Baliospermum montanum hydroxynitrile lyase catalyzed stereoselective synthesis of chiral cyanohydrins

A thesis submitted for the degree of

PHILOSOPHY OF DOCTORATE

By

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CERTIFICATE

This is to certify that the thesis entitled ***"Baliospermum montanum hydroxynitrile lyase catalyzed stereoselective synthesis of chiral cyanohydrins"*** submitted by Nisha Jangir to University of Hyderabad, Hyderabad, for the award of the degree of Doctor of Philosophy is bonafied record of research work carried out by her 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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DECLARATION

I, **Nisha Jangir**, hereby declare that this thesis entitled ***"Baliospermum montanum hydroxynitrile lyase catalyzed stereoselective synthesis of chiral cyanohydrins"*** 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.

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Signature of the student

Name: Nisha Jangir

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Signature of the Supervisor

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This is to certify that this thesis entitled ***"Baliospermum montanum hydroxynitrile lyase catalyzed stereoselective synthesis of chiral cyanohydrins"*** submitted by **Ms. Nisha Jangir** bearing registration number **15LBPH10** in partial fulfilment of the requirements for award of Doctor of Philosophy in the Department of Biochemistry, School of Life Sciences, is a bonafide work carried out by her under my supervision and guidance.

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A. Published in the following publications:

1. Nisha Jangir, Dheeraj Sangoji, and Santosh Kumar Padhi, *Baliospermum montanum* hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins in a biphasic solvent. *Biocatal Agric Biotechnol.*, **2018**, *16*, 229-236 (Chapter-4 and part of Chapter 3).

2. Nisha Jangir, Santosh Kumar Padhi, Immobilized *Baliospermum montanum* hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins. *Bioorg. Chem.*, **2019**, *84*, 32-40 (Chapter-5 and part of Chapter 3).

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Further, the student has passed the following courses towards fulfilment of coursework requirement for Ph.D.

Course code	Name	Credits	Pass/Fail
BC 801	Analytical Techniques/Research methodology	4	Pass
BC 802	Biostatistics Research ethics and Data analysis	3	Pass
BC 803	Lab seminar and Record	5	Pass

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Abstract

Optically pure cyanohydrins are potential chiral molecules used in the synthesis of various pharmaceuticals, agrochemicals and bioactive compounds. They can be synthesized by different chemical and biocatalytic methods. Enzyme mediated kinetic resolution which includes asymmetric acylation and deacylation of racemic cyanohydrins by lipases and esterases, is one of the common biocatalytic methods to obtain chiral cyanohydrins. However, kinetic resolution has the disadvantage of giving maximum 50% yield of each enantiomer. The second biocatalytic method for synthesis of chiral cyanohydrins is enantioselective C-C bond formation which is usually carried out by hydroxynitrile lyases (HNLs).

Among the several HNLs known so far, the latest member of HNL from the α/β hydrolase fold is *Baliospermum montanum* hydroxynitrile lyase (*Bm*HNL). It is an (*S*)-selective HNL with high substrate preference for bulky aromatic aldehydes. Despite of its unique substrate selectivity, *Bm*HNL biocatalysis is limited with poor enantioselectivity. The aim of the present study is to investigate the *Bm*HNL catalyzed stereoselective synthesis of chiral cyanohydrins to improve the enantioselectivity and stability of the enzyme in biocatalysis.

To achieve the objectives, *Bm*HNL was subcloned. Its protein expression, purification was performed and it was characterized by SDS-PAGE and enzymatic assay. Racemic cyanohydrins to be used as internal analytical standards were synthesized. Eighteen racemic cyanohydrins were synthesized using literature based and modified protocols using different cyanide donors. Among them, six racemic cyanohydrins were synthesized using KCN as cyanide donor and another six were synthesized using trimethylsilylcyanide as cyanide source. Rest six cyanohydrins were prepared by a modified protocol using

acetone cyanohydrin as source of cyanide. All the cyanohydrins were characterized by ^{1}H and ^{13}C NMR. Chiral resolution of racemic cyanohydrins was carried out using high performance liquid chromatography (HPLC) with Chiralpak IB and IE chiral columns. Separation factor (α) and resolution factor (*Rs*) of all the HPLC chromatograms of the racemic cyanohydrin were calculated. HPLC chromatograms of sixteen racemic cyanohydrins have showed $\alpha > 1$ and $Rs > 1.5$, indicting good resolution of the enantiomers.

*Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins was carried out for the first time in a biphasic system. Toward this, standardization of reaction parameters/conditions such as different organic solvents and their percentage, substrate concentration, pH of buffer and temperature was carried out using crude enzyme. For each of the above optimization step, benzaldehyde conversion to (*S*)-mandelonitrile was used as the standard reaction. Using the optimized conditions, seventeen different aldehydes were converted into their corresponding (*S*)-cyanohydrins. In all, eight (*S*)-cyanohydrins reported first time here whereas fourteen were not tested for *Bm*HNL earlier.

To improve biocatalytic properties of the enzyme, *Bm*HNL was first time immobilized using cross-linking method. Cross-linked enzyme aggregates of *Bm*HNL i.e. CLEA-*Bm*HNL was prepared and characterized by SDS-PAGE and scanning electron microscopy. The enzymatic activity recovery of CLEA-*Bm*HNL was found to be 41.6%. The reactions conditions for CLEA-*Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins were optimized using benzaldehyde as a standard substrate. CLEA-*Bm*HNL produced (*S*)-mandelonitrile in very high % ee i.e. 99.8. Using the optimized conditions, eleven different (*S*)-cyanohydrins were synthesized with good % ee. Among them, eight cyanohydrins have not been synthesized by any CLEA-*Bm*HNL, and nine were synthesized by *Bm*HNL for

the first time. The reusability of CLEA-*Bm*HNL could be reused for five cycles without loss of % ee of (*S*)-mandelonitrile.

Effect of different biocatalytic conditions on the stability and activity of *Bm*HNL was studied. At its optimum pH 5.5, temperature 20 ºC, and buffer concentration (100 mM citrate phosphate pH 5.5) it showed half-life of 554 to 690 h, which is the maximum half-life among all α/β hydrolase fold HNLs. Addition of sucrose to *Bm*HNL's biocatalysis has increased its half-life by fivefold, while addition of sorbitol or glycerol increased ~ 9 fold specific activity. Among the polyols, glycerol addition to *Bm*HNL's biocatalysis has showed >99% ee of (*S*)-mandelonitrile in its synthesis. This study was extended to the synthesis of (*S*)-3,5-dimethoxy mandelonitrile and (*S*)-3-phenoxymandelonitrile, a precursor of pyrethroid, an insecticide. Effect of organic solvents and different temperatures on secondary structure of *Bm*HNL was studied with circular dichroism and it was observed that the secondary structure of protein was least affected by both.

Keywords: Cyanohydrins, Enantoselective C-C bond formation, *Baliospermum montanum* hydroxynitrile lyase, Biphasic system, Immobilization, Biocatalysis, Half-life.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURE

ABBREVIATIONS

α	Alpha
AcCN	Acetone cyanohydrin
AcN	Acetonitrile
AcHNL	Acidobacterium capsulatum Hydroxynitrile lyase
Ala	Alanine
CPBA	alpha-cyano-3-phenoxybenzyl alcohol
ACE	Angiotensin-converting enzyme
AtHNL	Arabidopsis thaliana hydroxynitrile lyase
Asp	Aspartic acid
β	Beta
BmHNL	Baliospermum montanum Hydroxynitrile lyase
bp	base pair
BpHNL	Burkholderia phytofirmans Hydroxynitrile lyase
% conv	% conversion
CAL-A	Candida antarctica lipase-A
CAL-B	Candida antarctica lipase-B
CCL	Candida cylindracea lipase
C-C	Carbon-carbon
ChuaHNL	Chamberlinius hualienensis Hydroxynitrile lyase
CD	Circular dichroism
csp	cold shock protein
J	coupling constant
CLEA	Cross-linked enzyme aggregates
DtHNL	Davallia tyermanii hydroxynitrile lyase
dNTP	deoxy nucleotide tri-phosphate
D	Dextrorotatory
DCM	dichloromethane
DEAE	Diethylaminoethyl
DIPE	Diisopropyl ether
DMF	dimethyl formamide
dH2O	distilled waster
dd	doublet of doublet
ee	Enantiomeric excess
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
γ	Gamma
GABOB	Gamma-amino beta-hydroxybutanoic acid
GABA	Gamma-aminobutyric acid
GET	Glucose-EDTA-Tris

GtHNL	Granulicella tundricola Hydroxynitrile lyase
HbHNL	Hevea brasiliensis hydroxynitrile lyase
HPLC	High performance liquid chromatography
His	Histidine
h	Hour
HCN	Hydrogen cyanide
HNL	Hydroxynitrile lyase
IPA	Isopropanol
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo dalton
kg	Kilogram
l	Lamda
L	Levorotatory
LuHNL	Linum usitatissimum Hydroxynitrile lyase
LiCl	lithium chloride
L	Litre
LB	Luria-bertani broth
Lys	Lysine
MeHNL	Manihot esculenta hydroxynitrile lyase
MHz	mega hertz
MeO	Methoxy
µg	microgram
µL	microlitre
mg	milligram
mL	millilitre
mM	millimolar
mmol	millimole
min	Minutes
M	Molar
MWCO	Molecular weight cutoff
m	multiplate
ng	nanogram
nm	nanometer
n-BA	n-butyl acetate
Ni-NTA	Nickel-Nitrilotriacetic acid
NMR	Nuclear magnetic resonance
ω	Omega
OD	Optical density
π-π	Pai-pai
ppm	parts per million
PeHNL	Passiflora edulis Hydroxynitrile lyase
Ph	Phenyl
Phe	Phenylalanine

PMSF	phenylmethylsulfonyl fluoride
FaHNL	Phlebodium aureum Hydroxynitrile lyase
pmol	picomole
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PPL	porcine pancreatic lipase
KCN	Potassium cyanide
KPB	potasssium phosphate buffer
PaHNL	Prunus amygdalus hydroxynitrile lyase
PSL-C	Pseudomonas cepacia lipase
PsmHNL	Pseudomonas mephitica Hydroxynitrile lyase
PSL	Pseudomonas species lipase
RT	Retention time
rpm	revolutions per min
SEM	Scanning electron microscope
Ser	Serine
s	Singlet
NaHCO3	Sodium bicarbonate
Na2SO4	Sodium sulphate
SDS	Sodiumdodecyl sulphate
SbHNL	Sorghum bicolor Hydroxynitrile lyase
TBME	*tert*-butyl methyl ether
tert	tertiary
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TMSCN	Trimethylsilylcyanide
UV	Ultra voilet
U	Unit
Val	Valine
Vis	Visible
XaHNL	Ximenia americana Hydroxynitrile lyase
XfHNL	Xylella fastidiosa Hydroxynitrile lyase

Chapter 1

Introduction

1.1. Importance of optically pure cyanohydrins

Optically pure cyanohydrins are resourceful building blocks in the synthesis of pharmaceuticals, agrochemicals and other biologically active compounds [1–10]. They are multifunctional optically active compounds having a nitrile and a hydroxyl group attached to the same carbon center. Because of these diverse functional groups, they are used in the synthesis of several industrially important chiral intermediates. In a few instances, the pharmacological principle of a drug also incorporates chiral cyanohydrins as a constitutive structural element [7]. With wide and potential industrial applications of the chiral cyanohydrins, there is an increasing demand for these optically pure molecules. Enantiopure cyanohydrins are precursors for α-hydroxycarboxylic acids, α-hydroxy aldehydes, and vicinal amino alcohols, which have thus become accessible in the stereochemically pure form [9]. The major application of chiral cyanohydrins can be classified into three categories:

a) Synthesis of pharmaceuticals

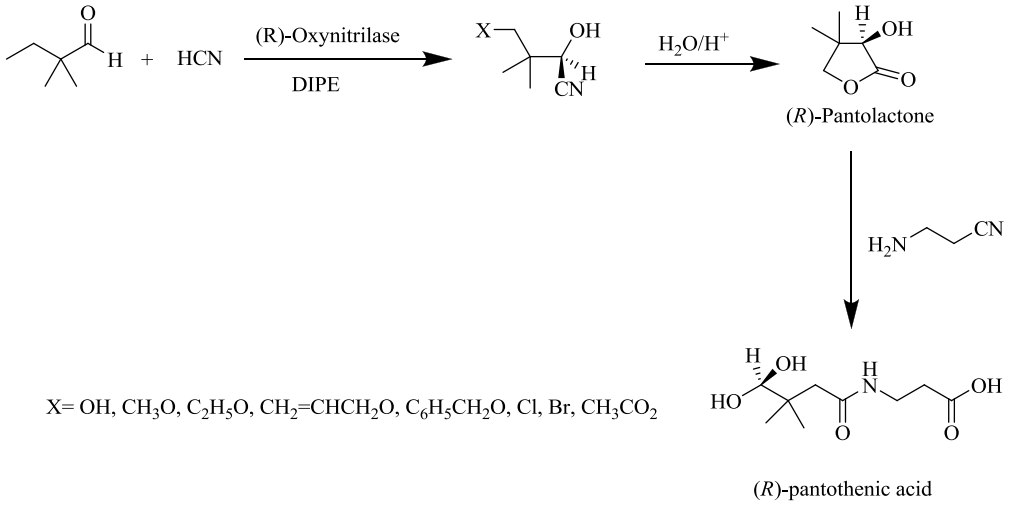
b) Synthesis of agrochemicals

c) Synthesis of versatile synthetic chiral intermediates and fine chemicals

1.1.1. Chiral cyanohydrins in pharmaceuticals

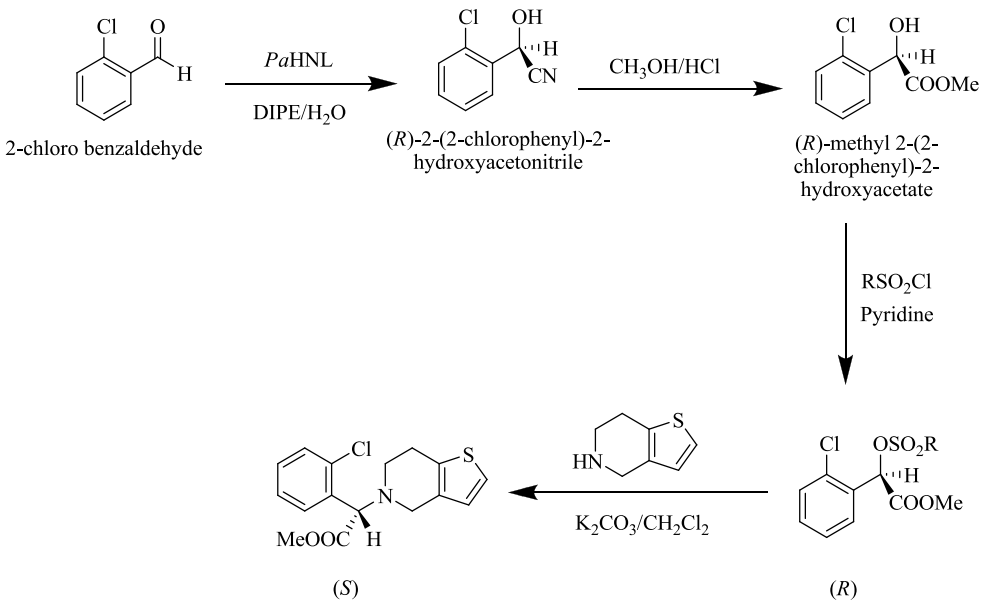
In pharmaceuticals industries, optically pure cyanohydrins are used as important intermediates for the synthesis of various kinds of drugs and other therapeutic agents. For example (*R*)-pantolactone is synthesized from β-substituted pivalaldehyde cyanohydrins

(**Scheme 1.1**). (*R*)-pantolactone is a key intermediate for the synthesis of (*R*)-panthenol, a bactericide [11]. The same optically pure molecule also acts as a starting compound for the synthesis of (*R*)-pantothenic acid (**Scheme 1.1**) [11,12] a constituent of coenzyme A as well as in the preparation of (*R*)-pantotheine (growth factor) [11].



Scheme 1.1: Chemo-enzymatic synthesis of (*R*)-pantolactone and (*R*)-pantothenic acid [11]

Clopidogrel (Plavix) is an useful inhibitor in blood clotting (anti-platelet) [13,14]. Production of the blockbuster Clopidogrel (Plavix) is a convincing example of the synthetic potential of optically pure (*R*)–2-chlorobenzaldehyde cyanohydrin (**Scheme 1.2**) [13,14]. *Threo*-3-aryl-2,3-dihydroxypropanoic acids, derived from cyanohydrins of aryl aldehydes are intermediate of diltiazem, a cardiac drug (calcium channel blocker) which works by relaxing muscles of heart and blood vessels. The drug is also used to treat high blood pressure and hypertension [15].



Scheme 1.2: Stereoselective synthesis of the blockbuster drug Clopidogrel (Plavix) [14]

Chiral cyanohydrins are important building blocks for the synthesis of *β*-adrenergic blocking agents (*β*-blockers), which are drugs showing marked efficacy in hypertension, cardiac arrhythmias, migraine headaches and other disorders related to the sympathetic nervous system. Examples of some *β*-blockers are atenolol, metoprolol, bisoprolol and propranolol (**Figure 1.1**) [16].

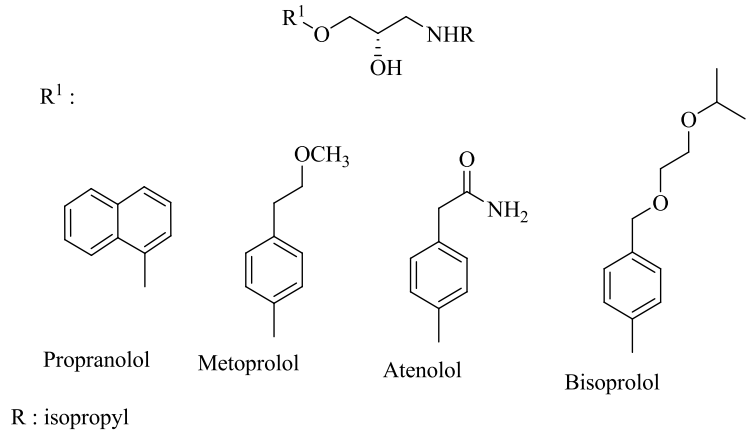
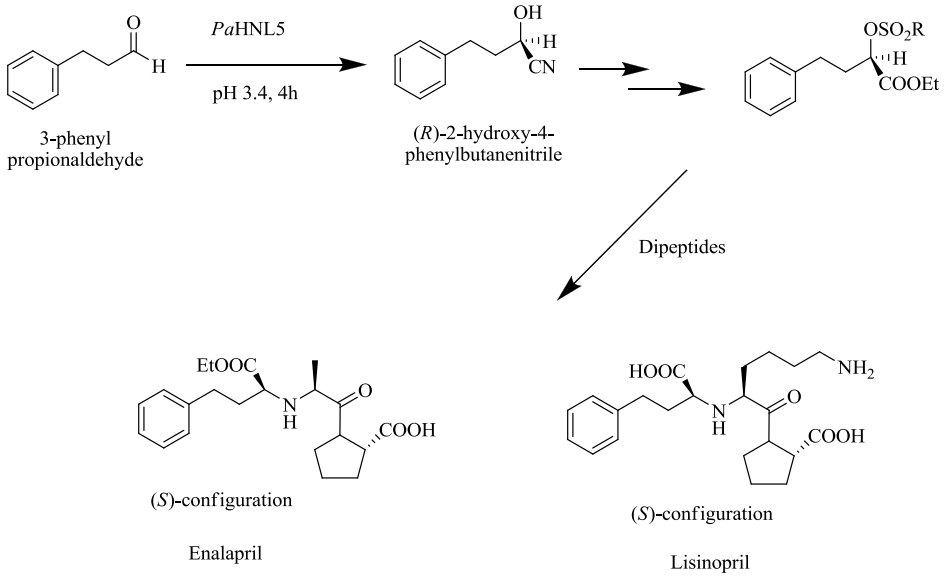


Figure 1.1: Typical structures of (*S*)-β-blockers with the aryl substituents: propranolol, metoprolol, atenolol, and bisoprolol [16]

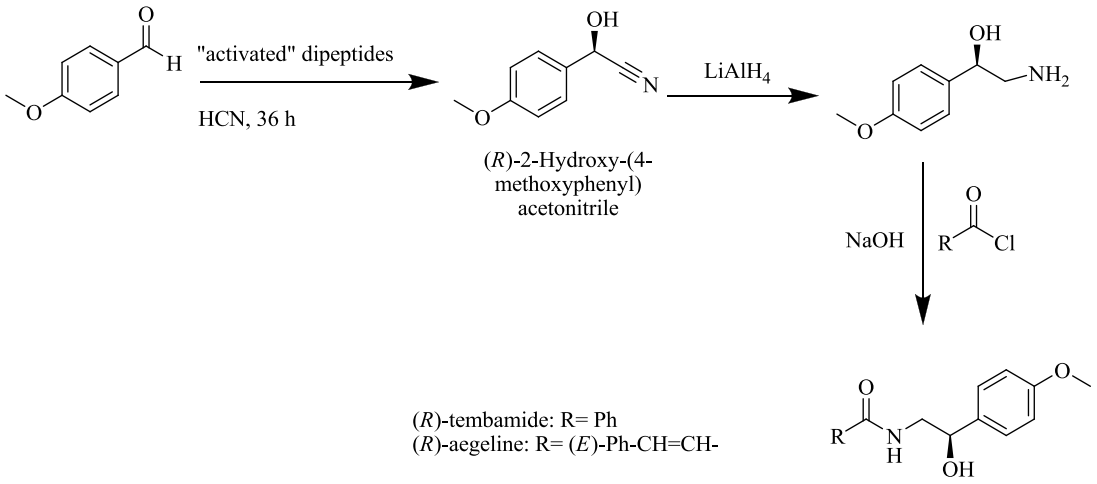
Another class of pharmaceuticals that can be synthesized through optically active cyanohydrins are angiotensin-converting enzyme (ACE) inhibitors [9,17,18]. (*R*)-3-phenylpropionaldehyde cyanohydrin is an important starting cyanohydrin to prepare ACE inhibitors like enalapril and lisinopril (**Scheme 1.3**) [9,17,18].



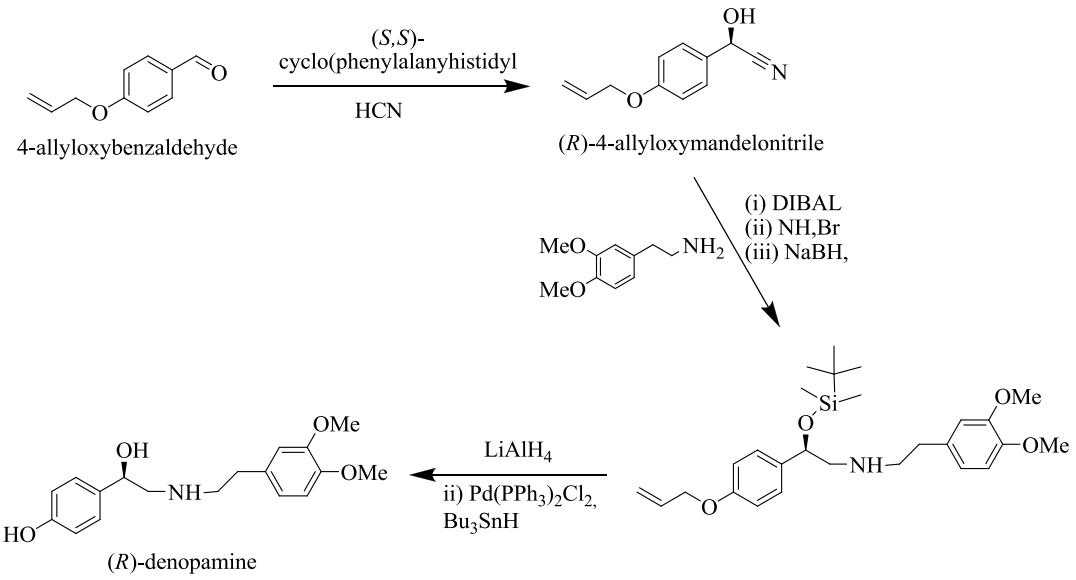
Scheme 1.3: Stereoselective synthesis of ACE-inhibitors [18]

(*R*)-2-Hydroxy-(4-methoxyphenyl)acetonitrile serves as a starting component in the preparation of *R*-(-)-aegeline and *R*-(-)-tembamide. Aegeline and tembamide are hydroxyamides with stereogenic center which possess adrenaline–like activity. Aegeline also shows hypoglycemic activity (**Scheme 1.4**) [1,9].

(−)-Denopamine is a *β*-receptor agonist for treating congestive heart failure. *para*-methoxy or *para*-allyloxybenzaldehyde cyanohydrin serves as a potent intermediate in the synthesis of (*R*)-denopamine (**Scheme 1.5**) [1,2,9].



