low K_M) to the substrate 2-phenylpropionaldehyde (PPA), 2.6-fold higher turnover number (k_{cat}) and 4.76-fold higher k_{cat}/K_M than the wild type in the synthesis of total amount of NPB (all the four diastereomers), hence proved to be efficient than the wild type. Towards the synthesis of (2*S*,3*S*)-NPB, the variant has shown a lower K_M to the substrate PPA, 1.73-fold higher k_{cat} and 2.8-fold higher k_{cat}/K_M than the wild type. Similarly, F179L has shown higher efficiency towards the synthesis of (2*S*,3*R*)-NPB. It showed 10.57-fold higher k_{cat} and 14-fold higher k_{cat}/K_M compared to the wild type. Therefore, F179L has proven its higher catalytic efficiency not only in the synthesis of total amount of NPB,

but also towards the synthesis of the regular stereoisomer (2*S*,3*R*)-NPB and the uncommon stereoisomer (2*S*,3*S*)-NPB. This study has shown that a single variant F179L in *At*HNL has efficiently performed the diastereocomplementary synthesis of β-aryl-α-hydroxynitroalkanes via DKDNR and produced two stereoisomers in high enantiopurity.

Future prospects:

- We have demonstrated the *At*HNL catalyzed retro-Henry reaction in the preparation of a number of (*S*)-β-nitroalcohols. This method of retro-Henry reaction can be further exploited to prepare opposite enantioselective products using other HNLs.
- One of the major limitations of *At*HNL catalyzed retro-Henry reaction is substrate inhibition by the enzyme at higher aldehyde concentrations. This limits the process to prepare enantioenriched β-nitroalcohols in large scale. Protein engineering of the enzyme may address the substrate inhibition issue. The other possible solution is to in-situ remove the aldehyde from the reaction mixture by a chemical or enzymatic approach without altering the catalytic activity of the *At*HNL.
- In the current study we prepared a number of (*S*)-β-nitroalcohols. Some of them are precursors to pharmaceutical intermediates. Their potential industrial application could be exploited.
- To make the *At*HNL catalyzed retro-Henry reaction a feasible biocatalytic approach, (*S*)-β-nitroalcohols could be prepared in preparative scale and commercialization of these fine chemicals could be carried out.
- Preparation of a diverse range of (*S*)-β-nitroalcohols can be done using *At*HNL and its variants.

- Our immobilized *At*HNL could not be recycled in the retro-Henry reaction. Hence, other immobilization methods can be studied to address (a) the recyclability of the enzyme in the biocatalysis, and (b) minimize the substrate inhibition.
- The celite-*At*HNL being active at low pH 4.5 to 5.0 in the retro-Henry reaction, can be exploited in related cascade, and chemo-enzymatic syntheses that may require acidic pH.
- *At*HNL can be engineered to accept a wide variety of racemic β-nitroalcohols in the retro-Henry reaction.
- In the present study we have developed DKDNR for the first time using *At*HNL variants and carried out DKR in the synthesis of β-aryl-α-hydroxynitroalkane stereoisomers. This process needs further investigation to understand the molecular mechanism of how the F179L variant synthesized the (2*S*,3*R*)-NPB and the uncommon stereoisomer (2*S*,3*S*)-NPB. We have demonstrated the DKDNR in the synthesis of a single product only. The application of this method in the synthesis of a number of β-aryl-α-hydroxynitroalkane stereoisomers can be studied.

LIST OF PUBLICATIONS BASED ON RESEARCH WORK

a) In refereed international journals

1. Ayon Chatterjee, **D.H. Sreenivasa Rao,** Santosh Kumar Padhi. One-pot enzyme cascade catalyzed asymmetrization of primary alcohols: Synthesis of enantiocomplementary chiral β-nitroalcohols, *Adv. Synth. Catal*., **2021**, 363, 5310–5318. https://doi.org/10.1002/adsc.202100803. ISSN:1615-4169, Impact factor: **5.837**, Published: 08.09.2021.

2. **D.H. Sreenivasa Rao**, Ayon Chatterjee, Santosh Kumar Padhi. Biocatalytic approaches for enantio and diastereoselective synthesis of chiral β-nitroalcohols, *Org. Biomol. Chem.*, **2021**, 19, 322-337. https://doi.org/10.1039/D0OB02019B. ISSN: 1477-0520 (print); 1477-0539 (web). Impact factor: **3.876** Published: 27.11.2020.