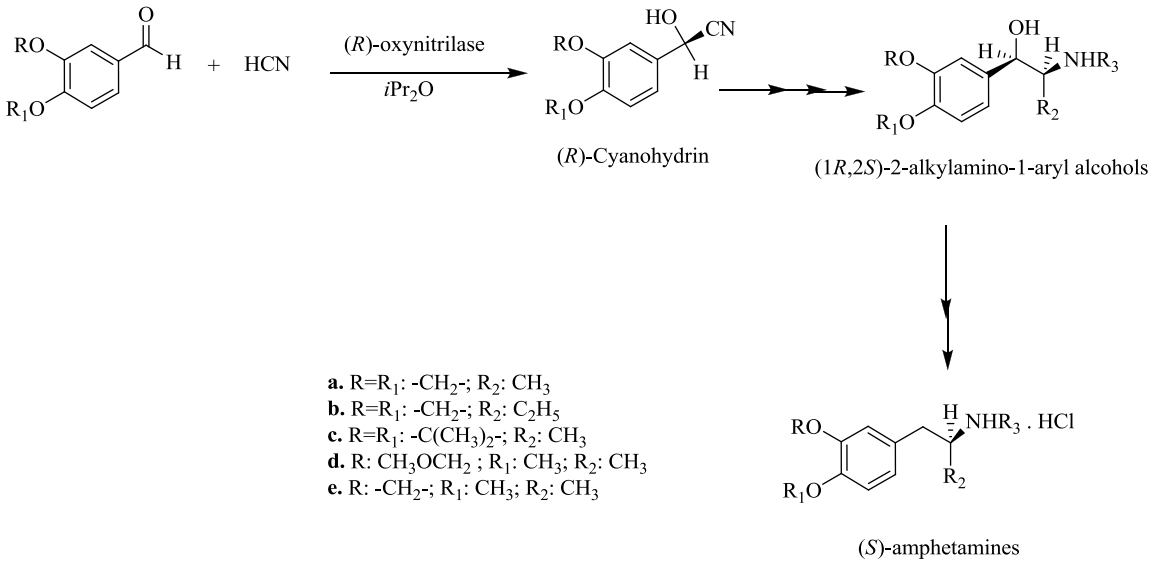
Scheme 1.4: Synthesis of *R*-(-)-aegeline and *R*-(-)-tembamide from (*R*)-cyanohydrin of 4-methoxybenzaldehyde [1]



Scheme 1.5: Synthesis of cardiac drug (*R*)-denopamine [2]

2-Amino-1-aryl alcohols are synthesized by the addition of Grignard reagent on chiral cyanohydrins followed by hydrogenation (**Scheme 1.6**). These amino alcohols are the major precursors of (*S*)-amphetamines. Amphetamines represent one of the medically

important drugs used in the psycho-therapeutical applications and also reported to produce stimulant and hallucinogen-like effects in humans [9,19].

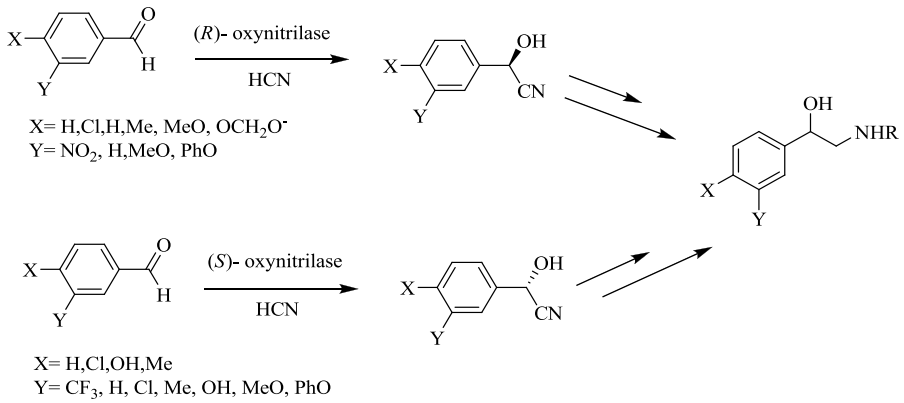


Scheme 1.6: Synthesis of (*S*)-amphetamines from an (*R*)-cyanohydrin [19]

In addition to therapeutic use, the drug Mureidomycin A, a deoxysugar with cyanohydrin of (*S*)-butenal works as an inhibitor of peptidoglycan synthesis. It is a competitive inhibitor of translocase 1 that catalyzes the first reaction in the biosynthesis of bacterial peptidoglycan. Mureidomycin A possesses the antimicrobial activity/ inhibitor effect especially to Pseudomonas family [9,20]. Further, in this context, *β*-lactam cyanohydrin derivatives are reported to synthesize polyhydroxylated piperidines which are also known as azasugars and iminosugars and act as glycosidase and glycoprotein-processing enzyme inhibitor as they mimic sugars [9,21]. Therefore these inhibitors have potential use in cancer, diabetes and viral infections [9,21,22]. Cyanohydrins of 3,5-*di*isopropoxybenzaldéhyde, 4-isopropoxybenzaldehyde, and 2-hydroxybenzaldehyde are used in the synthesis of stemofurans e.g. 2-(4-hydroxyphenyl)-6-hydroxybenzofuran, 2-

(3,3-dihydroxyphenyl)-6-hydroxybenzofuran, and 2-(4-hydroxyphenyl)-benzofuran having antifungal, antibacterial, therapeutical and cosmetic applications [9,23].

Chiral cyanohydrins are used in the synthesis of some important adrenergic drugs such as 2-amino-1-phenylethanols. Adrenaline is one example of 2-amino-1-phenylethanol. The key precursors of these drugs are 2-amino-, 2-halo-, 2-azido-1-phenylethanol and 2-hydroxycarboxylic acid which are prepared from their corresponding cyanohydrins (**Scheme 1.7**) [24].

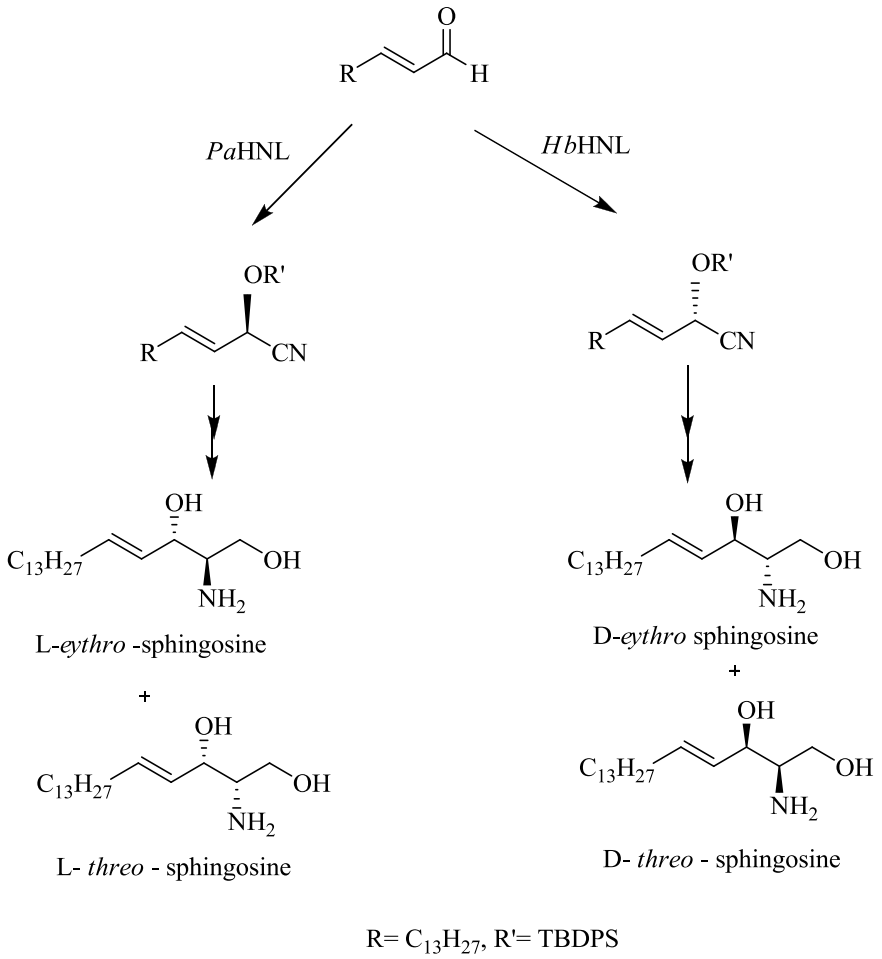


Scheme 1.7: Chemo-enzymatic synthesis of 2-amino-1-phenylethanol via chiral cyanohydrins [24]

Chiral 1-cycloalkyl-1-hydroxy-1-phenyl cyanohydrins i.e. (2-cyclobutyl-2-hydroxy-2-phenylacetonitrile and derivatives) are the key building block for (*S*)-oxybutynin and other anti-muscarinic agents [8,9] which are frequently used in the treatment of overactive bladder by blocking the release of acetylcholine.

Sphingosines are long chain 2-amino-1,3-diols. They have been synthesized from both (*R*) and (*S*)-2-octenal cyanohydrins (**Scheme 1.8**). Sphingosines are present in phospholipids

of nervous tissue and cell membrane. They are key molecules of glycosphingolipids which are used in the detection of cancer as biological markers. Sphingosines are potent inhibitors of protein kinase C [25].



Scheme 1.8: Chemo-enzymatic synthesis of L- and D-sphingosines [25]

Enantiopure pentafluorobenzaldehyde cyanohydrin is a key intermediate in the synthesis of GABOB (γ-amino β-hydroxybutanoic acid) [9,25] and it is an analogue neurotransmitter of GABA (γ-aminobutyric acid) (**Figure 1.2**).

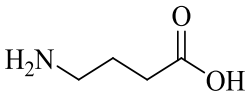


Figure 1.2: Neurotransmitter GABA

Naturally occurring mitosanes (e.g. mitomycins A, B, and C, porfiromycin and miitiromycine) containing an aziridine ring, have antibiotic activity. Stereoselective synthesis of 2,3-disubstituted *trans*-aziridines has been carried out using enantiopure mandelonitrile, 4-bromomandelonitrile and other cyanohydrins [27].

Enantiopure pentoses are building blocks of nucleoside analogues having potential antiviral and antitumor activity [28]. L-sugars have shown many pharmaceutical applications and they also have less cytotoxicity than D-sugars. Chiral cyanohydrins have been used in the *de novo* synthesis of pentoses of L-sugars. The key intermediate cyanohydrins for the synthesis of pentoses are cyanohydrins of 4-*O*-protected 4-hydroxybut-2-enals or cyanohydrins of (*E*)-4-allyloxybut-2-enal, 4-benzyloxybut-2-enal, 4-methoxymethoxybut-2-enal, 4-*tert*-butyldimethylsilyloxybut-2-enal [28].

Ephedrine (**Figure 1.3**) is a common drug molecule used in the treatment of low blood pressure. It is a β-amino alcohol with two chiral centers. Ephedrine is usually prepared from TMS-ether of mandelonitrile. The substrate is converted into ephedrine by Grignard reaction, transamination and reduction [14].

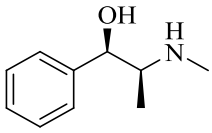
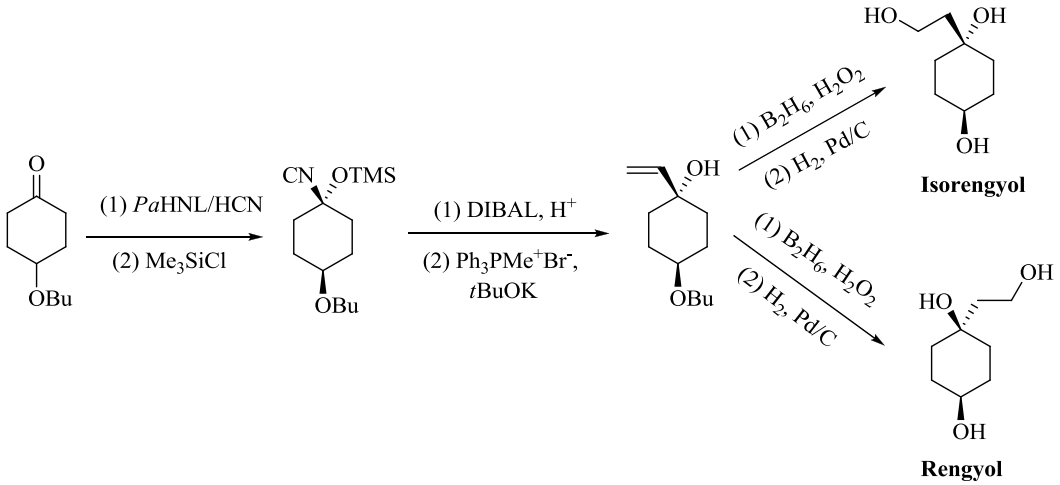


Figure 1.3: (1*R*, 2*S*)-Ephedrine

Chiral cyanohydrins are also used as a precursor in the preparation of Rengyol and Isorengyol (**Scheme 1.9**) which have anti-inflammatory, antibacterial and antiemetic properties. Kobler and Effenberger reported their chemo-enzymatic synthesis using 4-hydroxy derivatives of cyclohexanone which were converted into corresponding cyanohydrins using *Prunus amygdalus* hydroxynitrile lyase (*Pa*HNL) [14,29].



Scheme 1.9: Chemo-enzymatic synthesis of Rengyol and Isorengyol from 4-butoxycyclohexanone [14]

Epothilones are pharmaceutically important molecules with antitumor activity. They are known to bind and stabilize microtubules. Their preparation is reported by hydrolysis of nitrile group of (2*R*,3*E*)-2-hydroxy-3-methyl-4-(2-methyl-1,3-thiazol-4-yl)-3-butenenitrile which on further reduction with DIBAL and another subsequent reduction results in the production of epothilones A and B [14,30].

Psymberin 1 (**Figure 1.4**) is a cytotoxin which is used as a therapeutic agent in the treatment of tumor. Its synthesis has been described starting from a chiral cyanohydrin [14,31].

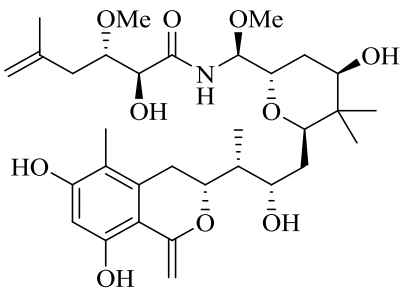
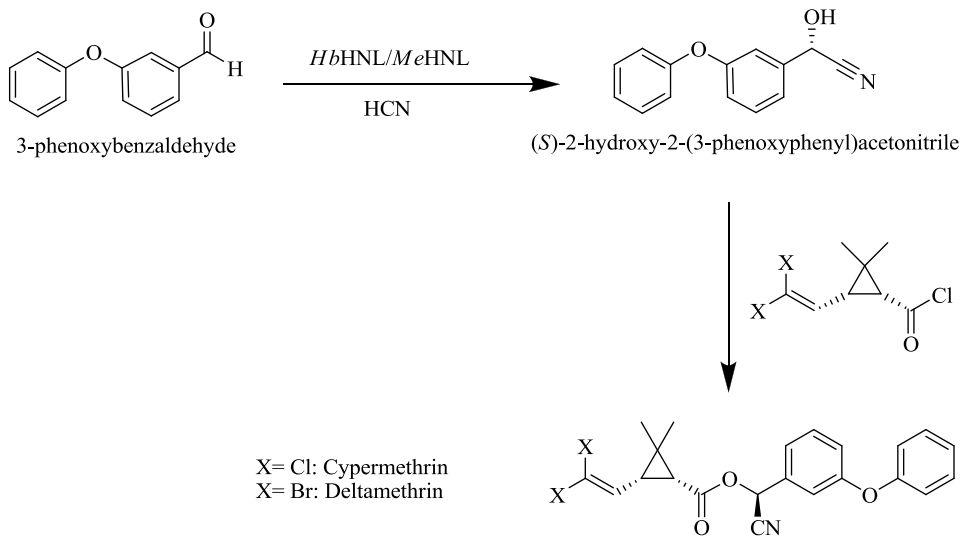


Figure 1.4: Psymberine 1

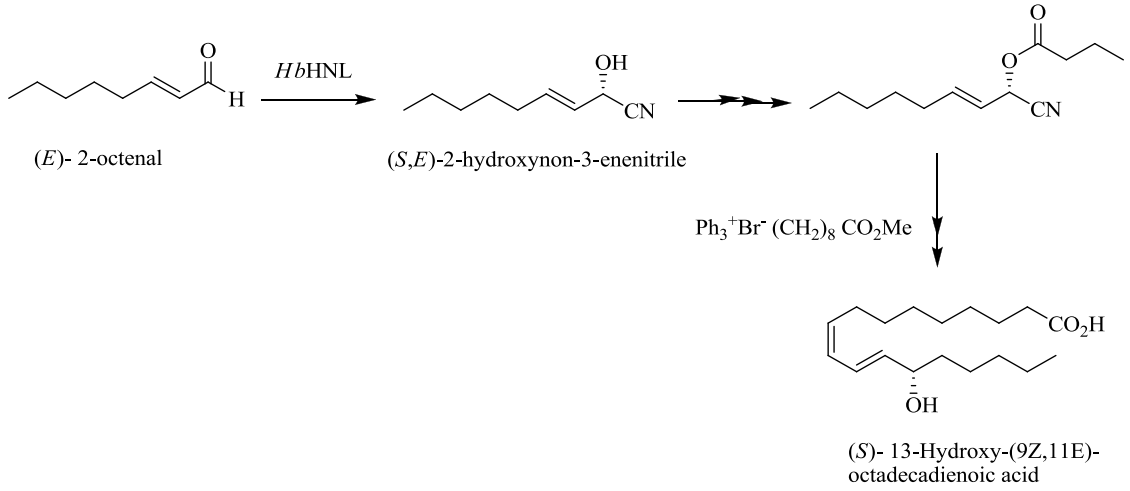
1.1.2. Application of chiral cyanohydrins in agrochemicals

The insecticidal activities of cyanohydrin esters are well known. The esters of chiral cyanohydrins with chiral pyrethrum acids are the most important insecticides [6]. Synthetic pyrethroids are commonly used in agriculture practices for protecting the crops from insects. Most of the commercially available and important pyrethroids contain (*S*)-3-phenoxybenzaldehyde cyanohydrin as an alcohol component. One of the well-known biocatalytic method for the production of this (*S*)-cyanohydrin is through HNL-catalyzed addition of HCN to 3-phenoxybenzaldehyde e.g. *Hevea brasiliensis* hydroxynitrile lyase (*Hb*HNL) or *Manihot esculenta* hydroxynitrilelyase (*Me*HNL) has been used as catalysts (**Scheme 1.10**) [5,6,14].

(*S*)-13-Hydroxyoctadeca-(9*Z*, 11*E*)-dienoic acid (13-(*S*)-HODE) is an oxygenated metabolite of linoleic acid, harboring many biological properties such as antirice blast activity (inhibits spore germination of the blast fungus) and calcium ionophoric activity. This compound is synthesized from (*E*)-2-octenal cyanohydrin as a precursor. This optically pure cyanohydrin has been synthesized by *Hb*HNL (**Scheme 1.11**) [32].



Scheme 1.10: Synthesis of pyrethroids using HNLs [14]

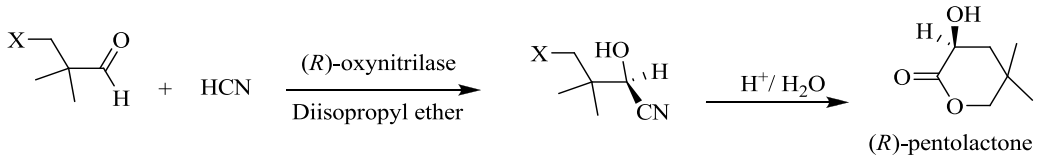


Scheme 1.11: Chemo-enzymatic synthesis of 13-(*S*)-HODE [32]

1.1.3. Application of optically pure cyanohydrins in fine chemicals

Optically pure cyanohydrins are important synthons not only in pharmaceuticals and agrochemicals but also in the synthesis of many potential fine chemicals and biologically active small molecules. Some of the examples are described in this section.

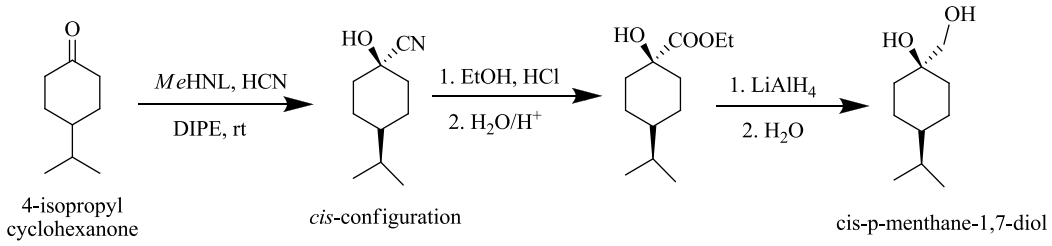
(*R*)-pantolactone which is a chiral building block and a precursor for many pantolactone derivatives is prepared using hydroxyl pivalaldehyde (*R*)-cyanohydrin (**Scheme 1.12**) [33]. (*R*)-pantolactone acts as a precursor in the synthesis of (*R*)-pantothenic acid (vitamin B5), a constituent of coenzyme A [11].



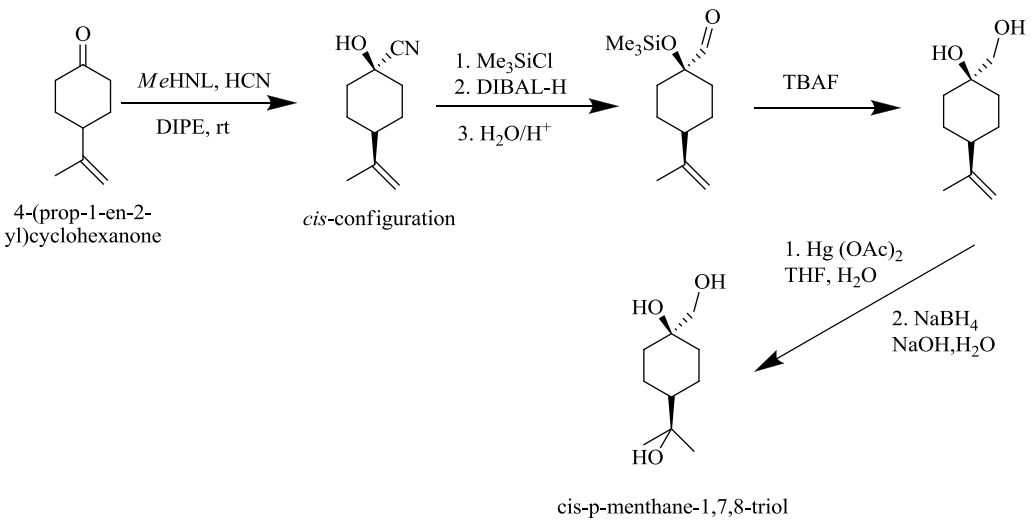
X: OH, CH_3O, C_2H_5O, CH_2=$CHCH_2O$, Cl, Br, CH_3CO_2

Scheme 1.12: Chemo-enzymatic synthesis of (*R*)-pantolactone [11]

Chiral cyanohydrins are also used as building blocks in the synthesis of monoterpenes which are biologically active compounds [34]. Chiral 4-isopropylcyclohexanone cyanohydrin and 4-(prop-1-en-2-yl)cyclohexanone are used in the synthesis of monoterpenes e.g. *cis*-*p*-menth-8-ene-1, 7-diol (**Scheme 1.13**) and *cis*-*p*-menthane-1,7,8-triol (**Scheme 1.14**) respectively [34].

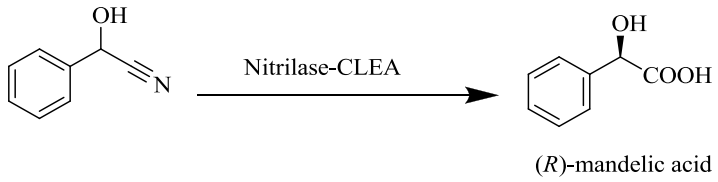


Scheme 1.13: Synthesis of *cis*-*p*-menthane-1,7-diol from 4-isopropylcyclohexanone [34]



Scheme 1.14: Synthesis of *cis*-*p*-menthane-1,7,8-triol from 4-isopropenylcyclohexanone [34]

Chiral cyanohydrins are converted into enantiopure α-hydroxyl carboxylic acids and their derivatives. Optically pure α-hydroxyl carboxylic acids play an important role in pharmaceuticals, chiral determination reagents, and as a resolving reagent. For example, in the synthesis of some pharmaceuticals drugs like semisynthetic penicillin, cephalosporin, antitumor agent and antiobesity drugs, (*R*)-mandelic acid (**Scheme 1.15**) works as a key intermediate [14,34,35].



Scheme 1.15: Hydrolysis of racemic mandelonitrile to (*R*)-mandelic acid by a nitrilase [14]

The reductive amination of nitrile group results in the formation of a potent precursor in the synthesis of chiral piperidones which are used in the preparation of biologically active molecules (**Figure 1.5**) [14,37].

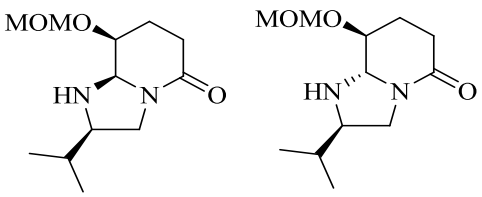


Figure 1.5: Piperidones

1.2. Asymmetric synthesis of cyanohydrins

Nucleophilic addition of nitrile anion to a carbonyl compound to synthesize cyanohydrin is a C-C bond formation reaction with wide potential applications. Although cyanohydrins are undoubtedly important synthons in organic chemistry but more often a single enantiomer of it involves in the preparation of final product. Extensive research in chiral cyanohydrins preparation has already been done by researchers. Based on the various reports, the different methods used in the preparation of enantiopure cyanohydrins can be categorized into three types:

a) Chemical method

b) Synthetic peptide based

c) Biocatalytic method

Before discussing the methods used in chiral cyanohydrins preparation, one must discuss the source of cyanide which is a crucial parameter in the synthesis of these chiral molecules. Hydrogen cyanide was the only cyanide donor in early research. However, its use in the reaction is associated with several disadvantages such as extreme toxicity, highly volatile and difficult handling in scale-up synthesis. Thus, there was a constant demand to discover new cyanide sources. Several alternative cyanide sources have been developed. Among them, TMSCN is most commonly used in the synthesis of cyanohydrins. It was used the first time used in 1973 to produce TMS-protected cyanohydrins [38–42]. TMSCN has

become the choice of cyanide source because of its easy handling and availability. It also produces protected cyanohydrins that avoid racemization of cyanohydrins. Having these advantages, it has some disadvantages also such as high flammability, toxicity and high price, which drives towards the usage of other alternative cyanide sources. Other cyanide sources are acetone cyanohydrin [43–46], metal cyanide salts, acyl cyanide, [47,48] cyanoformates [49,50] and cyanophosphonates [51,52]. The last four sources i.e. metal cyanide salts, acyl cyanide, cyanoformates, and cyanophosphonates are not true cyanide reagents as they are only different precursors to hydrogen cyanide or cyanide. Further, they require nucleophilic catalysts or solvent to produce cyanide in order to accomplish the reaction [42]. The rapid hydrolysis of TMSCN by water or alcohols [53] results in HCN production which proves that TMSCN is a true cyanating agent. Apart from TMSCN, other silyl cyanide agents have also been reported [54,55]. They are less reactive than TMSCN but are known to produce cyanohydrins which are less susceptible/sensitive to hydrolysis. The cyanide source is essential in the asymmetric synthesis of cyanohydrins as the addition of cyanide is directly associated in the stereodetermining state [42].

1.2.1. Asymmetric synthesis of cyanohydrins by chemical catalysts

There exist several established methods for synthesis of chiral cyanohydrins by chemical catalysts. Transition metal complexes are common catalysts in the enantioselective synthesis of cyanohydrins. Titanium-based complexes are the most explored catalyst in enantioselective addition of cyanide to carbonyl compounds. Other than titanium complexes, boron, vanadium, aluminum, tin, magnesium, yttrium, lanthanide, manganese, bismuth, zirconium, cobalt and rhenium based complexes have also been explored in the asymmetric synthesis of cyanohydrins [42,56,57].

1.2.2. Asymmetric synthesis of cyanohydrins by synthetic peptides

In nature, biochemical reactions are usually carried out by enzymes. Enzymes are chiral polypeptides and their catalysis is chemo-, regio-, and stereoselective in nature. Motivated with the natures catalysts, synthetic peptides [42,56–59] have been synthesized by mimicking the enzyme. Oku *et al* reported the first-time asymmetric synthesis of mandelonitrile by addition of hydrogen cyanide to benzaldehyde using peptide catalysts. They used L-Histidine based linear and cyclic dipeptides for the reaction and observed that linear peptide gave very less % ee while cyclic dipeptides resulted in 10% ee. The linear peptide could be unfavorable to asymmetric synthesis because of its flexible and variable conformation, while the rigid structure of 2,5-piperazinedione ring in cyclic peptides favors the reaction. *cyclo*[(*S*)-alanyl-(*S*)-histidine] or *cyclo*[(*S*)-Ala-(*S*)-His] (**Figure 1.6**) was the peptide which gave the best result among the tested peptides. It produced (*R*)-mandelonitrile with 10% ee and 50% conversion [60]. Further, Oku and Inoue described the asymmetric synthesis of optically active mandelonitrile by enantioselective addition of HCN to benzaldehyde in presence of synthetic peptide, cyclo (L-Phe-L-His) which resulted in 90% ee of the product with 40% conversion in 30 min [61].

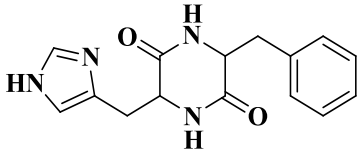


Figure 1.6: cyclo[(*S*)-alanyl-(*S*)-histidine]

Jackson *et al* prepared a series of cyclic peptides and used in asymmetric hydrocyanation of benzaldehyde and 3-phenoxybenzaldehyde. They found that (*S,S*)-cyclo(phenylalanylhistidy1) showed 100% ee and 83% yield in the synthesis of (*R*)-mandelonitrile in benzene while in case of 3-phenoxybenzaldehyde the product obtained

in 91% ee with 92% yield in toluene [62]. Ohno *et al* also studied the asymmetric trimethylsilylation of benzaldehyde in the presence of peptide-Me_3Al. They have tested different peptides to be used as a catalyst such as Nap-S-Val-S-Phgly-OMe, Nap-S-Val-S-Val-OMe, Nap-S-Val-S-Phe-OMe, Nap-S-Val-NHCy, Nap-S-Phe-S-Phe-OMe, Nap-S-Leu-S-Leu-OMe (Phgly: phenylglycine, Cy: cyclohexyl). Among them, Nap-S-Val-NHCy gave the best result with 69% ee and 95% yield for (*R*)-mandelonitrile [63].

1.2.3. Importance of enzyme catalysis

Enzymes are nature's evolved catalysts. They catalyze a broad range of biochemical transformations essential for the growth and survival of living organisms. They catalyze complex reactions with very high efficacy. Enzyme-catalyzed transformations are highly selective. They possess chemo-, regio-, and stereoselectivity in the reactions they catalyze. Further several enzymes have a high catalytic turnover which means the number of substrates converted to the product in a unit time is very high for the enzymes. Clearly, such high selectivity, turnover and efficacy are almost impossible to achieve using a chemical catalyst. Because of these important catalytic properties enzyme catalysis in organic chemistry and especially asymmetric synthesis has gained tremendous importance in the past several decades. Several advantages associated with enzyme catalysis compared to their chemical counterparts are given below.

- Enzyme catalysis follows green chemistry.
- Enzymes are environment-friendly catalysts and hence their use minimizes the use of hazardous chemical and metal catalysts.
- Enzymes increase the rate of a chemical reaction and remain unchanged after completion of the reaction.

- The enzyme catalysis, also known as biocatalysis can be performed in mild conditions such as pH and temperature which help in reducing or avoiding side reactions of the process.
- Biocatalysts are biodegradable.
- They can be reused by immobilizing them.
- The efficiency of biocatalysts can be improved or manipulated by enzyme engineering as well as by immobilization.

1.2.4. Asymmetric synthesis of cyanohydrins by biocatalytic methods

Biocatalytic synthesis of optically pure cyanohydrins is majorly carried out using the following two methods. They are (a) kinetic resolution and (b) enantioselective C-C bond formation. Kinetic resolution method usually involves catalysis by lipases and esterases, however also reported with whole cells while enantioselective C-C bond formation is usually carried out by hydroxynitrile lyases.

1.2.4.1. Kinetic resolution

Kinetic resolution is a process where one selective enantiomer of the racemic substrate undergoes faster reaction with the enzyme than the other enantiomer. This eventually helps in separation of the two enantiomers in different forms, one unreacted substrate enantiomer, and another converted product enantiomer. Preparation of optically active cyanohydrins using this method involves two types of biotransformations: (i) the enantioselective hydrolysis of racemic cyanohydrin esters, and (ii) the enantioselective esterification of racemic cyanohydrins.

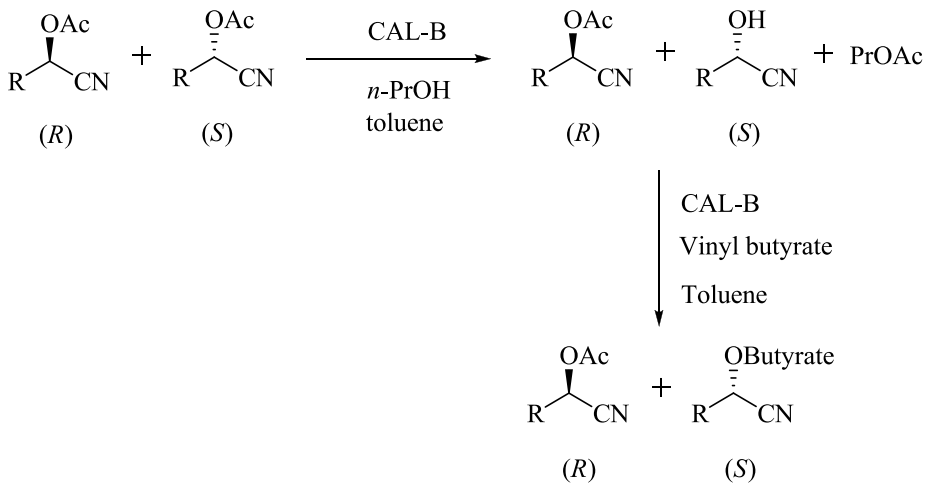
1.2.4.1.1. Enantioselective hydrolysis of racemic cyanohydrin esters

Resolution of racemates via lipase and esterase catalyzed kinetic resolution is one of the most attractive methods used to prepare enantiopure compounds as the enzymes used in this method don't require any cofactor for catalysis. The enzymes accept a broad range of substrates and often exhibit high enantioselectivity [64,65]. Moreover, a large number (>50) of lipases and esterases are commercially available.

Matsuo and Ohno described the synthesis of (*S*)-1-acetoxy-2-aryloxypropionitriles by enantioselective hydrolysis of the corresponding racemic mixture in the presence of *Pseudomonas sp*. lipase in pH 5 buffer solution. The resulted (*S*)-enantiomers were obtained in excellent enantiopurity i.e. up to 96.8% ee [66]. Ohta *et al* reported the asymmetric hydrolysis of different aryloxyacetaldehyde cyanohydrin acetates with different microorganisms such as *Bacillus sp*., *Candida tropicalis*, *Arthrobacter paraffineus*, and *Pichia miso* [67]. They tested these strains in the enantioselective hydrolysis of phenoxyacetaldehyde cyanohydrin acetate as a model substrate. Among them, *Bacillus* sp. KU 5185 gave the best results with the highest 45% yield and 95% ee of (*S*)-phenoxyacetaldehyde cyanohydrin acetate. They found 95% ee and 30% yield of the (*S*)-*p*-methylphenoxyacetaldehyde cyanohydrin acetate and 85% ee and 40% yield of the (*S*)-*m*-methylphenoxyacetaldehyde cyanohydrin acetate in case of hydrolysis of the corresponding racemic acetates. A similar enantioselective hydrolysis of l-cyanoalkyl acetates has been reported [68]. Screening of different microorganisms such as *Candida tropicalis, Penicillium notatum, Pseudomonas fluorescens* and *Aspergillus flavas* towards hydrolysis of 1-cyanohexyl acetate, identified *Candida tropicalis* with best results which were further screened for hydrolysis of other l-cyanoalkyl acetates to produce

corresponding (*S*)-cyanohydrins and (*R*)-cyanohydrin acetates. Racemic l-cyanoundecyl acetate on enantioselective hydrolysis gave (*S*)-l-cyanoundecyl acetate in 99% ee. Ohta *et al* also carried out microbial asymmetric hydrolysis of racemic l-cyano-1-methylalkyl acetates using *Pichia miso IAM 4682* [69]. The ketone cyanohydrin acetates on biocatalysis produced corresponding (*S*)-acetates and (*R*)-1-methyalkyl cyanohydrins. Almsick *et al* described the preparation of forty-seven chiral cyanohydrin acetates with 30-98% ee and 44-58% conversion [70]. They performed the enantioselective hydrolysis of racemates using an ester hydrolase from *Pseudomonas sp.* Schneider and co-workers have demonstrated the kinetic resolution of different racemic cyanohydrin acetates using *Pseudomonas sp.* lipase that produced a number of enantiopure cyanohydrin acetates with *E* up to ≥100 [70]. Hydrolysis of different aromatic cyanohydrin acetates has been reported by several research groups. Enantioselective hydrolysis has been carried out using lipases from different sources such as *Bacillus cogulans* [71,72], *Pseudomonas fluorescens* [70,73] etc with 70-98% ee of (*R*)-aromatic cyanohydrin acetates [74]. Mitsuda *et al* investigated the enantioselective hydrolysis of racemic *α*-cyano-3-phenoxybenzyl alcohol (CPBA) acetate using lipases from different sources i.e. *Achromobacter sp*., *Alcaligene sp*., *Arthrobacter sp*., *Chromobacterium sp*., *Pseudomonas sp*. and *Candida cylindracea* [75]. All the lipases gave (*R*)-CPBA acetate except *Arthrobacter* lipase which showed opposite enantioselectivity. It synthesized (*S*)-CPBA acetate in 99% ee. Effenberger *et al* investigated the hydrolysis of racemic cyanohydrin acetates with three lipases, *Pseudornonas fluorescens***,** lipase PS and lipase P [73]. These enzymes have cleaved (*S*)-cyanohydrin acetates which resulted in the formation of (*R*)-cyanohydrin acetates and (*S*)-cyanohydrins. They observed 48-98% ee of (*R*)-cyanohydrin acetates in case of aromatic

cyanohydrins while with aliphatic cyanohydrins, the % ee was negligible. Mitsuda *et al* studied the hydrolysis of *α*-cyano-3-phenoxybenzyl acetate with *Arthrobacter* lipase [75]. It produced (*S*)-*α*-cyano-3-phenoxybenzyl alcohol in very low yield. DEAE-Sephadex immobilized *Pseudomonas* sp. catalyzed kinetic resolution of racemic α-cyano-3-phenoxybenzyl acetate has been described in the preparation of (*S*)-*α*-cyano-3-phenoxybenzyl alcohol in 96% ee, and 75% yield [76]. *Pseudomonas fluorescens* lipase catalyzed kinetic resolution of racemic benzaldehyde cyanohydrin acetate has produced its (*S*)-cyanohydrin in up to 99% ee which was used to prepare (1*R*,cis,*αS*)-cypermethrin was synthesized, a pesticide [5]. Hanefeld *et al* performed kinetic resolution with a wide range of racemic aromatic cyanohydrin acetates using *Candida antarctica* lipase (CAL-B) that produced their corresponding chiral cyanohydrins with excellent enantiopurities [77]. The unprotected cyanohydrins are usually tend to degradation into corresponding carbonyl compound and HCN. In order to produce stable and protected chiral cyanohydrins, Veum *et al* have reported a process to produce both enantiomers of protected cyanohydrins using CAL-B catalyzed kinetic resolution of racemic aromatic cyanohydrin acetate coupled with esterification of the (*S*)-cyanohydrin with vinyl butyrate (**Scheme 1.16**) [78]. They hydrolyzed 4 different racemic cyanohydrin acetates in the presence of toluene and *n*-propanol at 60 °C using CAL-B. The resulted (*S*)-cyanohydrin was butyrylated by vinyl butyrate to produce (*S*)-cyanohydrin butyrate in 98% ee and 85% yield. Similarly, in case of 3-phenoxybenzaldehyde, the corresponding (*S*)-cyanohydrin butyrate was obtained in 96% ee and 67% yield [78].



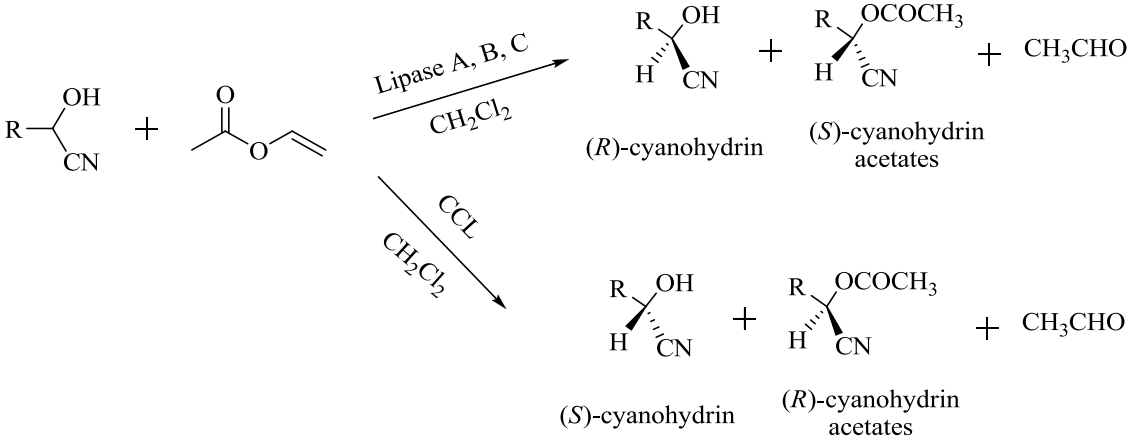
Scheme 1.16: Kinetic resolution of cyanohydrin acetates and protection of the resulted (*S*)-cyanohydrins [78]

Paizs *et al* described kinetic resolution of several optically active 10-alkyl-phenothiazin-3-ylcyanomethyl acetates using *Candida antarctica lipase* A (CAL-A) catalyzed enantioselective hydrolysis of the racemic substrates. It produced (–)-cyanohydrin acetates with high enantioselectivity ($E \geq 100$) [79].

1.2.4.1.2. Enantioselective esterification of racemic cyanohydrins

Among the several methods used in kinetic resolution, enantioselective esterification of a single enantiomer of a racemic cyanohydrin by lipase in an organic solvent or a biphasic system is commonly used to synthesize optically pure cyanohydrins. In the presence of a suitable acyl donor, appropriate organic solvent, and optimal reaction conditions, a lipase could selectively acylate to one enantiomer of the racemic secondary alcohol to produce the corresponding enantiopure ester, leaving the second unreacted enantiomer in the enantiopure form [15,66,80–83]. This is one of the effective methods to prepare protected cyanohydrins as unprotected cyanohydrins are not stable. Effenberger *et al* described enantioselective esterification of racemic cyanohydrins in dichloromethane using lipases

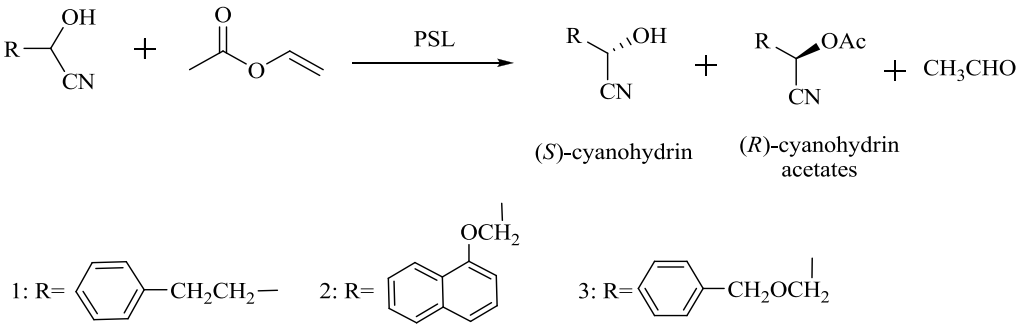
and vinyl acetate as an acylating agent. *Pseudomonas fluorescens* (A), *Pseudomonas cepacia* lipase (Lipase PS) (B) and Lipase P (C) produced (*S*)-cyanohydrin esters in 55-95% ee and (*R*)-cyanohydrins. However, in case of *Candida cylindracea* lipase (CCL), the products were produced with reverse enantioselectivity (**Scheme 1.17**) with 8% ee of (*R*)-cyanohydrin esters and 55% ee of (*S*)-cyanohydrin [73].



Scheme 1.17: Enantioselective acylation of aldehydes through lipase A, B, C and CCL [73]

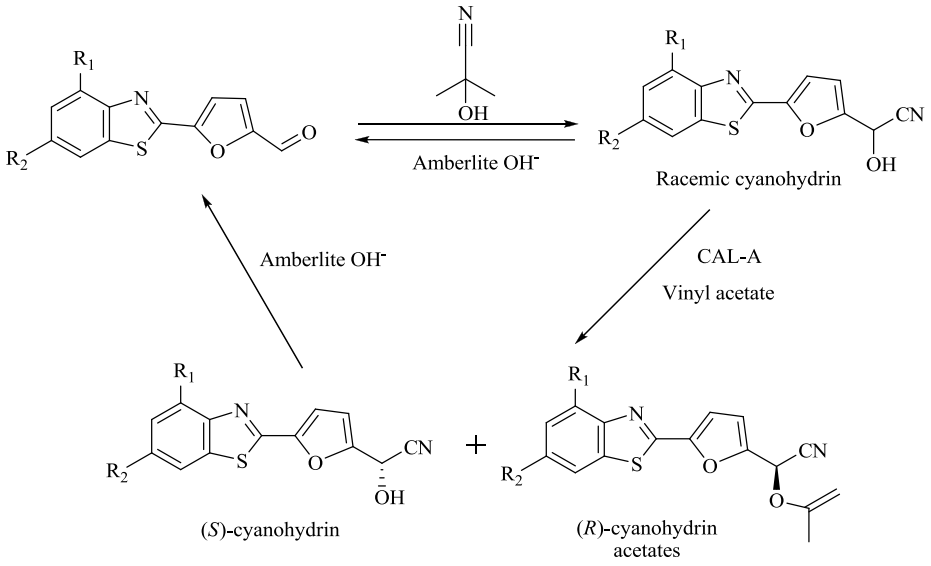
One of the major disadvantages of kinetic resolution is it can produce a maximum 50% yield of the desired enantiomer. Improvement in the yield can be achieved by biocatalytic dynamic kinetic resolution. The unwanted enantiomer is again converted into the racemic mixture which can be used as a substrate by the lipase or esterase for successive cycles. Kanerva *et al* prepared enantiopure cyanohydrin acetates by acylation of racemic cyanohydrins using lipases. The racemic cyanohydrins were acylated by vinyl butyrate in toluene using porcine pancreatic lipase (PPL), CCL and lipase PS. Among the selected lipases, PPL showed best results with 64-92% ee [14,84]. Inagaki and co-worker synthesized optically active cyanohydrin acetates from aldehydes. The aromatic aldehydes were converted into racemic cyanohydrins by the basic anion-exchange resin which have

undergone enantioselective acylation using isopropenyl acetate as an acylating agent by *Pseudomonas sp*. lipase that produced (*S*)-cyanohydrin acetates in 70-91% ee and 70-96% yield [14,56,85]. Wang *et al* prepared chiral cyanohydrin acetates by transesterification of racemic cyanohydrins via vinyl acetate using lipase from *Pseudomonas sp*. (PSL) (**Scheme 1.18**). These enantiopure cyanohydrins have been used as precursor in the synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate and (*S*)-propranolol [14,86].



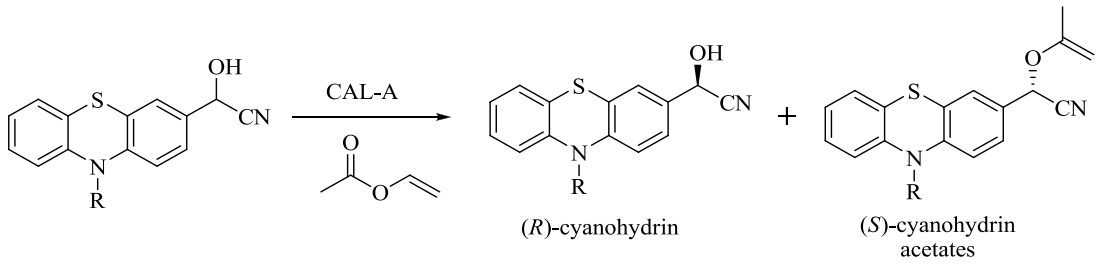
Scheme 1.18: *Pseudomonas sp.* Lipase-catalyzed enantioselective transesterification of racemic cyanohydrins [86]

Paizs *et al* synthesized enantiopure (*R*)-5-phenylfuran-2-ylcyanomethyl butanoates using a lipase-catalyzed dynamic kinetic resolution. The racemic cyanohydrins were prepared by adding acetone cyanohydrin to the aldehydes in the presence of basic Amberlite resin followed by acylation through CAL-A (**Scheme 1.19**). The resulting (*R*)-esters obtained in 57-96% ee [14,56,87].



Scheme 1.19: Lipase-catalyzed dynamic kinetic resolution furylbenzotiazol-based cyanohydrin [87]

Paizs *et al* prepared a series of (*S*)-10-alkylphenothiazin-3-ylhydroxyacetonitrile acetates by CAL-A catalyzed kinetic resolution where the corresponding (*S*)-enantiomers undergone acylation [79]. They have also described a dynamic kinetic resolution method to prepare the (*S*)-cyanohydrin acetates up to >99% ee and >92% yield. The dynamic kinetic resolution approach involved CAL-A catalyzed esterification of (*S*)-cyanohydrin along with Amberlite I-904 catalyzed racemization of uncatalyzed (*R*)-cyanohydrin to aldehyde, which subsequently converted to racemic cyanohydrin by the same Amberlite I-904 (**Scheme 1.20**). Repeated cycles of this catalysis in 24-48 h have produced the desired products [79].



Scheme 1.20: Enantioselective acylation of cyanohydrin by lipase [79]

A dynamic kinetic resolution approach with CAL-A and Amberlite have been used in the synthesis of (*R*)-furylbenzotiazol-based cyanohydrin acetates [87]. Another dynamic kinetic resolution based synthesis of (*R*)-5-phenylfuran-2-yl cyanomethyl butanoates has been reported by Paizs *et al*. This approach used *Pseudomonas cepacia* lipase (PSL-C) along with a basic Amberlite for kinetic resolution and racemization respectively [88]. Gotor and coworkers prepared (*S*)-ω-hydroxycyanohydrins and corresponding (*R*)-acetates using *Pseudomonas cepacia* lipase (PSL-C) and CAL-A catalyzed kinetic resolution of the racemic substrates [89]. Kinetic resolution of racemic mandelonitrile has been carried out using *Candida antarctica* lipase in an orbital shaker with microwave irradiation in toluene. This process has produced (*S*)-mandelonitrile acetate with up to >98% ee [90].