3. **D.H. Sreenivasa Rao**, Kummari Shivani, Santosh Kumar Padhi. Immobilized Arabidopsis thaliana hydroxynitrile lyase catalyzed retro-Henry reaction in the synthesis of (S)-β-nitro alcohols, Appl. Biochem. Biotechnol., **2021**, 193, 560-576. https://doi.org/10.1007/s12010-020-03442-3. Impact factor: **2.926** Published: 12.10.2020, ISSN- 02732289

4. **D.H. Sreenivasa Rao**, Santosh Kumar Padhi. Production of (*S*)-β-nitro alcohols by enantioselective C-C bond cleavage with an (*R*)-selective Hydroxynitrile lyase. *ChemBioChem*. **2019**, 20, 371-378. https://doi.org/10.1002/cbic.201800416, Impact factor: **3.164**. Published: 08.11.2018.

b) Manuscripts communicated

1. Badipatla Vishnu Priya, **D. H. Sreenivasa Rao**, Rubina Gilani, Surabhi Lata, Nivedita Rai, Mohd. Akif, Santosh Kumar Padhi. Enzyme engineering improves catalytic efficiency and enantioselectivity of hydroxynitrile lyase for promiscuous retro-nitroaldolase activity. *Bioorg. Chem.*, Under review.

c) Presentations in conferences

1. **D.H. Sreenivasa Rao**, Dheeraj Sangoji, Santosh Kumar Padhi "Production of (*S*)-β-nitro alcohols by a (*R*)-selective hydroxynitrile lyase via enantioselective C-C bond cleavage"-

Presented in "**International Chemical Biology Society, 8th Annual Conference on Navigating Translational Discoveries"** 2nd -4th November 2019, at CSIR-IICT, Hyderabad. (P-73)

2. **D.H. Sreenivasa Rao**, Dheeraj Sangoji, Santosh Kumar Padhi "Biocatalytic retro-Henry reaction, a new route to prepare (*S*)-β-nitro alcohols"-Presented in "**National Seminar on "Biomolecular Interaction in Development and Diseases**" 26th -28th September 2019, at School of Life Sciences, University of Hyderabad. (Abstract book page no 62)
3. **D.H. Sreenivasa Rao***, Dheeraj Sangoji, Santosh Kumar Padhi "Biocatalytic retro-nitroaldol reaction, a new enzymatic route to prepare (*S*)-β-nitro alcohols" - Presented in "**BioQuest 2019**" School of Life Sciences, University of Hyderabad, Hyderabad, India from 11th March 2019 (Best Oral Presentation award, 2nd prize).
4. Kummari Shivani*, **D.H.Sreenivasa Rao**, Santosh Kumar Padhi "Immobilization of Hydroxynitrile lyase and its Biocatalytic Application in the Synthesis of Chiral β-nitro alcohols" - Presented in "**BioQuest 2019**" School of Life Sciences, University of Hyderabad, Hyderabad, India from 11th March 2019 (Best Poster Presentation award, 1st prize, Poster No: 34).
5. Nivedita Rai*, **D. H. Sreenivasa Rao**, Lipika Pattanayak, Badipatla Vishnu Priya, Surya Narayan Rath, and Santosh Kumar Padhi, "A theoretical and experimental study on two different reactions catalyzed by the same catalytic site of a α/β-hydrolase fold hydroxynitrile lyase" - Presented in "**BioQuest 2017**" School of Life Sciences, University of Hyderabad, Hyderabad, India from 12th – 13st October 2017 (Poster P-63).
6. **D.H. Sreenivasa Rao***, Dheeraj Sangoji, and Santosh Kumar Padhi, "Preparation of optically pure β-nitro alcohols by biocatalytic stereoselective C-C bond cleavage of a racemic mixture" - Presented in "**BioQuest 2017**" School of Life Sciences, University of Hyderabad, Hyderabad, India from 12th – 13st October 2017 (Poster P-39).

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Sustainable Biocatalytic Synthesis of (S)-β-Nitro Alcohols using a Promiscuous (R)-Selective Hydroxynitrile Lyase

by Sreenivasa Rao D H

Sustainable Biocatalytic Synthesis of (S)-β-Nitro Alcohols using a Promiscuous (R)-Selective Hydroxynitrile Lyase

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12. Whether a summary of the dissertation enclosed: Yes.

Supervisor(s) Signature: 24/12/2021
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Signature of the Head of the Department.
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HYDERABAD-500 046.

Signature of the Dean, of the School :

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