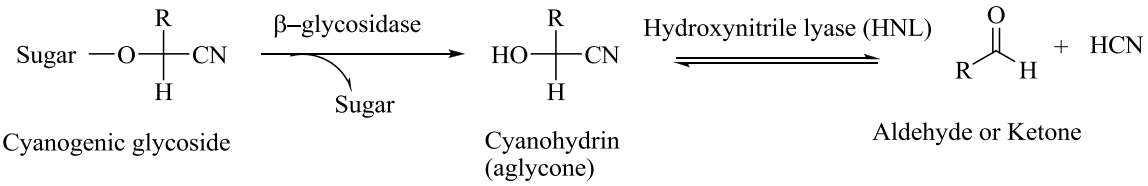
Lipase and esterase catalyzed kinetic resolution provides wide scope to prepare enantiopure cyanohydrins. However, the process is limited by the maximum theoretical yield of 50%. It uses racemic cyanohydrins whose preparation requires an additional step. Further, it produces an unwanted enantiomer in a derivative form which again needs an extra reaction for the deprotection of the protected hydroxyl group. The dynamic kinetic resolution could provide higher yield and enantiopurity however it also uses racemic cyanohydrins as substrate and an additional chemical catalyst for racemization.

1.2.4.2. Enantioselective C-C bond formation

The second and important biocatalytic method to synthesize chiral cyanohydrins involves enantioselective C-C bond formation between electrophilic carbonyl carbon and nucleophilic cyanide. This reaction is catalyzed by hydroxynitrile lyases.

1.2.4.2.1. Hydroxynitrile lyase

Hydroxynitrile lyases (HNL, E.C. 4.1.2.X, X = 10, 11, 46 and 47) are the class IV enzymes, mainly found in higher plants. They are also reported in some bacteria, fungi, ferns, lichens, and arthropods. Along with β-glycosidase, HNL catalyzes the catabolism of cyanogenic glycosides in higher plants, (**Scheme 1.21**) [9]. This decomposition of cyanohydrin produces corresponding carbonyl substrate and releases HCN. This process is known as cyanogenesis. Plants use the released HCN to protect them from herbivores and pathogens as part of their defense mechanism [91]. Although releasing of HCN is a common defense mechanism in plants, the number of available HNLs are large. Apart from cyanogenesis, HNLs catalyze the reverse reaction. They catalyze the addition of cyanide anion to carbonyl center to synthesize optically pure cyanohydrins. This biotransformation plays an important role due to its synthetic utility and importance of the chiral cyanohydrins as described earlier.



Scheme 1.21: Catabolism of cyanogen glycoside to carbonyl compound and HCN

1.2.4.2.2. Classification of HNLs

The different HNLs discovered so far can be classified into several groups based on their (I) enantioselectivity, (II) presence of co-factor and (III) protein structure (**Figure 1.7**).

(I). Based on the enantioselectivity or absolute configuration of the product formed, HNLs are classified into two classes:

(a). (*R*)-selective HNLs: They produce (*R*)-cyanohydrins. Ex: *Arabidopsis thaliana* HNL (*At*HNL), *Pa*HNL), *Linum usitatissimum* HNL (*Lu*HNL).

(b). (*S*)-selective HNLs: They produce (*S*)-cyanohydrins. Ex: *Baliospermum montanum* HNL (*Bm*HNL), *Hb*HNL, *Me*HNL and *Sorghum bicolor* HNL (*Sb*HNL).

(II). Based on the co-factor presence they can be categorized into the following two types:

(a). FAD-containing HNLs: These HNLs contain FAD cofactor however the requirement of the cofactor for catalysis has not been proved so far. Example: *Pa*HNL and other *Prunus* sp. HNLs.

(b). Non-FAD based HNLs: These HNLs do not have any FAD with them. List of HNLs without the presence of FAD in them are, (*R*)-specific HNLs: *At*HNL, *Phlebodium aureum* HNL (*Fa*HNL), *Lu*HNL, *Passiflora edulis* HNL (*Pe*HNL) and *Xylella fastidiosa* HNL (*Xf*HNL), and (*S*)-specific HNLs: *Sorghum bicolor* HNL (*Sb*HNL), *Ximenia americana* HNL (*Xa*HNL), *Hb*HNL, *Me*HNL and *Bm*HNL.

(III). Based on the protein structure HNLs can be categorized into six superfamilies [92].

(a). α/β Hydrolase fold: Each enzyme of this superfamily have a core of an α/β-sheet rather than a barrel. Thus these enzymes contain 8 β-strands connected by 6 α-helices and a conserved catalytic triad (nucleophile-histidine-aspartate). HNLs from α/β hydrolase fold superfamily include *Hb*HNL *Me*HNL, *At*HNL, and *Bm*HNL.

(b). Serine carboxypeptidase-like: *Sb*HNL is a single example of this family. Similar to serine carboxypeptidases, it exists in a heterodimeric form which is made-up by two pairs of chain A and B. Both chains are linked with glycosylated cysteine. *Sb*HNL contains catalytic triad Asp, His, and Ser similar to other HNLs [93].

(c). FAD-containing oxidoreductase: HNLs from *Prunus* sp. are major examples in this category. This class of HNLs shows maximum similarity (30% sequence similarity) with glucose-methanol-choline (GMC)-oxidoreductase but they do not show any oxidase activity [94].

(d). Zn^{2+} dependent alcohol dehydrogenase-like: *Lu*HNL is only an example of this family. It shows structure similarity to alcohol dehydrogenases (ADHs) which catalyze the oxidation and reduction of a wide variety of alcohols and aldehydes. *Lu*HNL and ADHs also show conserved Zn^{2+} binding domain but *Lu*HNL doesn't exhibit any ADH activity [95].

(e). Cupin fold: HNLs belonging to this superfamily show maximum identity with cupin fold superfamily proteins as they contain conserved barrel domain. However, cupin fold HNLs show HNL activity. Cupin fold HNLs are majorly bacterial HNLs e.g. *Burkholderia phytofirmans* HNL (*Bp*HNL), *Pseudomonas mephitica* HNL (*Psm*HNL), *Granulicella tundricola* HNL (*Gt*HNL), and *Acidobacterium capsulatum* HNL (*Ac*HNL) [10,96].

(f). Dimeric α+β barrel superfamily: *Pe*HNL is only one example of this family, consisting of a central β-barrel in the middle of a dimer [97].

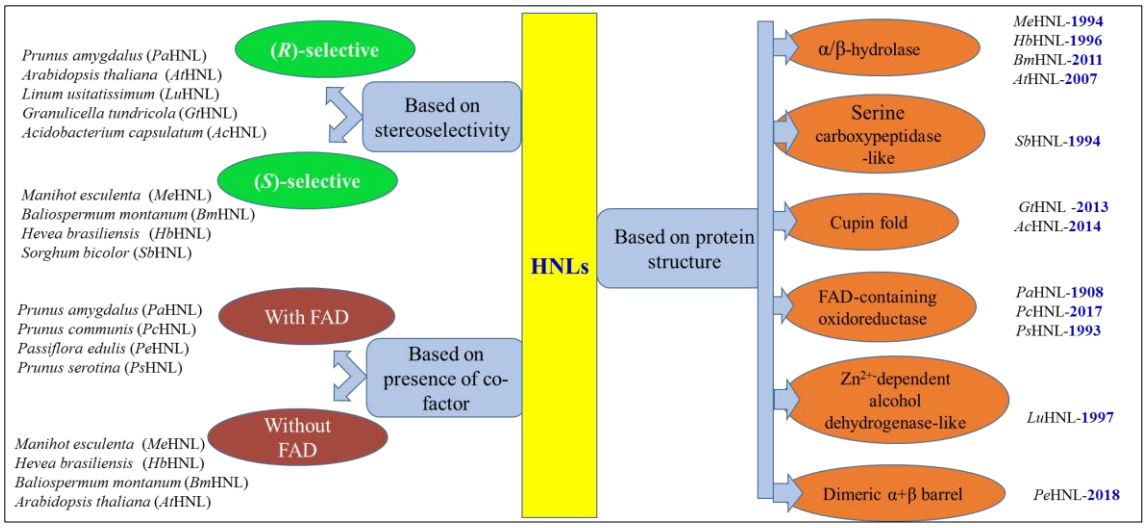


Figure 1.7: Overview of classification of HNLs, the year mentioned next to each HNL represents it's year of discovery

1.2.4.2.3. HNL catalyzed synthesis of optically active cyanohydrins

Application of HNLs in the enantioselective synthesis of cyanohydrins has been known for a long time. Both (*R*)- and (*S*)-cyanohydrins have been synthesized by using the corresponding stereoselective HNLs. Thus, asymmetric synthesis of cyanohydrins using HNLs can be divided into two categories (a) synthesis by (*R*)-oxynitrilase and (b) synthesis by (*S*)-oxynitrilase.

1.2.4.2.3a. (*R*)-oxynitrilases in the synthesis of optically active cyanohydrins

First asymmetric synthesis of mandelonitrile from benzaldehyde and HCN was published by L. Rosenthaler in 1908 [9,57,98]. They described the use of HNL from almond. Afterward, in the 1960s, E. Pfeil *et al* purified the HNL from bitter almond and used it in asymmetric addition of HCN to benzaldehyde. They tested the enzyme in asymmetric synthesis of cyanohydrins with aliphatic, aromatic as well as unsaturated aldehydes apart from benzaldehyde. HNL from bitter almond (*Pa*HNL) had produced (*R*)-mandelonitrile in 86% ee [14,56,98]. HNL from bitter almond with cellulose-based ion exchanger has

been used in the synthesis (*R*)-mandelonitrile in 95% yield with 97% ee [9,56,99]. While the earlier described methods of *Pa*HNL catalyzed synthesis of cyanohydrin were tested in aqueous and biphasic system consisting of buffer and alcohol, Effenberger *et al* reported the biotransformation using this enzyme in ethyl acetate. They further esterified the resulted chiral products to restrict them from undergoing racemization. They observed that (*R*)-cyanohydrins were obtained in higher % ee (up to 99%) in ethyl acetate as compared to biocatalysis in water/ethanol [100]. Improve % ee was observed when *Pa*HNL catalyzed synthesis of cyanohydrins was carried out in isopropyl ether [56,101]. Kiljunen and T. Kanerva isolated novel (*R*)-oxynitrilase from almond meal, apple, and cherry. They synthesized (*R*)-cyanohydrins of various aldehydes in 0.1 M tartrate buffer pH 5.4 and DIPE with high % ee and yield [102,103]. *Pa*HNL catalyzed synthesis of chiral cyanohydrins have been used as precursors in preparation of many important chiral synthons [104–111]. Few more HNLs have been discovered from Prunus sp. and similar plant sources and used in the synthesis of chiral cyanohydrins. They are *Prunus serotine* (*Ps*HNL) [112], *Prunus amygdalus turcomanica* (*Pat*HNL) [113], *Prunus mume* (*Pm*HNL) [114,115], *Amygdalus pedunculata* Pall (*AP*HNL) [116], *Passiflora edulis* (*Pe*HNL) [97,117,118], *Prunus communis* (*Pc*HNL) [119], HNL from wild apricot i.e. *Prunus armeniaca* L. (*Pars*HNL) [120–122], *Prunus serotina* var. capulli) and peach (*Prunus persica*) HNL [123].

Asano and co-workers discovered new HNLs from different plant sources. They isolated (*R*)-HNLs from *Eriobotrya japonica* (*Ej*HNL), *Chaenomeles sinensis* (*Cs*HNL), *Prunus persica* (*Pp*HNL) and *Sorbus aucuparia* (*Sa*HNL) [9,124].

Other than HNLs from *Prunus* species, many other (*R*)-HNLs have been reported e.g. *At*HNL, *Lu*HNL, *Cucumis melo* (melon, a noncyanogenic plant) HNL [9], *Poiteria sapota* (mamey) HNL [125], *Nandina domestica* Thunb HNL (*Nd*HNL-L) [92], HNL from the white rabbit's foot fern *Davallia tyermanii* (*Dt*HNL) [126,127], HNL from fern *Phlebodium aureum* HNL (*Pha*HNL) [128], bacterial HNLs *Psm*HNL, *Bp*HNL [96], manganese-dependent *Gt*HNL [129,130] and *Ac*HNL [131]. They all have been characterized and studied in the synthesis of (*R*)-cyanohydrins.

Martina Pohl *et al* have explored *At*HNL, the first (*R*)-selective HNL with α/β hydrolase fold [132]. It accepts a broad range of aromatic aldehydes to produce the corresponding (*R*)-cyanohydrins. They have also demonstrated its use in organic synthesis [133].

In 1988 Conn *et al* discovered *Lu*HNL whose natural substrate is acetone cyanohydrin [134]. *Lu*HNL catalyzed the synthesis of sixteen (*R*)-cyanohydrins with low to high % ee. Among them, small cyanohydrins of aliphatic substrates were obtained in >90% ee while large substrates such as cinnamaldehyde were with <10% ee [9,57].

Dadashipour *et al* discovered the first arthropod HNL i.e. *Chamberlinius hualienensis* HNL (*Chua*HNL) from a millipede [135]. Using *Chua*HNL, they synthesized a number of (*R*)-cyanohydrins with excellent % ee. Yamaguchi *et al* discovered ten new HNLs from millipedes and observed highest HNL activity with *Parafontaria tonominea* (*Pton*HNL) for the synthesis of (*R*)-mandelonitrile [136].

1.2.4.2.3b. (*S*)-oxynitrilases in the synthesis of optically active cyanohydrins

Unlike several (*R*)-selective HNLs, there exist only a few (*S*)-selective HNLs. These enzymes catalyze the synthesis of (*S*)-cyanohydrins from their prochiral aldehydes/ketones. They belong to α/β hydrolase fold superfamily and are FAD

independent HNLs. The first (*S*)-HNL was isolated from *Sorghum vulgare* (var. Honey Drip) in 1961 during a study of biosynthesis of dhurrin [9,57,98]. The enzyme showed cyanohydrin cleavage activity. It was later biocatalytically characterized by Bove and Conn. They developed a spectrophotometric method to monitor decomposition of 4-hydroxymandelonitrile which is a natural substrate for the enzyme [9,57,98,137,138]. Effenberger and co-workers first time described the asymmetric synthesis of (*S*)-cyanohydrins using *Sb*HNL in 54-97% ee [139]. The enantiopure cyanohydrins were further converted into (*S*)-*α*-hydroxycarboxylic acids. Niedermeyer and Kula also reported *Sb*HNL catalyzed synthesis of (*S*)-cyanohydrins in citrate buffer of pH 3.25 to 4.50. They observed very high e.g. 96-99% ee of aromatic cyanohydrins while the enzyme was unable to catalyze the reaction with aliphatic aldehydes [140]. Kiljunen and Kanerva studied *Sb*HNL catalyzed synthesis of (*S*)-cyanohydrins and optimized the reaction conditions. The biocatalysis carried out in DIPE at 5 °C produced (*S*)-mandelonitrile with 97% ee [103]. The cloning and characterization of *Sb*HNL has been reported by Wajant *et al* [93,141] and it was crystallized in 2002 by Lauble *et al* [142].

*Hb*HNL and *Me*HNL are two other biocatalytically well characterized (*S*)-selective HNLs. Wajant *et al* isolated and purified *Me*HNL [9,57,98,143]. Later *Me*HNL has been cloned and overexpressed in *E.coli* [144]. The recombinant *Me*HNL has shown 25 fold higher specific activity compared to the natural enzyme. *Me*HNL has been widely exploited in the synthesis of various cyanohydrins of aliphatic, unsaturated and aromatic aldehydes in up to 98% ee and 100% yield, in a biphasic system [8,144–149]. *Me*HNL catalyzed (*S*)-mandelonitrile synthesis has been reported in 98% ee and 100% yield [144]. Wajant and Pfizenmaier have identified the potential active site residues of *Me*HNL [150]. Buhler and

co-workers studied wild type *Me*HNL and its W128A variant in the synthesis of (*S*)-cyanohydrins of various aliphatic, aromatic and unsaturated aldehydes as well as methyl and ethyl ketones. *Me*HNL showed >90% ee in case of aldehydes while in case of ketone, the % ee was >80% [151]. Immobilized *Me*HNL has been experimented in the synthesis of ketone and aldehyde cyanohydrins in 91-99% ee [152]. At pH>6, *Me*HNL catalyzed synthesis of (*S*)-3-phenoxymandelonitrile produced the product in 97% ee while with *Me*HNL-W128A the % ee was reduced to 85% [153].

*Hb*HNL is another important and widely used (*S*)-oxynitrilase, which was isolated form the rubber plant. It was partially purified and tested for HNL activity using acetone cyanohydrin and mandelonitrile cleavage [9,57,98,154]. Wajant and Foster have reported the purification and characterization of this enzyme [91] and later its crystal structure was solved [155,156]. Griengl *et al*. have demonstrated the use of *Hb*HNL in the synthesis of a number of enantiopure cyanohydrins and their application in organic synthesis [157–162]. Cloning and expression of *Hb*HNL has been reported by Hasslacher *et al* in *E.coli* and yeast [163,164].

Klempier and Griengl first time showed the use of *Hb*HNL in the synthesis of (*S*)-cyanohydrins of aliphatic aldehydes in 60-97% ee [165]. They also studied crude *Hb*HNL catalyzed synthesis of α,β-unsaturated (*S*)-cyanohydrins produced in 80-95% ee [166]. They further investigated the biocatalysis using aliphatic, aromatic and heteroaromatic aldehydes and prepared corresponding (*S*)-cyanohydrins in 77-99% ee with crude *Hb*HNL. Few aldehydes such as pyrrol-2-aldehyde, indole-3-carboxaldehyde, 2-pyridine aldehyde, 3-pyridine aldehyde, and 4-pyridine aldehyde were not converted into corresponding cyanohydrins. *Hb*HNL catalysis has produced (*S*)-mandelonitrile in >99% ee and 67%

yield while (*S*)-2-hydroxy-4-phenyl-(*E*)-but-3-enenitrile was obtained in 95% ee and 50% yield [157]. Veum *et al* prepared immobilized *Hb*HNL by sol-gel encapsulation and used it in the preparation of (*S*)-cyanohydrins from corresponding aldehydes [167]. This biocatalysis has produced (*S*)-cyanohydrins of furan-2-carbaldehyde and heptanal in 91-98% ee while in case of (*S*)-3-phenoxymandelonitrile, the % ee was >80. They also prepared (*S*)-cyanohydrin of 3-methylbutan-2-one with ~75% ee. *Hb*HNL catalyzed synthesis of heterocyclic (*R*)- and (*S*)-cyanohydrins has also been reported by others [161,168].

Solis *et al* discovered a new source of (*S*)-oxynitrilase i.e. Guanabana seed meal and used them in the preparation of (*S*)-cyanohydrins from aromatic, heteroaromatic and *α,β*-unsaturated aldehydes. They found 87% ee and 95% conversion in case of (*S*)-cyanohydrin of 2-furaldehyde, while only 50% ee and 10% conversion was observed with (*S*)-mandelonitrile. The enzyme did not catalyze asymmetric cyanohydrin synthesis in case of aliphatic aldehydes [169].

1.3. *Baliospermum montanum* HNL (*Bm*HNL)

Baliospermum montanum HNL is the latest member of (*S*)-selective HNL of α/β-hydrolase superfamily. *Bm*HNL has been purified from leaves of the plant *Baliospermum montanum*, a medicinal and cyanogenic plant belongs to the Euphorbiaceae family. Its common name is wild castor/wild croton. Asano and coworkers have reported *Bm*HNL while screening different plant extracts for HNL activity [124]. The same research group later reported cloning, expression and biocatalytic characterization of *Bm*HNL [170]. *Bm*HNL shares more than 50% sequence identity with *Me*HNL and *Hb*HNL, the other two (*S*)-selective HNLs of α/β-hydrolase superfamily (**Figure 1.8**). Its catalytic site contains Ser-His-Asp,

similar to *Me*HNL and *Hb*HNL. The hydrophobic amino acids located in active sites have been shown as * in **Figure 1.8**.

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BmHNL -MVSAHFILIHTICHGAWLWYKLIPLLQSAGHNATAIDLVASGIDPRQLEQIGTWEQYSE 59
HbHNL -MAFAHFVLIHTICHGAWIWHKLKPLLEALGHKVTALDLAASGVDPRQIEEIGSFDEYSE 59
MeHNL -MVTAHFVLIHTICHGAWIWHKLKPALERAGHKVTALDMAASGIDPRQIEQINSFDEYSE 59

BmHNL PLFTLIESIPEGKKVILVGESGGGINIALAAEKYPEKVSALVFHNALMPDIDHSPAFVYK 119
HbHNL PLLTFLEALPPGEKVILVGESCGGLNIAIAADKYCEKIAAAVFHNSVLPDTEHCPSYVVD 119
MeHNL PLLTFLEKLPQGEKVIIVGESCAGLNIAIAADRYVDKIAAGVFHNSLLPDTVHSPSYTVE 119

BmHNL KFSEVFTDWKDSIFSNYTY-GNDTVTAVELGDRTLAENIFSNSPIEDVELAKHLVRKGSF 178
HbHNL KLMEVFPDWKDTTYFTYTK-DGKEITGLKLGFTLLRENLYTLCGPEEYELAKMLTRKGSL 178
MeHNL KLLESLPDWRDTEYFTFTNITGETITTMKLGFVLLRENLFTKCTDGEYELAKMVMRKGSL 179

BmHNL FEQDLDTLPNFTSEGYGSIRRVYVYGEEDQIFSRDFQLWQINNYKPDKVYCVPSADHKIQ 238
HbHNL FQNILAKRPFFTKEGYGSIKKIYVWTDQDEIFLPEFQLWQIENYKPDKVYKVEGGDHKLQ 238
MeHNL FQNVLAQRPKFTEKGYGSIKKVYIWTDQDKVFLPDFQRWQIANYKPDKAYQVQGGDHKLQ 239

BmHNL ISKVNELAQILQEVANSASDLLAVA 263
HbHNL LTKTKEIAEILQEVADTYN------ 257
MeHNL LTKTEEVAHILQEVADAYA------ 258
```

Bold: Aromatic residues; * Hydrophobic residues located in the active site; Residues in catalytic triad.

Figure 1.8: Multiple sequence alignment of *Bm*HNL, *Hb*HNL, and *Me*HNL

Nakano *et al* have solved the crystal structure of *Bm*HNL [171]. Their study revealed the substrate entrance to the active site (**Figure 1.9**). Nine hydrophobic residues are found in the catalytic site, among them six are present in the entrance region. They are Phe121, Ser122, Trp128, Phe133, Val146, and Phe178.

The catalytic reaction mechanism of *Bm*HNL is proposed to be similar to other (*S*)-selective HNLs of α/β-hydrolase superfamily. Ser80 makes hydrogen bond with hydroxyl group and Lys236 interacts with the nitrile group of the mandelonitrile. The hydrophobic residues interact with benzene ring of (*S*)-mandelonitrile by π-π interaction. These hydrophobic residues, especially Phe121 and Phe178 are responsible for broad aromatic substrate preference of *Bm*HNL [171,172].

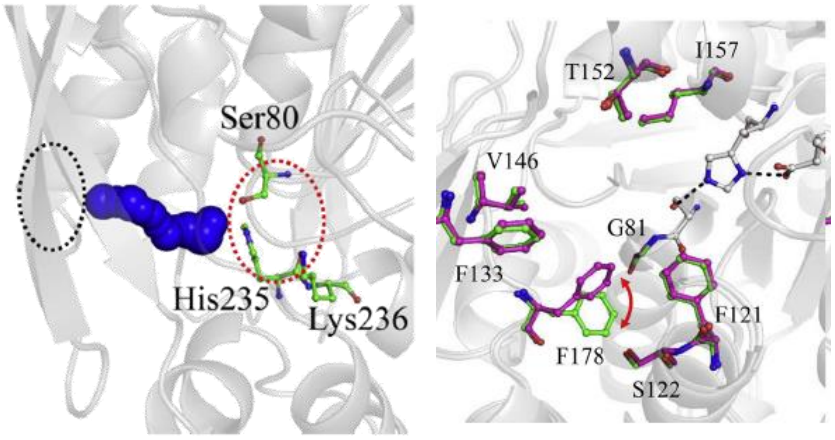

Figure 1.9: Left: Substrate entrance tunnel of *Bm*HNL. The entrance of the tunnel and the active site is shown as black and red dotted circles, respectively. Right: Stereoview of the apo1 and apo2 structures of *Bm*HNL (Image from S. Nakano *et al*. [171])

Substrate selectivity, stereoselectivity, and stability are some of the key parameters that remain important for a biocatalyst. *Bm*HNL has gained importance because of its ability to accept a broad range of aromatic bulky aldehydes and catalyze their conversion to corresponding chiral cyanohydrins. (*S*)-cyanohydrins of aromatic bulky aldehydes are used as precursors for the synthesis of several pharmaceuticals (**Figure 1.1**) and industrial products. Based on its substrate preference, we have selected *Bm*HNL for the present study. Further, it belongs to α/β hydrolase fold superfamily and does not require any cofactor for its stability like the FAD-containing HNLs.

However, two major limitations in *Bm*HNL catalytic synthesis of cyanohydrins that still remain unaddressed, and probably they are the reasons to make this enzyme less favorable for industrial applications. They are (a) *Bm*HNL shows poor enantioselectivity (low % ee) in the synthesis of corresponding chiral cyanohydrins of bulky aromatic aldehydes, and (b) *Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins is reported with less substrate

concentration (2.5 mM) which is not suitable for commercial production of chiral cyanohydrin. The common methods known to improve enantioselectivity of an enzyme include reaction/medium engineering, protein engineering, and enzyme immobilization. Biophysical characterization such as understanding the stability and activity of *Bm*HNL in various reaction conditions is also important as enzyme stability would affect its enantioselectivity in biocatalysis. Although molecular and biocatalytic characterization of *Bm*HNL has been carried out by Asano and co-workers but its biophysical characterization has not been studied.

Hence we aimed to study the *Bm*HNL catalyzed stereoselective synthesis of chiral cyanohydrins (a) in a biphasic system, and (b) using the immobilized enzyme. Biophysical characterization of *Bm*HNL is also investigated in order to understand the stability and activity of the enzyme.

1.4. Outline of the thesis

The goal of the thesis is to explore *Bm*HNL in the stereoselective synthesis of chiral cyanohydrins. Therefore, in chapter 2 subcloning and characterization of purified *Bm*HNL is summarized. Synthesis of internal standards i.e. racemic cyanohydrins and their characterization is described in chapter 3. In chapter 4 of this thesis, *Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins in a biphasic system is summarized while in chapter 5, stereoselective synthesis of chiral cyanohydrins using immobilized *Bm*HNL is described. In chapter 6, the biophysical characterization of purified *Bm*HNL is elaborated. Chapter 7 summarizes the entire work.

Objectives of the present study

1. *Baliospermum montanum* hydroxynitrile lyase subcloning, expression, purification, and characterization.
2. Chemical synthesis of racemic cyanohydrins.
3. *Baliospermum montanum* hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins in a biphasic solvent system.
4. Immobilized *Baliospermum montanum* hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins.
5. Study on increasing enzymatic stability and activity of *Baliospermum montanum* hydroxynitrile lyase in biocatalysis.

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Chapter 2

BmHNL subcloning, expression, purification and characterization

2.1. Introduction

HNLs are mostly found in higher plants while in the past decade several examples of their existence in other organisms like bacteria, arthropods, fungi, etc have been reported. They are industrially important enzymes due to their significant biocatalytic properties. They are used in the enantioselective synthesis of cyanohydrins which are building blocks in the production of many pharmaceuticals and agrochemicals. We aimed to study *Baliospermum montanum* HNL (*Bm*HNL) because of its unique biocatalytic properties and the limitations associated with it as described in the previous chapter. A critical study on *Bm*HNL to improve its biocatalytic properties needs preparation of a large amount of the enzyme. Isolation of *Bm*HNL from its natural source needs a large amount of the plant material i.e. leaves and the procedure of enzyme preparation is not only laborious but also time-consuming. Apart from that, purification of the natural enzyme may not be possible to achieve in large scale, which is required because the complete study warrants several experiments with the use of pure *Bm*HNL. Further, purification of the enzyme from the plant source in multiple times may not produce uniformity. The process would not be economic too. These reasons clearly show that purification of *Bm*HNL from the plant source to pursue our study is not a feasible process. Hence there is a requirement to develop a process to prepare *Bm*HNL in a cost-effective, simple and efficient manner to study and improve the biocatalytic properties of this enzyme. Preparation of recombinant protein by

the heterologous expression is a suitable solution to the above problems. Cloning/subcloning of gene and its expression in a suitable host facilitates the production of an effective amount of protein in less time. This procedure is usually cost-effective as well as easy.

In early work, HNLs were extracted from their natural sources, however, afterward genes of many HNLs have been cloned, and expressed in appropriate microorganisms to facilitate enzyme purification, characterization, and their biocatalytic studies. In 1994, Hughes *et al* have cloned cDNA of *Me*HNL for the first time [1]. Later it's cloning has been reported in pQE4 expression vector and transformation in *E.coli* M15[pREP4] cells [2]. They observed that 80 L culture of M15-*Me*HNL has produced 4000 U of *Me*HNL while to get the same amount of enzyme 100-200 kg of cassava leaves would be needed. *Me*HNL has been overexpressed in several *E. coli* strains as well as in yeast [3], *E. coli* strain M15[pREP4] [2,4,5], *E. coli* BL21(DE3) at low temperature using vector pET-21a [6], *E. coli* JM109 & BL21 (DE3) [7,8] and *Saccharomyces cerevisiae* (yeast) [9]. Cloning of the first (*S*)-HNL i.e. *Sb*HNL has been reported by Wajant *et al* in λZAP II plasmid following by its transformation in *E.coli* XL1-Blue cells. It has been further subcloned into pBluescript plasmid [10].

Although *E. coli* is extensively used as a host for the recombinant protein production due to its availability, less life-span and its non-pathogenic nature but the production of active recombinant protein depends upon nature of protein and culture conditions as well. There have been many instances where HNLs expressed in an *E. coli* strain have produced insoluble proteins [11–14]. This issue has been addressed by expression of HNLs in *E.coli* at low temperature [6,13,14] or use of another host such as yeast. Expression of HNLs in

Saccharomyces cerevisiae and *Pichia pastoris* have been reported which resulted in completely soluble and active HNL protein production [9,11,14,15]. Hasslacher *et al* showed the heterologous expression of *Hb*HNL in *Pichia pastoris* that resulted in a high yield of the enzyme i.e. ~22 g/liter [15].

*Bm*HNL, the (*S*)-oxynitrilase which is also the focus of the current thesis has been discovered in 2005 [16] where its purification was done from the plant leaves. Later Dadashipour *et al* in 2011 [13], have described its cloning, protein expression, purification, and biocatalytic characterization. Despite of this information, we here aimed to clone *Bm*HNL, express in different expression vectors with different *E. coli* strains, and purify the enzyme to carry out biocatalytic studies.

2.2. Objectives

- To carry out subcloning of *Bm*HNL into a suitable expression vector
- To optimize *Bm*HNL protein expression
- To purify, characterize *Bm*HNL and measure its HNL specific activity.

2.3. Materials and methods

2.3.1. Materials

All the chemicals used were molecular biology grade and obtained commercially. Buffers, antibiotics such as ampicillin, kanamycin, and other biochemical were purchased from Himedia Labs, Mumbai, India. The media used for culturing of *E. coli* bacterial strains were obtained from Himedia Labs, Mumbai, India. Enzymes such as T4 DNA ligase (catalog # M0202), Taq DNA polymerase (catalog # M0273), restriction enzymes i.e. *Bam*H1 (catalog # R0136) and *Sal*1 (catalog and M0138), and dNTPs used for PCR

amplification were obtained from New England Bio Labs, USA. The quick Gel Extraction Kit was procured from Qiagen., USA. Protein staining dye (Coomassie Brilliant Blue G-250) was obtained from HiMedia Labs, Mumbai, India. Isopropyl-*β*-D-thiogalactopyranoside (IPTG) was purchased from BR-BIOCHEM and phenylmethylsulfonyl fluoride (PMSF) was bought from Himedia. Lysozyme was purchased from AMRESCO Inc., USA**.** The Ni-NTA agarose resin (catalog # 30210) was obtained from Qiagen., USA.

2.3.2. Primers

All oligonucleotides used were custom synthesized from Eurofins genomics India Private Limited, Bengaluru. DNA sequencing was carried out in the DNA Sequencing Facility at UDSC, Department of Biochemistry, University of Delhi South Campus, New Delhi, India.

2.3.3. Bacterial strains and vectors

Host strains such as *E. coli* DH5*α*, BL21 (DE3), BL21 (DE3) pLYS and BL21 (DE3) stars were a kind gift of Dr. Vishal Saxena, Birla Institute of Technology & Science, Pilani, Rajasthan, India. The bacterial strains were maintained as frozen stocks in 80% glycerol at –80 °C.

2.3.4. Expression vectors

Subcloning of *Bm*HNL was carried out in two expression system, pET28a and pCold1 in the present study. Cold-shock expression vector pCold I (catalog # 3361) was procured from TAKARA, Bio Inc. Japan. The pET vector, pET-28a (+) was a kind gift from Prof. Uwe T. Bornscheuer, University of Greifswald, Germany.

2.3.5. Synthetic genes

*Bm*HNL gene codon optimized for *E. coli* expression, cloned in pUC57 and the same gene without codon optimization cloned in pUC57 were purchased from Genescript, USA.

2.3.6. Bacterial growth media

E. coli strains were cultured in Luria-Bertani Broth (LB media contained casein enzymic hydrolysate 10 g/L, yeast extract 5 g/L, and sodium chloride 10 g/L) HiMedia Labs, Mumbai. LB agar plates prepared using 15 g/L of Luria Bertani Agar Miller, HiMedia (catalog no. M1151) Media were autoclaved before use and supplemented with ampicillin (100 μg/mL) or kanamycin (50 μg/mL), as required [17].

2.4. Methods

2.4.1. Subcloning of *Bm*HNL synthetic gene

A synthetic gene of *Bm*HNL (LOCUS: AB505969) cloned into pUC57 at *Bam*HI and *Sal*I sites was designed and ordered from GeneScript, USA, named as syntgene-1. Another synthetic gene designed as above except the *Bm*HNL gene was codon optimized for *E. coli* expression was also ordered from GeneScript, named as syntgene-2. The procured plasmids pUC57-syntgene-1 and syntgene-2 were centrifuged at 3100*g* for 1 min and suspended in 20 μL of autoclaved milli-Q water. Both the plasmid suspension were mixed properly with vortex for 2 minutes, spun for 1 minute and stored at –20 °C.

2.4.2. Preparation of bacterial competent cells

The appropriate bacterial *E. coli* strain [DH5α, BL21 (DE3), BL21 (DE3) pLys or (DE3) stars] was streaked onto an LB plate and grown overnight at 37 °C. A single colony was inoculated in 5 mL LB broth and incubated at 37 °C with 200 rpm shaking for 16 h. A 1% inoculum of this subculture was transferred into 100 mL of LB broth. The bacterial growth

was monitored until the optical density of the culture reached OD_{600} of 0.3-0.5. The cells were then centrifuged at 4951*g* for 8 min at 4 ºC, the pellet was suspended in a one-half volume of ice-cold 0.1 M $CaCl_2$ (16.66 mL) and incubated on ice for 20 minutes. Later the cells were collected by centrifugation at 4951*g* for 8 min at 4 ºC. The cell pellet was suspended in a one-tenth volume of ice-cold 0.1 M $CaCl_2$ containing 15% final concentration of glycerol and stored as 100 µL aliquots in a –80 °C freeze. (Modified protocol of Sambrook *et al*, 2001)

2.4.3. Plasmid transformation by heat shock method in bacteria

Competent *E. coli* cells prepared were thawed on ice for 15-20 min. A 1-2 µL of the appropriate plasmid DNA i.e. pUC57-*Bm*HNL syntgene-1 or syntgene-2 (10-100 ng) was added to the 100 µL of chemically competent *E. coli* DH5α cells (prepared in **2.4.2**) and incubated for half an hour on ice, followed by heat shock at 42 °C for 90 seconds. After heat shock, cells were chilled on ice for 2 minutes, 1 mL fresh LB was added into it and the cell suspension was incubated in an orbital shaker at 37 °C, 200 rpm for 45 min. Cells were then centrifuged at 2100*g*, 25 °C for 5 min and plated on LB-agar plate containing ampicillin. A control experiment containing no plasmid, only *E. coli* DH5α cells was performed in an identical manner. All plates were incubated at 37 °C for 12 to 16 h for the appearance of the transformed colonies.

2.4.4. Isolation of plasmid DNA

A single transformed *E. coli* DH5α colony containing syntgene-1 or syntgene-2 was inoculated to a 5 mL LB-ampicillin media. The culture was incubated at 37 °C, 200 rpm for 16 h, cells harvested at 4951*g* in a centrifuge for 5 min and both the plasmids were isolated by alkaline lysis method as described by Sambrook *et al* in 2001 [17] with certain

modifications as mentioned below. Briefly, the cell pellet was suspended in 100 µL of chilled solution I (GET buffer: 50 mM glucose, 10 mM EDTA and 25 mM Tris-Cl, pH 8.0) and mixed properly by vortex. Then 200 µL of solution II (1% SDS and 0.2 N NaOH) was added into the suspension and mixed properly by inverting the tube followed by incubation of 3-5 min at room temperature. After addition of 150 µL of solution III (3M Na-acetate buffer pH 5.2), the tube was incubated on ice for 3-4 minutes followed by centrifugation at 8600*g*, 4 °C for 10 minutes. The supernatant was collected in a fresh tube. 3-5 µL of RNase solution (20 mg/mL) was added into the supernatant and incubated at 37 °C for 30 minutes. An equal volume of phenol-chloroform (1:1) was added into the tube and centrifuged at 8600*g* for 10 minutes.

The supernatant or the upper phase was separated, mixed with twice the volume of chilled ethanol and one-tenth volume of sodium acetate and incubated at –20 °C for half an hour. Further, it was centrifuged at 8600*g* for 10 minutes and the pelleted plasmid was washed with 70% ethanol. The pellet was air dried at room temperature and suspended in 20-30 µL of 1xTE (10 mM Tris-Cl and 1 mM EDTA) buffer.

2.4.5. Quantification of DNA by spectrophotometry

DNA samples were diluted 60 fold in 1x TE buffer and the concentration was estimated by measuring the OD_{260} in a spectrophotometer using a Quartz cuvette. The following formula was used to calculate DNA concentration: OD_{260} of 1.0 = 50 µg/mL of double standard DNA.

2.4.6. Restriction digestion and generation of cohesive end fragments

The double digestion reaction mixture containing 10 µg of a vector (pET28a/pUC57-with *Bm*HNL syntgene-1 or syntgene-2 or pCold1/pUC57-with *Bm*HNL syntgene-1 or

syntgene-2), 10 U each of the restriction enzymes *BamH*I and *Sal*1 (0.5 µL, 20, 000 U/mL), 2.5 µL of 10X NEBuffer 3.1, and rest volume of sterile dH_2O up to 20 µL was incubated at 37 °C for 3 h. Digested products were analyzed by 0.8% agarose gel electrophoresis. After heat inactivated at 65 °C for 20 min, the reaction mixture was gel extracted by Qiagen Gel Extraction Kit. The purified double digested DNA fragments were eluted with 10 µL of elution buffer. Their concentration measured by UV-Visible spectrophotometer were found to be 50 ng/µL for pET28a/*BamH*1/*Sal*1, 40 ng/µL for pCold1/*BamH*1/*Sal*1, 20 ng/µL for *Bm*HNL syntgene-1/*BamH*1/*Sal*1 and 20 ng/µL for *Bm*HNL syntgene-2/*BamH*1/*Sal*1 respectively.

2.4.7. Ligation

Ligation of the double digested product *Bm*HNL syntgene-1/*BamH*1/*Sal*1 and *Bm*HNL syntgene-2/*BamH*1/*Sal*1 into the pET28a-*BamH*I-*Sal*1 was done as follows. Ligation reaction mixture contained 1 µL of 10X ligase buffer, 1:5 molar ratio of vector: insert, 0.5 µL of T4 DNA ligase and rest volume with sterile dH_2O up to 10 µL. The reaction was carried out in a thermocycler using the programme: 25 °C for 2 h, 18 °C for 4 h, 16 °C for 3 h, 12 °C for 3 h, 10 °C for 2 h, 70 °C for 10 min and finally stored at 4 °C. Ligation of *Bm*HNL syntgene-1/*BamH*1/*Sal*1 and *Bm*HNL syntgene-2/*BamH*1/*Sal*1 into pCold1-*BamH*I-*Sal*1 was also carried out as above. Ligated products were transformed into *E.coli* BL21(DE3) chemically competent cells. The transformed *E. coli* cells with pET28a-*Bm*HNL syntgene-1 and pET28a-*Bm*HNL syntgene-2 were plated into LB-Kan plates while pCold1-*Bm*HNL syntgene-1 and pCold1-*Bm*HNL syntgene-2 into LB-Amp plates.

2.4.8. Colony PCR for pET28a-*Bm*HNL syntgene-1 and 2

Colony PCR was done in a PCR machine (BIORAD) using 10 of randomly selected transformed colonies from ligation as templates. The reaction mixture contained 1 µL of an *E. coli* colony suspended in nuclease-free water, 6.6 µL nuclease-free water, 0.2 µL Taq DNA polymerase (20 U/µL), 1 µL forward primer (10 pmol/µL), 1 µL reverse primer (10 pmol/µL), 0.2 µL dNTPs (10 mM), and 1 µL 10X reaction buffer. PCR was carried out at 95 °C (5 min) followed by 35 cycles of 95 °C (1 min), 54 °C (90 s), 72 °C (1 min) and final extension using 72 °C (5 min).

Detail sequence of the primers used for colony PCR are as follows:

Syntgene 1, forward primer: TATGGCTAGCATGACTGG,

Syntgene 1, reverse primer: AGCAGCCGGATCTCAGTG;

Syntgene 2, forward primer: TAGCGGATCCATGGTGTC, and

Syntgene 2 reverse primer: AAGATTGTCGACTTACAGG.

Primers designed for pET28a-*Bm*HNL syntgene-1 were vector specific and primers for pET28a-*Bm*HNL syntgene-2 were overlapping primers.

2.4.9. Colony PCR for pCold1-*Bm*HNL syntgene-1

Colony PCR was carried out using 10 of randomly selected colonies along with control colony. Part of a colony was suspended in 50 µL autoclaved milli Q water. The cell suspension was lysed at 65 °C for 15 min. A 2 µL of cell suspension was used as a template along with 6.6 µL nuclease-free water, 0.2 µL Taq DNA polymerase (5 U/ µL), 1.0 µL forward primer of 10 pmol/µL concentration (GAAGGTAGGCATATGGAG (pCold1-*Bm*HNL seq f.) and 1.0 µL reverse primer of 10 pmol/ µL concentration CCAAATGGCAGGGATCTTAGA (pCold1-*Bm*HNL seq r), 0.2 µL dNTPs (10mM), and

1 µL 10X reaction buffer. The above solution was amplified using a PCR machine (Biorad) under conditions: 95 °C for 5 min for initial cell breakage, 35 cycles of 1 min at 95 °C for denaturation, 90 sec at 48 °C for annealing, 1 min at 72 °C for extension and final extension using 72 °C for 5 min.. Colony PCR amplicons were analyzed by agarose gel electrophoresis (0.8%) and then for all ligated products glycerol stocks were prepared.

2.4.10. Colony PCR for pCold1-*Bm*HNL syntgene-2

Colony PCR of the corresponding colonies was carried out using the similar protocol as **2.4.9**, except the annealing temperature was 50 °C, and the forward primer used was: TAGAGGGATCCATGGTGTC (pCold1-*Bm*HNL Syn2 f.) and the reverse primer was: CTGGACTGCAGGTCGACTTA (pCold1-*Bm*HNL Syn2 r).

Ligation was further confirmed by restriction digestion of ligated products (pCold1-*Bm*HNL syntgene-1 and pCold1-*Bm*HNL syntgene-2).

2.4.11. *Bm*HNL gene sequencing

The recombinant plasmids (pCold1-*Bm*HNL syntgene-1 and pCold1-*Bm*HNL syntgene-2) were purified using alkaline lysis method and the gene was sequenced completely. Both *Bm*HNL inserts were sequenced using sequencing primers as mentioned in **2.4.8**.

2.4.12. Optimization of protein expression of recombinant *Bm*HNL

2.4.12.1. Bacterial strains: Expression of *Bm*HNL was carried out in three different *E.coli* strains e.g. BL21(DE3), BL21(DE3)pLysS and BL21 star (DE3). These all are B strains which lack Lon protease (cytoplasm) and OmpT protease (outer membrane). DE3 means that these strains contain λDE3 lysogen which carries gene for T7 RNA polymerase under control of the lacUV5 promoter. However, Lac repressor mechanism is not a tightly

regulated system, so a basal level expression of T7 RNA polymerase may be there. For expression of recombinant protein, IPTG is required.

pLysS strains express T7 lysozyme, which suppresses basal expression of T7 RNA polymerase prior to induction, thus stabilizing pET recombinants encoding target proteins that affect cell growth and viability while BL21 Star strain contain rne131 mutation which enhances the expression. The mutated rne gene (rne131) encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA, resulting in an increase in mRNA stability.

2.4.12.2. Expression vectors: In the present study, two expression vectors were used i.e. pET28a and pCold1. pET28a is a bacterial expression vector with T7 lac promoter, N-terminal His-tag, thrombin cleavage site, C-terminal His-tag, and kanamycin resistance. The second vector pCold1 is a cold shock expression vector. It has *csp*A promotor, N-terminal His-tag, 5'-UTR (untranslated region) and ampicillin resistance. Transcription from the cspA promoter can occur at 37 °C, but translation is not efficient because the adjacent 5' UTR is very unstable at this temperature. When the temperature is decreased from 37 °C to 15 °C, the 5'-UTR adopts a highly stable secondary structure. This results in improved translation efficiency, allowing extremely efficient protein synthesis at 15 °C [18].

2.4.12.3. Protein expression of *Bm*HNL in pET28a

A single colony of *E.coli* BL21 (DE3) carrying pET28a-*Bm*HNL syntgene-1 or 2 was inoculated into 5 mL of LB broth with 50 µg/mL kanamycin at 37 °C, 200 rpm. After 16 h, it was transferred to 20 mL of fresh medium in 1:100 ratio and incubated at 37 ºC until the OD_{600} reached 0.5 to 0.6. The *Bm*HNL protein was induced with IPTG to a final conc. of 1 mM and was further incubated at different temperatures (37, 30 and 18 °C). A time

course analysis was done by taking 1 mL of aliquots at different time intervals. Each aliquot was centrifuged at 17949*g* for 1 min at 4 °C and the pellet was suspended in 1X SDS sample buffer i.e. 62.5 mM Tris-HCl pH 6.8, 2.5% SDS, 0.002% bromophenol blue, 0.7 M (5%) β-mercaptoethanol, and 10% glycerol (1 mL/gm of pellet). Cells were disrupted by sonication (Microson^{TM} Ultrasonic cell disruptor) at 20 KHz for 1 min. The cell suspension was centrifuged at 4 °C, 14500*g* for 5 min. The supernatant was analyzed by 12% SDS-PAGE using medium range pre-stained protein marker (BR-BIOCHEM) and stained by Coomassie Brilliant Blue R-250.

2.4.12.4. Protein expression of *Bm*HNL in pCold1

A single colony of *E.coli* BL21 (DE3) having pCold1-*Bm*HNL syntgene-1 or 2 inoculated into 5 mL of LB broth with 100 µg/mL ampicillin was incubated at 37 °C, 200 rpm. After 14-16 h, it was transferred to 20 mL of fresh medium in 1:100 ratio and incubated at 37 °C until the OD_{600} reached 0.4 to 0.6. The *Bm*HNL protein was induced with IPTG to a final conc. of 1 mM after cold-shock of 2 h and was further incubated at 200 rpm, 18 °C. A time course analysis was performed as per the protocol mentioned in **2.4.12.3**.

2.4.13. Protein purification and characterization

Protein purification is necessary for characterization of a protein. Protein purification is carried out by different chromatographic techniques like size exclusion chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography etc. The *Bm*HNL protein is tagged with $(His)_6$/polyhistidine-tag so the protein was purified by affinity chromatography using Ni-NTA agarose resin.

2.4.13.1. *Bm*HNL purification by affinity chromatography

A 100 mL culture of *E.coli* BL21 (DE3)-pCold1-*Bm*HNL syntgene-1 and *Bm*HNL syntgene-2 carried out separately as per the protocol in **2.4.12.4**, produced 0.8 g and 0.92 g of cell pellet respectively. Cell pellet was suspended in 20 mM potassium phosphate buffer (KPB) pH 7.0 according to 15% w/v of pellet. Lysozyme in final concentration of 1 mg/mL and protease inhibitor PMSF in final concentration of 1 mM were added into the cell suspension. Cells were disrupted by sonication at 25 kHz for 10 minutes on ice. The lysate was centrifuged at 14500*g*, 4 °C for 60 min. Cell debris were removed and the cell lysate was purified by Ni-NTA agarose in an affinity chromatography. 10 mL of the Ni-NTA resin was poured into polypropylene column (34×112 mm, 23 mL spin column), mixed properly by inverting the column repeatedly and allowed the resin to settle down. The resin was pre-equilibrated with 20 mL equilibration buffer (50 mM KPB pH 7.0, 10 mM imidazole, and 300 mM NaCl). This step was repeated twice. The buffer was drained from the column without drying the resin. Then the cell lysate was added to the resin followed by shaking for 45 minutes at 4 °C in a rocker for proper binding. The unbound lysate was collected as flow-through (FT). Subsequently, the resin was washed with 20 mL of wash buffer (50 mM KPB pH 7.0, 20 mM imidazole, and 300 mM NaCl). This step was repeated twice. Then protein was eluted using 150 ml of elution buffer (50 mM KPB pH 7.0, 150 mM imidazole, and 300 mM NaCl). The final elution carried out using a buffer containing a high concentration of imidazole (50 mM KPB pH 7.0, 300 mM imidazole, and 300 mM NaCl). All steps were carried out at 4 °C. All the fractions were analyzed by SDS-PAGE (12% polyacrylamide gel) and quantitated by Nanodrop. For removal of imidazole from eluted protein, dialysis was carried out by using pre-treated membrane (20

kDa MWCO). Subsequently, the dialyzed protein was concentrated using 10,000 MWCO Amicon tubes (Merck Millipore) and the enzyme was stored at 4 °C.

2.4.14. HNL assay

The HNL assay was carried out in a microtitre plate using Multiskan Go UV-Visible spectrophotometer (Thermo Fisher Scientific, No. 1510-02398C). Each well of the microtitre plate contained total 200 µL of reaction mixture. The assay mixture contained 175 µL of 50 mM citrate-phosphate buffer pH 5.0, 5 µL of enzyme (1.2 µg) and 20 µL of 70 mM mandelonitrile pre-dissolved in 5 mM citrate-phosphate buffer pH 3.15. The assay was monitored for the degradation of mandelonitrile into benzaldehyde by observing the absorbance at 280 nm for 10 min. A control experiment was carried out by the same manner contained the corresponding volume of 20 mM KPB pH 7.0 instead of *Bm*HNL. Another control experiment was performed with only enzyme without substrate, as protein absorbs at 280 nm (**Figure 2.9**).

The specific activity of enzyme was calculated using formula

$$c = A/\varepsilon b$$

$$U/mg = \frac{[(Abs)rxn - (Abs)cont] \text{ X reaction volume}}{\varepsilon \text{ b X mg enzyme}}$$

(Abs)rxn: Absorbance of reaction, (Abs)cont: Absorbance of control, ε: molar extinction coefficient, b: path-length

2.5. Results

2.5.1. Subcloning of *Bm*HNL synthetic genes into pET28a expression vector

2.5.1.1. Transformation of pUC57-*Bm*HNL syntgene-1 and pUC57-*Bm*HNL syntgene-2 in *E.coli* DH5*α* cells and isolation of the plasmids

Both of the synthetic gene based pUC57 plasmids were successfully transformed into *E.coli* DH5*α* competent cells in LB-agar-ampicillin plates. This was evidenced by a lawn of colonies seen in the plates. Using the method described in the "methodology" section **2.4.4** both of these plasmids were isolated using alkaline lysis method and quantified by UV-Vis spectroscopy at 260, 280 nm. The concentration was found to be 2670 and 5308 ng/μL respectively.

2.5.1.2. Double digestion followed by ligation

All plasmids pET28a, pUC57-*Bm*HNL syntgene-1, and pUC57-*Bm*HNL syntgene-2 were double digested by *BamH*1 and *Sal*1 restriction enzymes. The DNA gel pictures (**Figure 2.1**) show the proper size of the digested DNA fragments. Ligation of pET28a/*BamH*1/*Sal*1 with pUC57-*Bm*HNL syntgene-1/*BamH*1/*Sal*1 and pUC57-*Bm*HNL syntgene-2/*BamH*1/*Sal*1 was carried out as per methodology. The ligation products were transformed into *E.coli* DH5*α* cells.

2.5.1.3. Colony PCR to confirm ligation

Colony PCR of the transformed *E.coli* DH5*α* colonies carrying ligated products was carried out as per the protocol given in the "Methodology" and was confirmed by the DNA agarose gel electrophoresis (**Figure 2.2**). Colonies (no: 1, 2 and 7 of this figure were used to isolate plasmids (pET28a-*Bm*HNL syntgene-1 and pET28a-*Bm*HNL syntgene-2). Both the plasmids were successfully transformed into *E.coli* BL21 (DE3). This was confirmed by a

number of colonies seen in the LB-agar-kanamycin plate in the experiment that was associated with appropriate control.

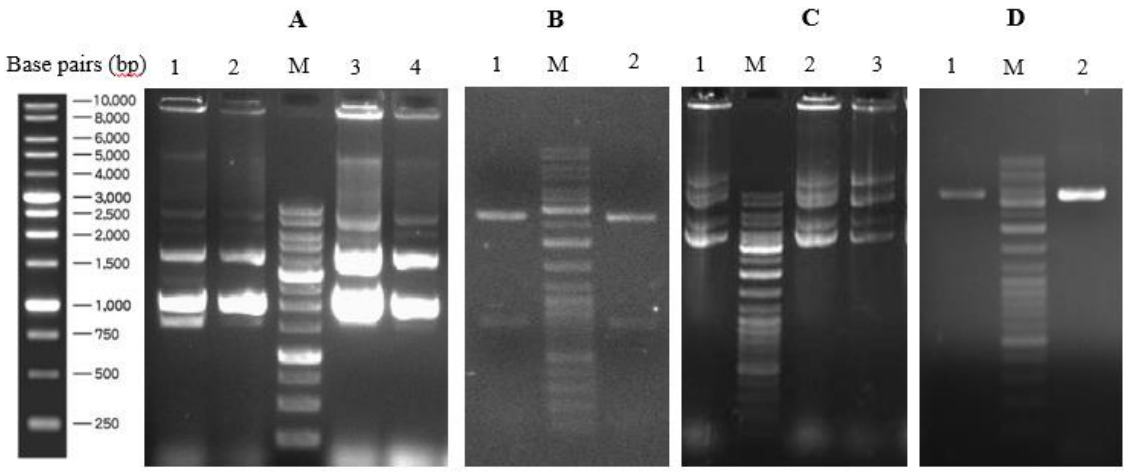


Figure 2.1: DNA gel of plasmids nd their double digestion. **A:** 1 & 2: pUC57- *Bm*HNL syntgene-1 plasmid, M: Marker, 3 & 4: pUC57-*Bm*HNL syntgene-2 plasmid; **B:** 1: pUC57- *Bm*HNL syntgene-1/*BamH*1/*Sal*1 (expected ~780 bp), M: Marker, 2: pUC57-*Bm*HNL syntgene-2/*BamH*1/*Sal*1 (expected ~780 bp); **C:** 1, 2 & 3: pET28a plasmid, M: Marker; **D:** 1 & 2: pET28a/*BamH*1/*Sal*1 (expected ~5.4 kbp), M: Marker

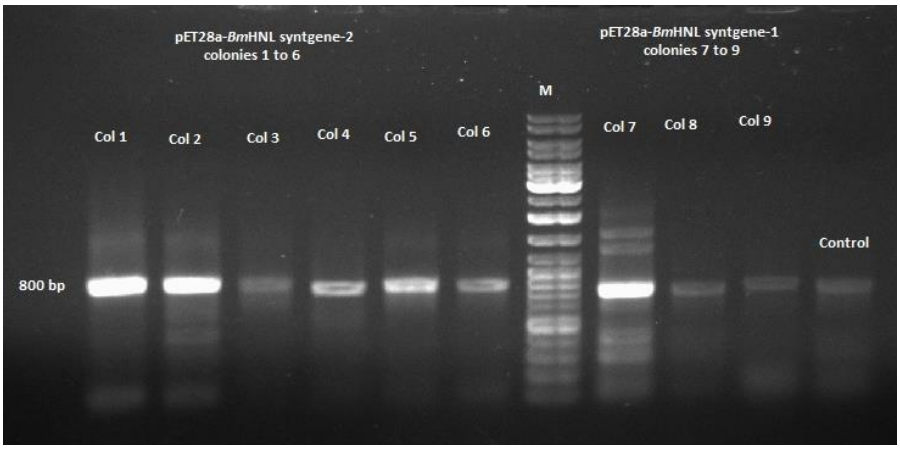


Figure 2.2: Colony PCR products showing amplified ~800 bp DNA fragment equivalent of *Bm*HNL syntgene-1 or *Bm*HNL syntgene-2

2.5.2. Subcloning of *Bm*HNL synthetic genes in pCold1 expression vector

*Bm*HNL syntgene-1 and *Bm*HNL syntgene-2 were successfully subcloned from the parent pUC57 vector into pCold1 vector, followed by transformation of the resulted pCold1-*Bm*HNL syntgene-1 and pCold1-*Bm*HNL syntgene-2 plasmid in *E.coli* BL21(DE3) host cells. Colonies (**no: 4** in **figure 2.3 C** were used to isolate plasmid and both the plasmids successfully transformed into *E.coli* BL21 (DE3). Subcloning was confirmed by DNA sequencing of the *Bm*HNL gene of the resulted ligated plasmids. Some of the experimental results toward the cloning such as DNA gel electrophoresis picture of (i) plasmids, isolated by alkaline lysis method (ii) double digested plasmids, (iii) colony PCR of the ligated colonies (iv) confirmation of ligation by double digested ligated plasmids (pCold1-*Bm*HNL syntgene-1 and 2) are represented below (**Figure 2.3** & **2.4**):

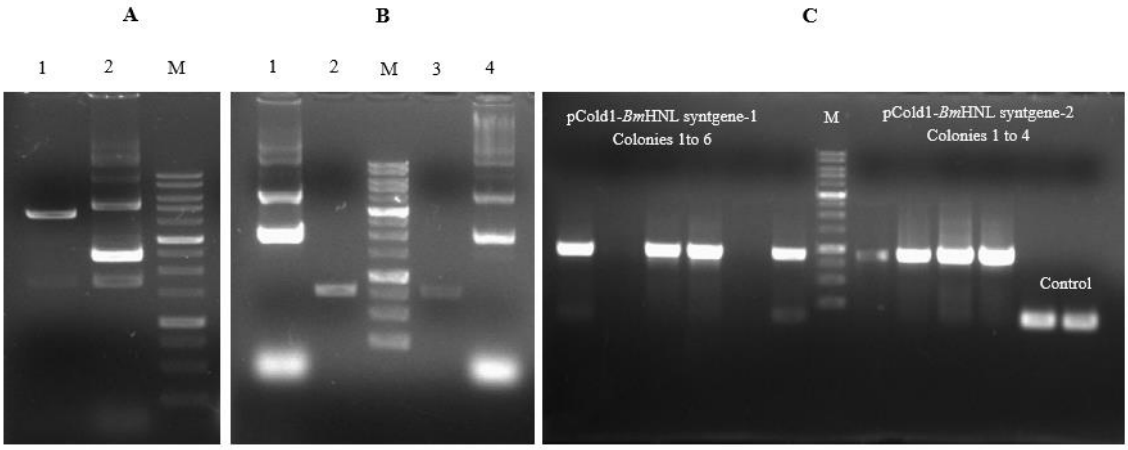


Figure 2.3: A: 1. pCold1/*BamH*1/*Sal*1 (expected ~4.4 kbp), 2: Undigested pCold1 plasmid, M: Marker; **B:** 1: Undigested pUC57-*Bm*HNL syntgene-1, 2: pUC57- *Bm*HNL syntgene-1/*BamH*1/*Sal*1 (expected ~780 bp), M: Marker, 3: pUC57-*Bm*HNL syntgene-2/*BamH*1/*Sal*1 (expected ~780 bp), 4: Undigested pUC57-*Bm*HNL syntgene-2; **C:** Colony PCR products showing amplified ~800 bp DNA fragment equivalent to *Bm*HNL syntgene-1 and *Bm*HNL syntgene-2 respectively.

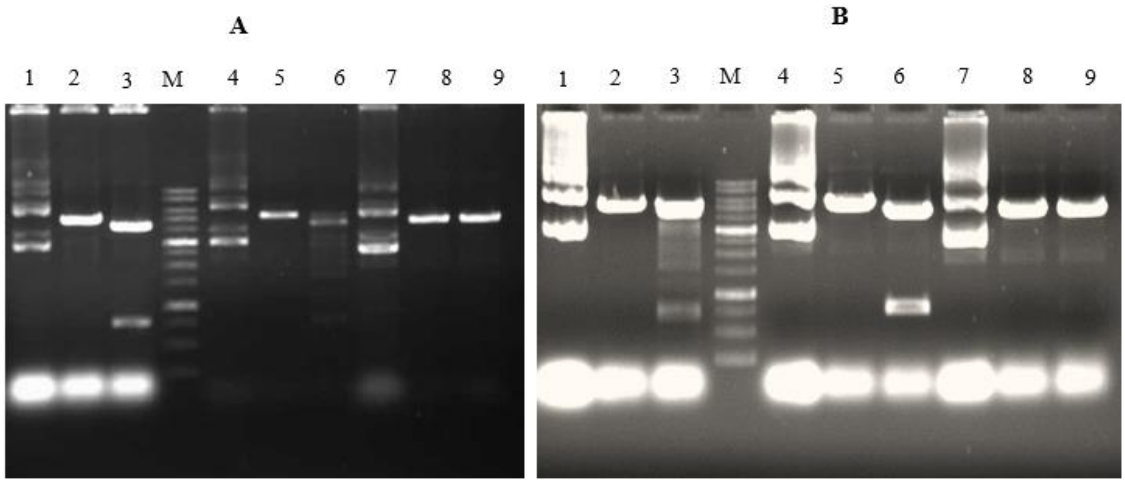


Figure 2.4: A: confirms pCold1-*Bm*HNL syntgene-1 plasmid. Lanes 1 to 3 are related to colony no. 4. 1: isolated undigested plasmid, 2: plasmid/*Bam*H1, 3: plasmid/*Bam*H1/*Sal*1; M: marker, 4 to 6 are related to colony no. 6, 4: isolated undigested plasmid, 5: plasmid/*Bam*H1, 6: plasmid/*Bam*H1/*Sal*1; 7 to 9 are related to colony no. 1 from control, 4: isolated undigested plasmid, 5: plasmid/*Bam*H1, 6: plasmid/*Bam*H1/*Sal*1; **B:** confirms pCold1-*Bm*HNL syntgene-2 plasmid. Lanes 1 to 3 are related to colony no. 3. 1: isolated undigested plasmid, 2: plasmid/*Bam*H1, 3: plasmid/*Bam*H1/*Sal*1; M: marker, 4 to 6 are related to colony no. 4, 4: isolated undigested plasmid, 5: plasmid/*Bam*H1, 6: plasmid/*Bam*H1/*Sal*1; 7 to 9 are related to colony no. 1 from control, 4: isolated undigested plasmid, 5: plasmid/*Bam*H1, 6: plasmid/*Bam*H1/*Sal*1 (These colony numbers are based on colony PCR image).

2.5.3. Protein expression of *Bm*HNL syntgene-1 and *Bm*HNL syntgene-2

2.5.3.1. Expression of *Bm*HNL syntgene-1 and *Bm*HNL syntgene-2 in pET28a

*Bm*HNL gene was expressed in pET28a system at 37 °C, 200 rpm with 1 mM IPTG induction as mentioned in methodology section **2.4.12.3**. Protein expression was checked by SDS-PAGE analysis (**Figure 2.5**).

Very less protein expression was observed in *E. coli* BL21(DE3), so experiments for optimization of expression of *Bm*HNL proteins were also performed by varying post induction time and temperature but none of the conditions improved expression of *Bm*HNL.

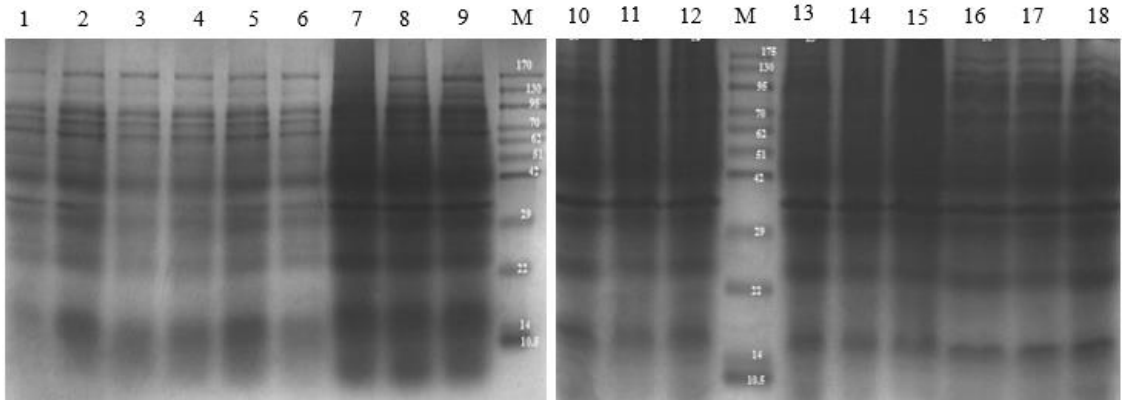


Figure 2.5: Protein expression of pET28a-*Bm*HNL in *E. coli* BL21 (DE3). 1: un-induced pET28a, 2: un-induced pET28a-*Bm*HNL syntgene-1, 3: un-induced pET28a-*Bm*HNL syntgene-2, 4: pET28 induced (zero h, 37 °C), 5: pET28-*Bm*HNL syntgene-1 induced (zero h, 37 °C), 6: pET28-*Bm*HNL syntgene-2 induced (zero h, 37 °C), 7: pET28 induced (2 h, 37 °C), 8: pET28-*Bm*HNL syntgene-1 induced (2 h, 37 °C), 9: pET28-*Bm*HNL syntgene-2 induced (2 h, 37 °C), 10: pET28a induced (6 h, 37 °C), 11: pET28-*Bm*HNL syntgene-1 induced (6 h, 37 °C), 12: pET28-*Bm*HNL syntgene-2 induced (6 h,37 °C), 13: pET28a induced (18 h, 37 °C), 14: pET28-*Bm*HNL syntgene-1 induced (18 h, 37 °C), 15: pET28-*Bm*HNL syntgene-2 induced (18 h, 37 °C), 16: pET28a induced (24 h, 37 °C), 17: pET28-*Bm*HNL syntgene-1 induced (24 h, 37 °C), 18: pET28-*Bm*HNL syntgene-2 induced (24 h, 37 °C).

In order to improve the expression of the *Bm*HNL gene, both the ligated plasmids pET28-*Bm*HNL syntgene-1 and pET28-*Bm*HNL syntgene-2 were also expressed in *E.coli* BL21-

star strain and BL21-pLys strain at 37 °C and for different time intervals. Protein expression was checked in SDS-PAGE from zero to 24 h (**Figure 2.6**). In all the above conditions, poor protein expression was observed. Only pET28a vector without *Bm*HNL was used as a control. No clear band of induced *Bm*HNL was observed in SDS-PAGE gel.

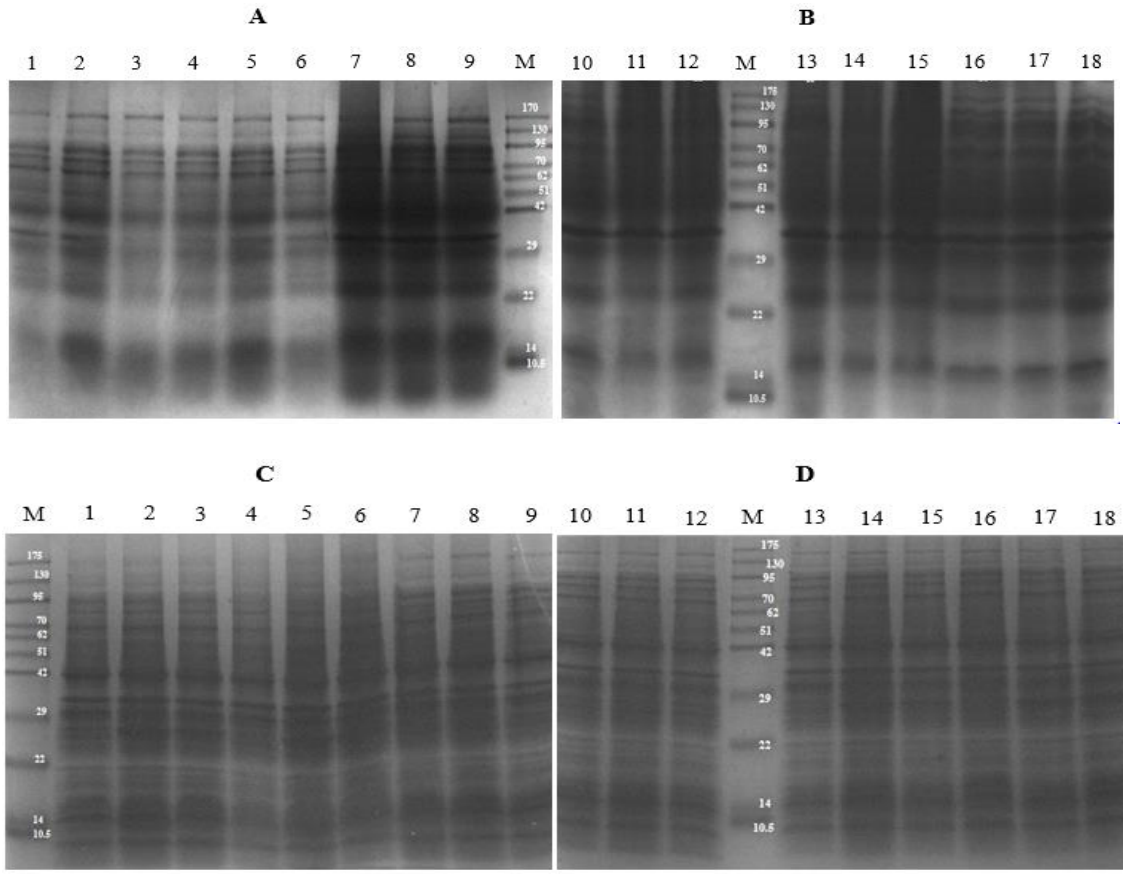


Figure 2.6: (A & B represents protein expression of pET28a-*Bm*HNL in *E. coli* BL21 (DE3)-pLys strain and **C & D** represents protein expression in *E. coli* BL21 (DE3)-star strain)**. A:** 1: un-induced pET28a, 2: un-induced pET28a-*Bm*HNL syntgene-1, 3: un-induced pET28a-*Bm*HNL syntgene-2, 4: pET28 induced (Zero h, 37 °C), 5: pET28-*Bm*HNL syntgene-1 induced (Zero h, 37 °C), 6: pET28- *Bm*HNL syntgene-2 induced (Zero h, 37 °C), 7: pET28a induced (2 h, 37 °C), 8: pET28a-*Bm*HNL syntgene-1 induced (2 h, 37 °C), 9: pET28a-*Bm*HNL syntgene-2 induced (2 h, 37 °C), **B:** 10: pET28a induced

(6 h, 37 °C), 11: pET28-*Bm*HNL syntgene-1 induced (6 h, 37 °C), 12: pET28- *Bm*HNL syntgene-2 induced (6 h, 37 °C), 13: pET28a induced (18 h, 37 °C), 14: pET28-*Bm*HNL syntgene-1 induced (18 h, 37 °C), 15: pET28-*Bm*HNL syntgene-2 induced (18 h, 37 °C), 16: pET28a induced (24 h, 37 °C), 17: pET28-*Bm*HNL syntgene-1 induced (24 h, 37 °C), 18: pET28-*Bm*HNL syntgene-2 induced (24 h, 37 °C). **C:** 1: un-induced pET28a, 2: un-induced pET28a-*Bm*HNL syntgene-1 , 3: un-induced pET28a-*Bm*HNL syntgene-2, 4: pET28 induced (2 h, 37 °C), 5: pET28- *Bm*HNL syntgene-1 induced (2 h, 37 °C), 6: pET28-*Bm*HNL syntgene-2 induced (2 h, 37 °C), 7: pET28 induced (4 h, 37 °C), 8: pET28-*Bm*HNL syntgene-1 induced (4 h, 37 °C), 9: pET28- *Bm*HNL syntgene-2 induced (4 h, 37 °C), **D:** 10: pET28a induced (8 h, 37 °C), 11: pET28-*Bm*HNL syntgene-1 induced (8 h, 37 °C), 12: pET28-*Bm*HNL syntgene-2 induced (8 h, 37 °C), 13: pET28a induced (21 h, 37 °C), 14: pET28-*Bm*HNL syntgene-1 induced (21 h, 37 °C), 15: pET28-*Bm*HNL syntgene-2 induced (21 h, 37 °C), 16: pET28a induced (24 h, 37 °C), 17: pET28-*Bm*HNL syntgene-1 induced (24 h, 37 °C), 18: pET28-*Bm*HNL syntgene-2 induced (24 h, 37 °C).

2.5.3.2. Expression of *Bm*HNL syntgene-1 and *Bm*HNL syntgene-2 in pCold1

Protein expression of *Bm*HNL syntgene-1 and syntgene-2 was carried out in pCold1 expression vector as described in **2.4.12.4. Figure 2.7** (**A, B** and **C**) shows SDS-PAGE analysis of both the proteins with 1 mM IPTG concentration and 15 °C at different time points. The expression profile has been checked from zero to 24 h. The protein expression increased with time. The expression was highest in 24 h. The protein expression was better in codon optimized *Bm*HNL compared to wild type *Bm*HNL. pCold1 vector without *Bm*HNL was used as a control in the experiment. There was no protein expression in control. Between pET28a and pCold1, the later has showed excellent protein expression

for both the proteins. This is probably due to the cspA promotor present in pCold1 vector that enhances the translation process in host cells.

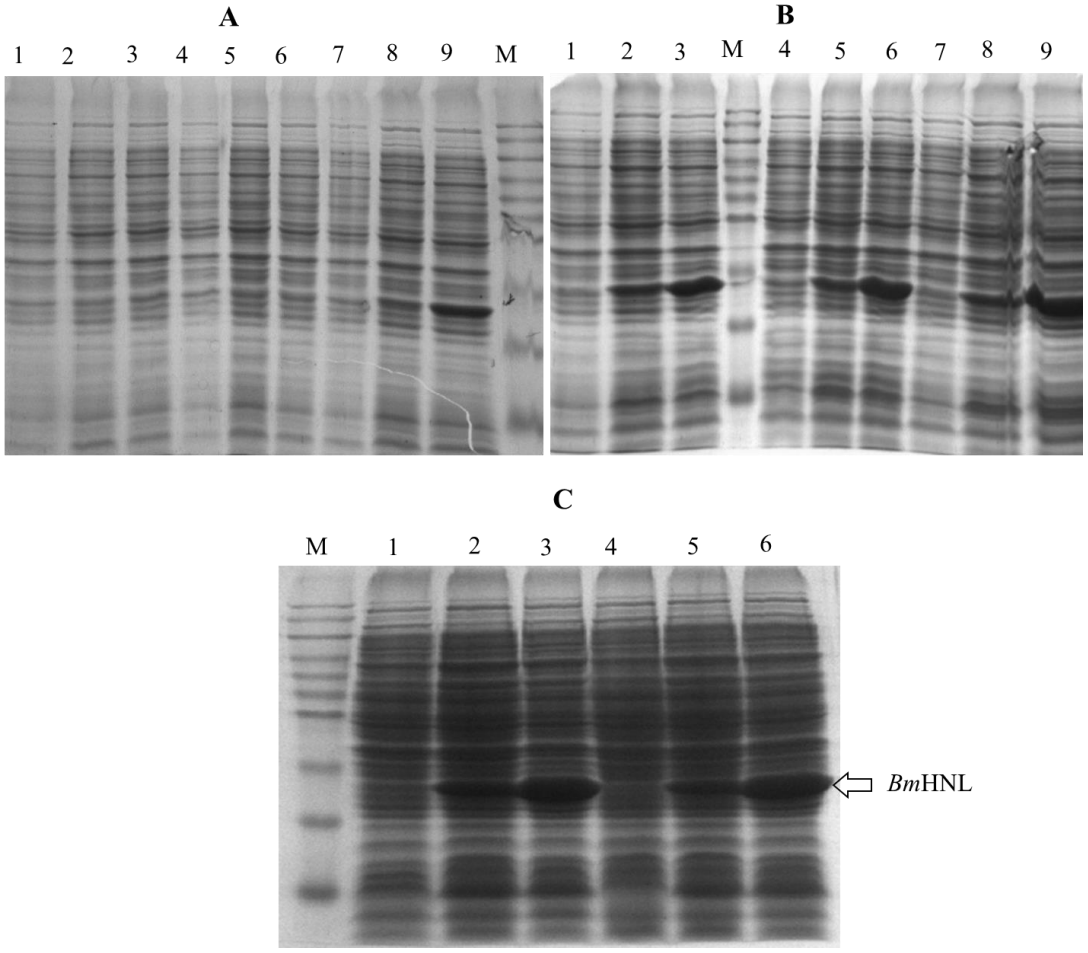


Figure 2.7: A: Protein expression of pCold1-*Bm*HNL in *E. coli* BL21(DE3); 1: un-induced pCold1, 2: un-induced pCold1-*Bm*HNL syntgene-1 , 3: un-induced pCold1-*Bm*HNL syntgene-2, 4: pCold1 induced (zero h, 18 °C), 5: pCold1-*Bm*HNL syntgene-1 induced (zero h, 18 °C), 6: pCold1-*Bm*HNL syntgene-2 induced (zero h, 18 °C), 7: pCold1 induced (2 h, 18 °C), 8: pCold1- *Bm*HNL syntgene-1 induced (2 h, 18 °C), 9: pCold1-*Bm*HNL syntgene-2 induced (2 h, 18 °C), M: marker. **B:** 1: pCold1 induced (4 h, 18 °C), 2: pCold1-*Bm*HNL syntgene-1 induced (4 h, 18 °C), 3: pCold1-*Bm*HNL syntgene-2 induced (4 h, 18 °C), M: marker, 4: pCold1 induced (6 h, 18 °C), 5: pCold1-*Bm*HNL syntgene-1 induced

(6 h, 18 °C), 6: pCold1-*Bm*HNL syntgene-2 induced (6 h, 18 °C), 7: pCold1 induced (7 h, 18 °C), 8: pCold1-*Bm*HNL syntgene-1 induced (7 h, 18 °C), 9: pCold1-*Bm*HNL syntgene-2 induces (7 h, 18 °C). **C:** M: Marker, 1: pCold1 induced (17 h, 18 °C), 2: pCold1-*Bm*HNL syntgene-1 induced (17 h, 18 °C), 3: pCold1-*Bm*HNL syntgene-2 induced (17 h, 18 °C), 4: pCold1 induced (24 h, 18 °C), 5: pCold1-*Bm*HNL syntgene-1 induced (24 h, 18 °C), 6: pCold1-*Bm*HNL syntgene-2 induced (24 h, 18 °C)

2.5.4. *Bm*HNL protein purification

*Bm*HNL protein was purified using the optimal conditions as obtained from above study. The pCold1 vector with *Bm*HNL has polyhistidine tag (His-tag). Expression of *Bm*HNL syntgene-1 was followed by protein purification using Ni-NTA column (Section **2.4.13.1**). After one step purification of *Bm*HNL by Ni-NTA column, the purified protein was analyzed by SDS-PAGE (**Figure 2.8 A & B**). The purified *Bm*HNL was eluted in 150 mM imidazole fractions. *Bm*HNL syntgene-2 was also purified as mentioned above using 150 mM imidazole for elution. All the fractions were checked in SDS-PAGE (**Figure 2.8 C**). The protein expression was more in *Bm*HNL syntgene-2 as compared to syntgene-1 (**Figure 2.7**) however, less protein was found in soluble form in case of *Bm*HNL syntgene-2 as compared to cell pellet. Presence of clear band of ~29 kDa for purified fraction confirms the presence of *Bm*HNL.

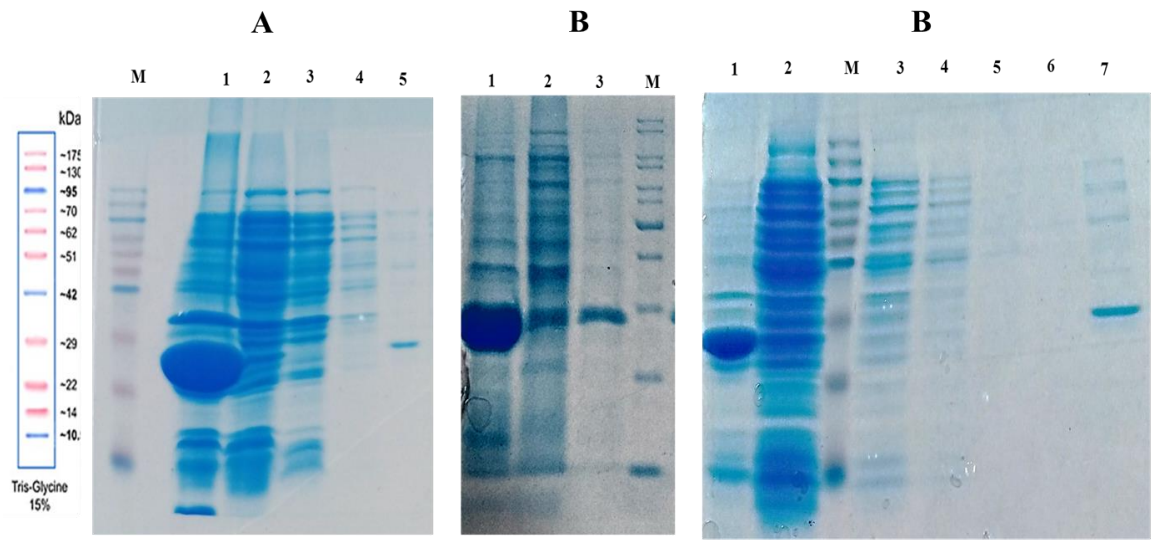
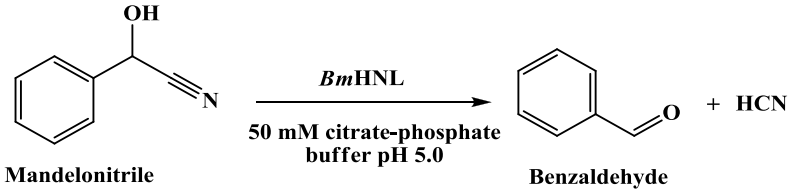


Figure 2.8: A: M: Marker, 1: Cell pellet (*E.coli* BL21(DE3) pCold1-*Bm*HNL syntgene 1), 2: Cell lysate, 3: Flow-through (FT), 4: Wash, 5: Elution 150 mM imidazole; **B:** 1: Cell pellet (*E.coli* BL21(DE3) pCold1-*Bm*HNL syntgene 1), 2: Cell lysate, 3: Elution 150 mM imidazole, M: Marker; **C:** 1: Cell pellet (*E.coli* BL21(DE3) pCold1-*Bm*HNL syntgene 2), 2: Cell lysate, M: Marker, 3: Flow through, 4: Wash 1, 5: Wash 2, 6: Elution 75 mM imidazole, 7: Elution 150 mM imidazole

2.5.5. HNL assay

The *Bm*HNL syntgene-1 and *Bm*HNL syntgene-2 purified from affinity chromatography were subjected to HNL assay as per **2.4.14**. **Figure 2.9** shows the absorbance at 280 nm vs. time of *Bm*HNL syntgene-1. Specific activity of purified *Bm*HNL syntgene-1 was found to be 43.2 U/mg while the reported specific activity was 49.3 U/mg by Dadashipour *et al* in 2011 [13]. Specific activity of purified *Bm*HNL syntgene-2 was found to be 17.4 U/mg.



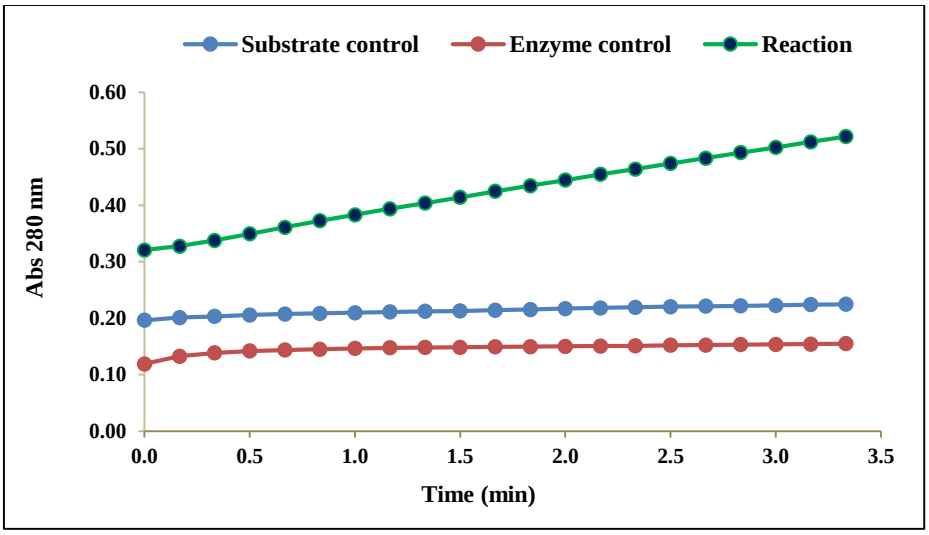


Figure 2.9: HNL assay using purified *Bm*HNL-syntgene-1

Based on the higher HNL activity, *Bm*HNL syntgene-1 in purified or crude form was used in the biocatalytic reactions of all further studies.

2.6. Discussion

Synthetic genes of *Bm*HNL wild type and its version with codon optimized for *E. coli* expression were ordered from GeneScript, USA. Both the synthetic genes were subcloned into pET28a and pCold1 vector after restriction digestion of the vector and insert. After ligation of the digested vector and insert, the ligated product was transformed into *E.coli* DH5*α* cells. The ligated products were confirmed by amplifying the cloned gene using transformed *E.coli* DH5*α* cell suspension as a template in a colony PCR. The subcloning was further confirmed by DNA sequencing of the plasmids i.e. pET28a-syntgene-1 and syntgene-2 and pCold1-syntgene-1 and syntgene-2. Expression of both *Bm*HNL syntgene-1 and syntgene-2 was almost negligible in pET28a expression vector. Optimization of pET28a-*Bm*HNL expression in *E.coli* BL21(DE3) with different concentration of inducer did not improve the protein expression. We also studied pET28a-*Bm*HNL expression in *E.coli* BL21 (DE3)pLysS and BL21(DE3) star host cells but the protein expression did not

improve in the later case also. *Bm*HNL expressed very well in pCold1 expression vector at 18 °C, 200 rpm for 24 h incubation as reported earlier [13]. Further *Bm*HNL protein was subjected for one-step affinity chromatography purification using Ni-NTA agarose resin. The purified protein was ultracentrifuged using 10 kDa MWCO Amicon tubes and concentrated protein was quantified in Nanodrop. The concentrations of purified *Bm*HNL syntgene-1 and syntgene-2 were 7.5 and 2.3 mg/mL. Specific activity of purified *Bm*HNL syntgene-1 and 2 were 43.2 and 17.4 U/mg respectively.

Dadashipour *et al* purified *Bm*HNL form leaves of *Baliospermum montanum* by ammonium sulfate precipitation followed by different column chromatography such as DEAE-Toyopearl 650M, Butyl-Toyopearl 650M, MonoQHR10/10, and Phenyl-SuperoseHR5/5. The purified fraction showed 28 U/mg activity towards synthesis of (*S*)-mandelonitrile. Further, they isolated mRNA from leaves of *Baliospermum montanum* and synthesized cDNA. They cloned the *Bm*HNL gene into expression vectors such as pRSET-B, pCold1 and pT7 Blue T-vector and transformed into various *E.coli* strains such as M109, BL21 (DE3), BL21 (DE3) codon plus RIL, and BL21 (DE3) pLysS. They observed the protein majorly as inclusion bodies in all strains except *E.coli* BL21 (DE3). In the later case, low level of *Bm*HNL soluble protein was obtained in both expression vectors i.e. pRSET-B and pCold1-*Bm*HNL. In case of pRSET-*Bm*HNL, activity of the the enzyme for synthesis of (*S*)-mandelonitrile was reported as 31 U/L of culture while pCold1-*Bm*HNL showed 175 U/L of culture. They reported specific activity of purified *Bm*HNL from pCold1-*Bm*HNL as 49.3 U/mg in mandelonitrile cleavage assay while it was 52 U/mg in synthesis of (*S*)-mandelonitrile [13]. Nakano *et al* expressed pCold1-*Bm*HNL in *E.coli*

BL21 (DE3) and purified by affinity column chromatography using Ni-Sepharose Fast Flow [19]. The purified protein was concentrated with Amicon Ultra to 15-30 mg/mL.

2.7. Conclusions

*Bm*HNL was successfully subcloned into pET28a and pCold1 expression vectors. The protein was expressed and purified by affinity column chromatography. Subsequently, the specific activity of purified protein was calculated using mandelonitrile cleavage assay.

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Chapter 3A

Synthesis of racemic cyanohydrins

3A.1. Introduction

Chiral cyanohydrins are key building blocks in the synthesis of many biologically active compounds such as α-hydroxy aldehydes, *β*-amino alcohols, *α*-azido nitriles, *β*-hydroxy aminoacids, and *α*-hydroxyacids. Synthesis of chiral cyanohydrins is reported using both chemical and biocatalytic methods. However, biocatalytic synthesis has advantages over chemical catalyst based synthesis i.e. high regio- and stereoselectivity, mild reaction conditions, biodegradable catalyst, etc. Biocatalytic synthesis of chiral cyanohydrins mainly involves two methods. They are, lipase/esterase catalyzed kinetic resolution of racemic cyanohydrins and HNL catalyzed nucleophilic addition of cyanide to a prochiral carbonyl center. Given the importance of chiral cyanohydrins, *Bm*HNL catalyzed synthesis of enantiopure cyanohydrins is the focus of this thesis. In order to analyze and characterize the biocatalytically synthesized cyanohydrins, we aimed to prepare racemic cyanohydrins. These racemic compounds were used as internal standards to confirm the biocatalytically synthesized chiral cyanohydrins and also to assign their absolute configuration by chiral HPLC. Several chemical methods have been reported in the literature for the synthesis of racemic cyanohydrins [1]. HCN, KCN, NaCN, TMSCN, and acetone cyanohydrins are commonly used as cyanide donor in the preparation of racemic cyanohydrins. We have synthesized nineteen racemic cyanohydrins using three different methods. The prepared racemic cyanohydrins were characterized by ^{1}H and ^{13}C NMR. HPLC chiral resolution of all these compounds was also carried out prior to use them as internals in biocatalytic study.

This chapter describes the detail of synthesis and characterization of the racemic cyanohydrins.

3A.2. Objectives of the present study

The major objectives for the present work are:

1. Synthesis of a series of racemic cyanohydrins to be used as internal standards to analyze the biocatalytically produced chiral cyanohydrins **(Figure 3A.1 and Table 3A.1)**.
2. Characterization of synthesized cyanohydrins by ^{1}H and ^{13}C NMR.

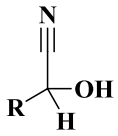


Figure: 3A.1

Table 3A.1: Racemic cyanohydrins used in the present study

S. No.	R	S. No.	R
1	2,4-di $MeOC_6H_3$	10	3-$PhO-C_6H_4$
2	2,3,4-tri $MeOC_6H_2$	11	3-$PhCH_2O-C_6H_4$
3	3,4,5-tri $MeOC_6H_2$	12	3-C_5H_4N
4	2-Naphthyl	13	3,5-di $MeOC_6H_3$
5	9-Anthranyl	14	2,5-di $MeOC_6H_3$
6	*trans*-PhCH=CH	15	4-$PhCH_2O-C_6H_4$
7	4-CH_2=CH-CH_2OPh	16	4-BrC_6H_4
8	Ph-CH_2	17	4-OHC_6H_4
9	Ph-CH(CH_3)	18	3-OHC_6H_4

3A.3. Different chemical methods of synthesis of racemic cyanohydrins

Synthesis of racemic cyanohydrins is usually carried out using different cyanide sources. Based on the cyanide sources we can describe racemic cyanohydrins preparation in three categories.

(i). KCN as cyanide donor

(ii). TMSCN as cyanide donor

(iii). Acetone cyanohydrin as cyanide donor

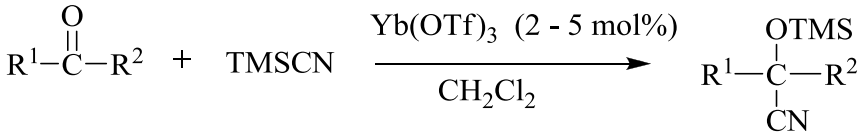
3A.3.1. Synthesis of racemic cyanohydrins using KCN

Smitskamp-Wilms *et al* prepared racemic cyanohydrins by addition of 3 equivalents of KCN pre-dissolved in water, to 1 equivalent of aldehyde dissolved in acetic acid and the reaction was carried out at room temperature [2]. This method has produced racemic cyanohydrins in >90% yield. Danieli *et al* synthesized racemic cyanohydrins of 2-phenylpropionaldehyde and 3-phenylbutyraldehyde using NaCN as a cyanide source. They carried out this reaction at room temperature for 1 h that resulted in 78% yield [3]. Gerrits *et al* followed a modified protocol of Smitskamp-Wilms *et al* and Danieli *et al* to prepare racemic cyanohydrins as it resulted in improved yield. They took 10 g of NaCN dissolved in water and adjusted its pH to 5.5 by addition of citric acid, followed by extraction with TBME. They added this TBME dissolved HCN into a mixture of 0.66 to 47.5 mmol of aldehyde, and 60 mL of 0.1 M citrate-phosphate buffer pH 6.8 to prepare racemic cyanohydrins in 90-99.8% conversion in 5-72 h [4]. Nanda *et al* followed a similar protocol reported by Gerrits *et al* to prepare a series of cyanohydrins [5]. Salama *et al* used tetrachlorosilane (TCS) and potassium cyanide (KCN) in the preparation of ketone cyanohydrins at 60-70 °C. It produced racemic cyanohydrins with 68-95% yield in 5-22 h

[6]. Avi *et al* synthesized racemic ketone cyanohydrins by addition of 1 equivalent of ketone pre-dissolved in *tert*-butyl methyl ether (TBME) and 2 equivalent of HCN with weakly basic ion-exchange resin (Amberlyst A-21). The reaction was carried out at room temperature with stirring for 5-24 h [7].

3A.3.2. Synthesis of racemic cyanohydrins by TMSCN

Racemic cyanohydrin synthesis using TMSCN as cyanide source is usually carried out in presence of Lewis acids, Lewis base, salts, organic catalyst and metal complexes as catalyst. Evans *et al* investigated Lewis acids catalyzed the synthesis of cyanohydrin from *n*-hexanal by comparing two Lewis acids ZnI_2 and KCN-18-crown-6. The latter case has resulted in cyanohydrin with 76% yield in 16 h [8]. Gassman and Talley prepared ketone cyanohydrins using TMSCN in presence of catalytic amount of ZnI_2 that produced in 89-99% yield [9]. Frohlich *et al* prepared ferrocene cyanohydrins by using TMSCN as a cyanide donor in the presence of ZnI_2. The reaction carried out for 1 h at 50 °C resulted in 82-100% yield of racemic ferrocene cyanohydrins [10]. A typical method reported by Gassman and Talley was followed by Csaba Paizs *et al* to prepare novel phenylfuran-based cyanohydrins that resulted in 81-93% yield [11]. Csaba Paizs *et al* synthesized phenothiazine-based cyanohydrins by using a known protocol described by Gassman and Talley. The trimethylsilyl cyanohydrins were cleaved by addition of Dowex 50WX8 which is a strong acidic ion exchange resin instead of HCl. This process resulted in 90-94% yield of cyanohydrins [12]. Yang and Wang reported a method of cyanohydrin synthesis using TMSCN with $Yb(OTf)_3$ as Lewis acid (**Scheme 3A.1**) [13] while Saravanan *et al* reported the synthesis of cyanohydrins by using another Lewis acid $Cu(OTf)_2$. They reported 70-80% yield of racemic cyanohydrins from aldehydes in 2-3 h [14].



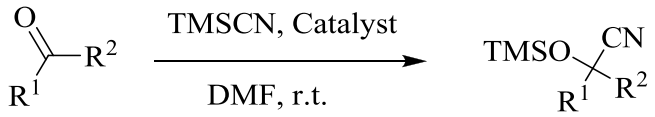
Scheme 3A.1: Synthesis of silyated cyanohydrin in the presence of Lewis acid $Yb(OTf)_3$

Iwanami *et al* reported a novel method of one-pot synthesis of cyanohydrin esters by addition of TMSCN to carbonyl compounds in the presence of 0.05 mol% of $FeCl_3$ [15]. Cyanohydrin esters were obtained with 56-94% yield in 3-12 h. De and Gibbs reported a method for synthesis of racemic cyanohydrins by using 1 mol% of vanadyl triflate as a catalyst and TMSCN. This method has resulted in 79-93% yield of cyanohydrins in 1-3 h [16]. Song *et al* reported preparation of cyanohydrins by addition of TMSCN to carbonyl compound in the presence of *N*-heterocyclic carbenes as catalyst, which resulted in 79-95% yield of corresponding racemic cyanohydrins in 10 min [17]. A nonionic strong base $P(RNCH_2CH_2)N$ (R = Me, *i*-Pr) has been used by Fetterly and Verkade for the synthesis of cyanohydrins. The catalyst proazaphosphatrane produced trialkylsilylation of carbonyl compounds with the best yield i.e. 89-99% in 0.5-2 h [18].

A few reports are also available in which uncatalyzed cyanation of carbonyl compounds has been reported. Manju and Trehan reported the addition of TMSCN to aldehydes in acetonitrile without any catalyst at 20-85 ºC which resulted in 33-95% yield of the racemic TMS-cyanohydrins in 3-18 h (**Scheme 3A.2**) [19].

Scheme 3A.2: Uncatalyzed addition of TMSCN to aldehydes

Kobayashi *et al* reported the synthesis of silylated cyanohydrins by addition of TMSCN to carbonyl compounds in the presence of Lewis bases such as amines, phosphines, arsine, etc at 0 °C [20]. Surya Prakash *et al* reported cyanosilylation of carbonyl compounds in DMF without the use of catalyst. They observed increased yield with nucleophilic catalysts such as carbonates and phosphates. The cyanohydrins were obtained in 77-90% yield without catalyst while in the presence of K_2CO_3, 80-96% yield of product was obtained in 5-150 min (**Scheme 3A.3**) [21].

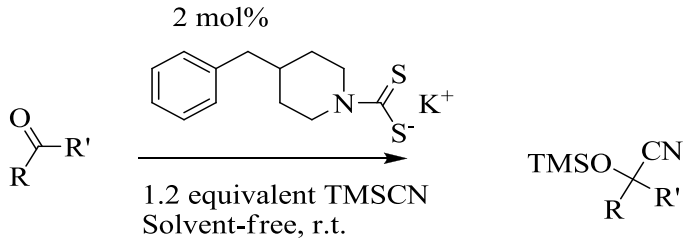


R^1 = alkyl, aryl; R^2 = H

Scheme 3A.3: Cyanosilylation of aldehydes in DMF

Kumaraswamy and Ankamma reported a method of preparation of protected racemic cyanohydrins without Lewis acid [22]. They have carried out the synthesis of racemic cyanohydrins in solvent polyethylene glycol (PEG-400) for 15 h that produced in 75-95% yield of cyanohydrins. Dekamin *et al* used potassium 4-benzylpiperidinedithiocarbamate (PBPDC) catalyzed the addition of TMSCN to various carbonyl compounds (**Scheme 3A.4**) that synthesized the corresponding TMS-cyanohydrins in excellent yield i.e. 62-100% in 5-240 min [23].

Kurono *et al* reported an efficient method for cyanosilylation of aldehydes and ketones in the presence of LiCl [24]. The resulted cyanohydrins from aldehydes were obtained with 90-99% yield in 0.2-1 h only. This method was efficient in comparison to others as the reaction was carried out in the absence of any solvent.



R = Aryl, Alkyl; R' = H or Alkyl

Scheme 3A.4: Synthesis of silyated cyanohydrin in presence of PBPDC

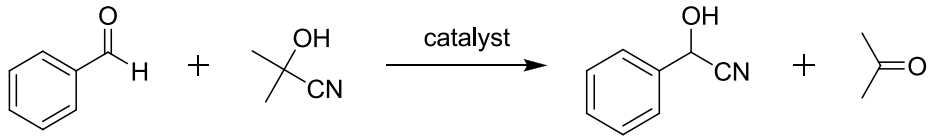
Synthesis of racemic cyanohydrin from ketones using TMSCN was reported by many research groups. Wang *et al* reported cyanosilylation of various ketones using 1,1,3,3-tetramethylguanidine as catalyst in solvent-free conditions [25] while Kim *et al* used cesium fluoride catalyst for the synthesis of ketone cyanohydrins [26]. The cyanosilylation of ketone using ZnI_2 is usually slow. Greenlee and Hangauer reported rapid addition of TMSCN to ketone by addition of potassium cyanide/18-crown-6 complex as catalyst [27].

3A.3.3. Synthesis of racemic cyanohydrins by acetone cyanohydrin

Acetone cyanohydrin is also an effective source of cyanide in the preparation of racemic cyanohydrins. There are a number of reports available describing racemic cyanohydrins preparation using acetone cyanohydrin as a cyanide source.

Inagaki *et al* prepared racemic cyanohydrins through transhydrocyanation of aldehydes with acetone cyanohydrin in presence of strong anion exchange resin Amberlite IRA904 during one-pot synthesis of chiral cyanohydrin acetate. from aldehydes [28]. This method has resulted in 89-100% conversion of racemic cyanohydrins [28]. Ohno *et al* reported the synthesis of cyanohydrins with acetone cyanohydrins in presence of lanthanoid (III) alkoxide as catalyst (**Scheme 3A.5**). They used several lanthanoid (III) alkoxides such as

La(O*i*-Pr)$_3$, Ce(O*i*-Pr)$_3$, Sm(O*i*-Pr)$_3$, and Yb(O*i*-Pr)$_3$ in the reaction that resulted in 86-91% yield of product in 30 min [1,29].

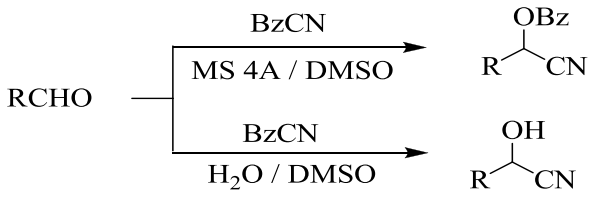


Catalyst: La(O*i*-Pr)$_3$, Ce(O*i*-Pr)$_3$, Sm(O*i*-Pr)$_3$, Yb(O*i*-Pr)$_3$

Scheme 3A.5: Synthesis of racemic mandelonitrile using acetone cyanohydrin in presence of lanthanoid (III) alkoxide as catalyst

3A.3.4. Synthesis of racemic cyanohydrins using other cyanide sources

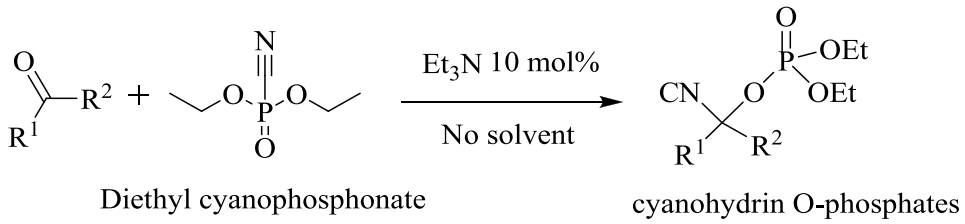
Watahiki *et al* reported a method of cyanohydrin synthesis without the use of any catalyst [30]. They reported the cyanobenzoylation and hydrocyanation of aldehydes without catalyst (**Scheme 3A.6**). The preparation of cyanohydrin esters was carried out by the addition of benzoyl cyanide in the presence of molecular sieve (MS 4A) at room temperature [30].



Scheme 3A.6: Synthesis of racemic protected cyanohydrins and free cyanohydrins without catalyst

Iwanami *et al* developed a method for preparation of cyanohydrin carbonates from various aldehydes [31]. The synthesis of cyanohydrin carbonates was carried out by the addition of cyanoformate in the presence of molecular sieve (MS 4A) which resulted in 62-97%

yield in 1-120 h [31]. Baeza *et al* reported a method to synthesize cyanohydrin *O*-phosphates by triethylamine-catalyzed addition of diethyl cyanophosphonate onto aldehydes and ketones in a solvent-free condition. The resulted cyanohydrins were obtained with 91-98% yield in 5 min (**Scheme 3A.7**) [32].



Scheme 3A.7: Synthesis of racemic *O*-phosphates cyanohydrin in a solvent-free medium

In this chapter, we synthesized 18 racemic cyanohydrins by using three different methods. In first method, we used KCN as cyanide donor to synthesize six racemic cyanohydrins. In the second method, TMSCN was used to synthesize six different racemic cyanohydrins and in the third acetone cyanohydrin was used as cyanide donor for the synthesis of six racemic cyanohydrins.

3A.4. Materials and methods

3A.4.1. Chemicals: Organic solvents such as hexane, ethyl acetate, dichloromethane, diethyl ether were purchased from Finar limited, India. Anhydrous sodium sulfate was purchased from AVRA synthesis Pvt. Ltd., India. HPLC grade solvents hexane and isopropanol were purchased from Rankem Pvt. Ltd., India. All aldehydes were synthesis grade. Phenylacetaldehyde, 2-phenylpropionaldehyde, 3-phenoxybenzaldehyde and 4-benzyloxybenzaldehyde were purchased from Alfa Aesar. 3-benzyloxybenzaldehyde, 2-naphthaldehyde, 2,5-dimethoxybenzaldehyde, and 3,5-dimethoxybenzaldehyde were purchased from sigma Aldrich, USA. 2,4-Dimethoxybenzaldehyde, 2,3,4-

trimethoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, *trans*-cinnamaldehyde, 4-allyloxybenzaldehyde, 3-pyridinecarboxaldehyde, 3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde were purchased from AVRA synthesis Pvt. Ltd., India. 9-Anthraldehyde and 4-bromobenzaldehyde were purchased from TCI chemicals Pvt. Ltd., India. Column chromatography was carried out using silica gel (100-200 mesh) procured from Sisco Research Laboratories (P) Ltd (SRL). Trimethylsilyl cyanide (TMSCN) was obtained from AVRA and KCN was taken from Department of Biochemistry, UoH.

All racemic cyanohydrins synthesized were characterized by ^{1}H and ^{13}C NMR spectroscopy (BRUKER 400 and 100.5 MHz NMR) with chloroform-d as solvent and tetramethylsilane as reference.

3A.4.2. Synthesis of racemic cyanohydrins

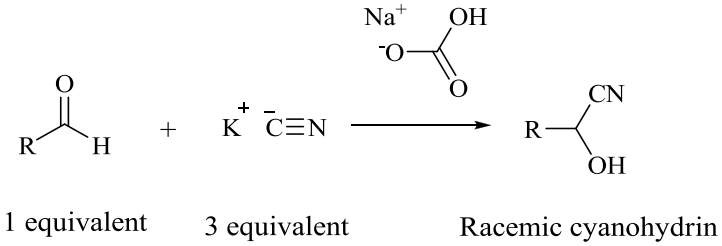
Synthesis of racemic cyanohydrins was performed using the following three different methods where the cyanide source (KCN, TMSCN and acetone cyanohydrin) is mainly varied.

3A.4.2.1. Synthesis of racemic cyanohydrins by KCN

Six racemic cyanohydrins i.e. cyanohydrins of 2,4-dimethoxybenzaldehyde, 2,4,5-trimethoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde, 2-naphthaldehyde, *trans*-cinnamaldehyde, and 4-allyloxybenzaldehyde were prepared using KCN as a cyanide donor (**Scheme 3A.8**) [2].

Five mmol of aldehyde was dissolved in 10 mL of glacial acetic acid. To this mixture, KCN solution which was prepared separately by dissolving 1.0 g (3 equivalent, 15 mmol) of it in 10 mL of water, was added. The whole reaction was stirred at 4 $^{\circ}$C. The conversion was monitored by TLC. After completion of the reaction, the reaction mixture was

neutralized by addition of saturated $NaHCO_3$ solution. The reaction mixture was extracted by diethyl ether with consecutive washes of water and saturated NaCl solution. The reaction mixture was dried over anhydrous Na_2SO_4 followed by evaporation of the solvent under reduced pressure. The reaction mixture was purified by flash chromatography with eluent hexane/ethyl acetate.



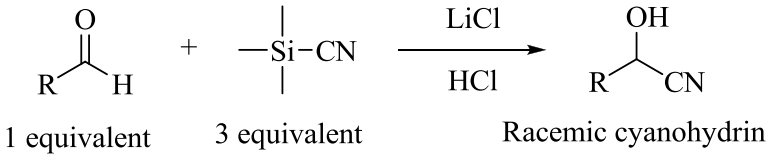
Scheme 3A.8: Synthesis of racemic cyanohydrins using potassium cyanide

3A.4.2.2. Synthesis of racemic cyanohydrins by TMSCN

Racemic cyanohydrins of 9-anthraldehyde, 2-phenylacetaldehyde, 2-phenylpropionaldehyde, 3-phenoxybenzaldehyde, 3-benzyloxybenzaldehyde and 3-pyridinecarboxaldehyde were prepared by using trimethylsilylcyanide (TMSCN) as a cyanide source and lithium chloride (LiCl) as a catalyst in solvent free medium (**Scheme 3A.9**) [24,33]

Ten mmol of aldehyde was taken in a 50 mL round bottom flask followed by addition of 3 equivalents of TMSCN at low temperature, to avoid vigorous reaction. To this mixture, 2-3 drops of 100 mM LiCl pre-dissolved in THF was added. Reaction was continued by stirring at 25 °C and monitored by TLC. After completion of the reaction, 5 mL of ethyl acetate was added, subsequently, the TMS group was cleaved by adding 1 N HCl with vigorous stirring at 25 °C. The reaction mixture was extracted using ethyl acetate with

repeated washes of saturated $NaHCO_3$ and brine. The reaction mixture was dried over anhydrous Na_2SO_4, evaporated the solvent under reduced pressure and products purified by flash chromatography.



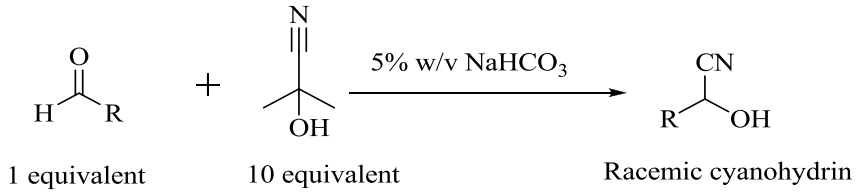
Scheme 3A.9: Synthesis of racemic cyanohydrins using trimethylsilyl cyanide and catalyst lithium chloride.

3A.4.2.3. Synthesis of racemic cyanohydrins by acetone cyanohydrin

Six aromatic aldehydes e.g. 3,5-dimethoxybenzaldehyde, 2,5-dimethoxybenzaldehyde, 4-benzyloxybenzaldehyde, 4-bromobenzaldehyde, 3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde were converted into corresponding racemic cyanohydrins using acetone cyanohydrin as a cyanide donor (**Scheme 3A.10**) using a modified protocol mentioned by Galletti *et al* [34]. The racemic cyanohydrins were used as analytical HPLC standards.

To a 100 mL round bottom flask containing 10 mL of 5% w/v aqueous $NaHCO_3$ solution, 10 mmol of aldehyde and its ten equivalents (100 mmol) of acetone cyanohydrin were added. In order to minimize the dissociation of the cyanohydrins formed, 20 mL of DCM was also added to the above mixture. The reaction mixture was stirred at 25 °C and monitored by TLC. The reaction was stopped by acidifying the mixture with 1N HCl after 1 h. Extraction was carried out in DCM with consecutive washes of water. The reaction

mixture was dried over anhydrous Na_2SO_4 followed by evaporation of the solvent under reduced pressure and purified by flash chromatography.



Scheme 3A.10: Synthesis of racemic cyanohydrins using acetone cyanohydrin

3A.5. Results

The synthesis of racemic cyanohydrins which were planned to be used as internal standards in the present work was carried out using three methods as mentioned in the experimental section above. The conversion of aldehydes into racemic cyanohydrins (1-18) was 95-100% while yields were 22 to 76%.

3A.5.1. Synthesis of racemic cyanohydrins using KCN

Synthesis of racemic cyanohydrins using KCN is a commonly used method. Cyanohydrins **1-6** (**Scheme 3A.8** and **Table 3A.2**) were synthesized using the procedure reported by Smitskamp-Wilms *et al* and Danieli *et al* [2,3]. The method involves hydrocyanation of aldehydes in the presence of acidic medium. Cyanohydrin **1** i.e. 2-hydroxy-2-(2,4 dimethoxyphenyl)acetonitrile was synthesized with 95% conversion of product from aldehyde in 2.5 h while the purified product was obtained in 43.65% yield. Similarly, cyanohydrin **2** was synthesized in 12 h that resulted in ~65% yield after purification. Cyanohydrins **3** and **6** were prepared in reaction time of 12 h, with 48.22 and 24.7% yields respectively. Synthesis of **4** and **5** was accomplished in 24 h in 22 and 25% yield respectively.

Table 3A.2: Synthesis of racemic cyanohydrins using KCN

S. No.	R	Time (h)	Yield%
1	2,4-di $MeOC_6H_3$	2.5	43.65%
2	2,3,4-tri $MeOC_6H_2$	12	64.91
3	3,4,5-tri $MeOC_6H_2$	12	48.22
4	2-Naphthyl	24	22
5	*trans*-PhCH=CH	24	25
6	4-CH_2=CH-CH_2OPh	12	24.69

3A.5.2. Synthesis of racemic cyanohydrins using TMSCN

Use of TMSCN in the synthesis of racemic cyanohydrins in the presence of LiCl is an efficient method [24]. We have successfully synthesized cyanohydrins **7-12** using this method (**Scheme 3A.9** and **Table 3A.3**). Cyanohydrins **7** and **12** were obtained in ~24% yields in a short reaction time of 1.5 to 4 h. Other three cyanohydrins **8-10** were obtained in 40-46% yield. While synthesis of **8** was done in only 1 h, **9** and **10** took a longer reaction time of 14 h. Racemic 3-benzyloxy mandelonitrile was synthesized in 68% yield in only 1.5 h.

Table 3A.3: Synthesis of racemic cyanohydrins using TMSCN

S. No.	R	Time (h)	Yield%
7	9-Anthranyl	1.5	23.87
8	Ph-CH_2	1	40
9	Ph-CH(CH_3)	14	45
10	3-PhO-C_6H_4	14	46.01
11	3-PhCH_2O-C_6H_4	1.5	68
12	3-C_5H_4N	4	24

3A.5.3. Synthesis of racemic cyanohydrins using acetone cyanohydrin

Our third method used to synthesize racemic cyanohydrins was with acetone cyanohydrin. We used a modified protocol mentioned by Galletti *et al* [34]. Synthesis of cyanohydrins **13-18** was achieved using this method (**Scheme 3A.10** and **Table 3A.4**) in 1-5 h. Cyanohydrin **15** was obtained in 20% yield in only 1 h and the cyanohydrins **13** was obtained in 41.5% yield in 5 h. Racemic cyanohydrin **14** was synthesized in ~51% yield in 5 h. Synthesis of three other products **16-18** was achieved in 58-76% yield in 1-2 h reaction time.

Table 3A.4: Synthesis of racemic cyanohydrins using acetone cyanohydrins

S. No.	R	Time (h)	Yield%
13	3,5-di MeOC_6H_3	5	41.5
14	2,5-di MeOC_6H_3	5	50.82
15	4-PhCH_2O-C_6H_4	1	20
16	4-BrC_6H_4	1	76.35
17	3-OHC_6H_4	2	58
18	4-OHC_6H_4	2	68

3A.5.4. Spectroscopic characterization of cyanohydrins 1-18

Spectroscopic data of compound matches with the reported data in literature.

1. **2-hydroxy-2-(2,4-dimethoxyphenyl)acetonitrile** [5]

   Yield: 43.65%

   ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 – 7.24 (m, 1H), 6.56 – 6.43 (m, 2H), 5.54 (s, 1H), 3.9 (s, 3H), 3.83 (s, 3H).

   ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 162.23, 157.99, 129.14, 119.08, 116.61, 104.75, 99.15, 60.05, 55.77, 55.53.

2. **2-hydroxy-2-(2,3,4-trimethoxyphenyl)acetonitrile** [5]

   Yield: 64.91%

   ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (d, *J* = 8.6 Hz, 1H), 6.68 (d, *J* = 8.0 Hz, 1H), 5.46 (s, 1H), 4.09 (s, 3H), 3.89 (d, *J* = 2.8 Hz, 6H).

   ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 155.45, 151.63, 141.95, 122.80, 121.84, 119.42, 106.99, 61.44, 60.86, 60.75, 56.11.

3. **2-hydroxy-2-(3,4,5-trimethoxyphenyl)acetonitrile** [5]

   Yield: 48.22%

   ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.70 (s, 2H), 5.46 (s, 1H), 3.86 (s, 6H), 3.83 (s, 3H).

   ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 153.50, 138.48, 131.25, 118.94, 103.70, 63.53, 60.90, 56.19.

4. **2-hydroxy-2-(naphthalen-2-yl)acetonitrile** [21,33,35,36]

   Yield: 22%

   ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (s, 1H), 7.96 – 7.91 (m, 3H), 7.62-7.59 (m, 3H), 5.73 (s, 1H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 133.66, 132.95, 132.61, 129.36, 128.35, 127.83, 127.23, 126.96, 126.16, 123.73, 118.96, 63.76.

5. **(*E*)-2-hydroxy-4-phenylbut-3-enenitrile** [4]

   Yield: 25%

   ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45-7.29 (m, 5H), 6.93 (d, *J* = 15.9 Hz, 1H), 6.28 (dd, *J* = 15.9, 5.9 Hz, 1H), 5.18 (d, *J* = 5.5 Hz, 1H).

   ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 135.14, 134.80, 129.06, 128.84, 127.09, 122.39, 118.37, 61.81.

6. **2-(4-(allyloxy)phenyl)-2-hydroxyacetonitrile** [5]

   Yield: 24.69%

   ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.45 (d, *J* = 8.0 Hz, 2H), 6.98 (d, *J* = 8.7 Hz, 2H), 6.07 (m, 1H), 5.46 (m, 2H), 5.33 (s, 1H), 4.58 (m, 2H).

   ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 159.57, 132.74, 128.31, 127.77, 119.15, 118.10, 115.25, 68.91, 63.11

7. **2-(anthracen-9-yl)-2-hydroxyacetonitrile** [21]

   Yield: 23.87%

   ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (s, 1H), 8.50 (d, *J* = 9.0 Hz, 2H), 8.09-8.03 (m, 2H), 7.67 – 7.54 (m, 4H), 6.99 (s, 1H).

   ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 131.45, 130.88, 129.55, 129.44, 127.51, 125.36, 125.06, 123.17, 119.56, 60.55.

8. **2-hydroxy-3-phenylpropanenitrile** [33]

   Yield: 40%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41-7.29 (m, 5H), 4.65-4.62 (m, 1H), 3.13 (d, *J* = 6.5 Hz, 2H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 133.97, 129.71, 128.92, 127.82, 119.47, 62.19, 41.31.

9. **2-hydroxy-3-phenylbutanenitrile** [3]

Yield: 45%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.29 (m, 5H), 4.50 (d, *J* = 6.5 Hz, 1H), 3.22 – 3.14 (m, 1H), 1.49-1.47 (d, *J* = 7.3 Hz, 3H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 139.56, 128.89, 128.21, 127.98, 127.96, 127.81, 119.02, 66.45, 44.07, 16.16.

10. **2-hydroxy-2-(3-phenoxyphenyl)acetonitrile** [5]

Yield: 46.01%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.25 (m, 3H), 7.20 (m, 1H), 7.19 – 7.16 (m, 2H), 7.07 – 7.05 (m, 3H), 5.48 (s, 1H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 158.15, 156.40, 137.14, 130.63, 130.02, 124.01, 121.05, 119.76, 119.64, 119.30, 119.01, 118.74, 116.71, 63.18.

11. **2-hydroxy-2-(3-benzyloxyphenyl)acetonitrile**

Yield: 68%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47-7.35 (m, 7H), 7.04 (d, *J* = 8.7 Hz, 2H), 5.46 (s, 1H), 5.11 (s, 2H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 159.19, 136.84, 136.49, 130.29, 128.67, 128.18, 127.63, 122.69, 119.14, 116.18, 113.14, 70.18, 63.09.

12. **2-Hydroxy-2-(pyridin-3-yl)acetonitrile** [37]

Yield: 24%

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (s, 1H), 8.55 (d, *J* = 1.1 Hz, 1H), 7.87 (d, *J* = 7.9 Hz, 1H), 7.39-7.35 (dd, *J* = 7.9, 5.0 Hz, 1H), 5.26 (s, 1H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 148.28, 147.13, 135.20, 134.88, 123.95, 118.70, 62.63.

13. 2-hydroxy-2-(3,5-dimethoxyphenyl)acetonitrile [5]

Yield: 41.5%

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.62 (d, *J* = 2.2 Hz, 2H), 6.45-6.44 (d, *J* = 2.1 Hz, 1H), 5.41 (s, 1H), 3.77 (s, 6H), 4.38 (broad, s, 1H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 161.14, 137.45, 119.04, 104.61, 101.55, 63.24, 55.53.

14. 2-hydroxy-2-(2,5-dimethoxyphenyl)acetonitrile [5]

Yield: 50.82%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.02 (d, *J* = 2.6 Hz, 1H), 6.94 – 6.88 (m, 2H), 5.59 (s, 1H), 3.89 (s, 3H), 3.79 (s, 3H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 153.79, 150.80, 124.55, 118.83, 115.73, 113.84, 112.38, 60.21, 56.23, 55.89.

15. 2-hydroxy-2-(4-benzyloxyphenyl)acetonitrile [5]

Yield: 20%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.37 (m, 7H), 7.04 (d, *J* = 8.6 Hz, 2H), 5.45 (s, 1H), 5.11 (s, 2H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 159.85, 136.45, 128.71, 128.37, 128.21, 127.82, 127.51, 119.05, 115.48, 70.17, 63.21.

16. 2-hydroxy-2-(4-bromophenyl)acetonitrile [5,38]

Yield: 76.35%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52-7.49 (d, *J* = 12 Hz, 2H), 7.35 – 7.33 (d, *J* = 8.4 Hz, 2H), 5.46 (s, 1H).

^{13}C NMR (100.5 MHz, $CDCl_3$) δ 134.58, 132.11, 128.33, 122.60, 118.73, 65.02.

17. 2-hydroxy-2-(3-hydroxyphenyl)acetonitrile

Yield: 58%

^{13}C NMR (100.5 MHz, DMSO) δ 158.08, 139.00, 130.29, 121.02, 117.26, 116.22, 113.57, 62.18.

18. 2-hydroxy-2-(4-hydroxyphenyl)acetonitrile [4,38]

Yield: 68%

^{1}H NMR (400 MHz, DMSO) δ 7.31 (d, *J* = 8.3 Hz, 2H), 6.83 (d, *J* = 8.3 Hz, 2H), 5.58 (s, 1H), 3.45 (broad, s, 1H).

^{13}C NMR (100.5 MHz, DMSO) δ 158.39, 128.52, 128.12, 124.26, 121.26, 115.85, 62.04.

3A.6. Discussion

The addition of cyanide to carbonyl compounds is one of the most potent methods for the synthesis of cyanohydrins in organic chemistry. Various cyanide sources, such as HCN, NaCN, KCN and different trialkylsilyl cyanides have been reported for the nucleophilic addition of cyanide to carbonyl compounds. We used KCN, TMSCN and acetone cyanohydrin as source of cyanide for the synthesis of a number of racemic cyanohydrins. Six different racemic cyanohydrins were prepared by using cyanide from KCN. The % yield of racemic cyanohydrins synthesized by this method varied from 22 to ~65. Synthesis of 2,4-dimethoxymandelonitrile was achieved in 43.65% yield while in case of 2,3,4-trimethoxymandelonitrile the yield was 64.91%. Further 3,4,5-trimethoxybenzaldehyde, 2-naphthaldehyde and 4-allyloxybenzaldehyde was also

converted into corresponding cyanohydrins with 48.22, 22 and 25.69% yield respectively. Prakash *et al* described the synthesis of 2-hydroxy-2-(naphthalene-2-yl)acetonitrile trimethylsilyl ether [21]. They obtained the product in 80% yield without any catalyst while it was 90% in presence of K_2CO_3. Fetterly and Verkade also reported the synthesis of racemic 2-hydroxy-2-(naphthalene-2-yl)acetonitrile with 99% yield [18].

While we have achieved the synthesis of racemic cyanohydrin of *trans*-cinnamaldehyde in 25% yield after purification, Gerrits *et al* reported 98% conversion and 97% yield of racemic (*E*)-2-hydroxy-4-phenylbut-3-enenitrile in 48 h reaction time [4]. Yang and wang prepared racemic (*E*)-2-hydroxy-4-phenylbut-3-enenitrile by using 5 mol% of $Yb(OTf)_3$ as an efficient catalyst and obtained the product in 81% yield in 2 h [13]. Saravanan *et al* reported the synthesis of the same compound in the presence of $Cu(OTf)_2$ and obtained it in 75% yield in 3 h [14]. Kobayashi *et al* reported 98% yield of racemic (*E*)-2-hydroxy-4-phenylbut-3-enenitrile trimethylsilyl ether [20]. Manju and Trehan reported the addition of trimethylsilyl cyanide to cinnamaldehyde in acetonitrile without the use of a catalyst which resulted in 92% yield [19].

Kurono *et al* reported the synthesis of same cyanohydrin ether using LiCl and obtained 97% yield of it [24] while Evans *et al* reported 99% yield of same cyanohydrin ether [8]. Dekamin *et al* used potassium 4-benzylpiperidinedithiocarbamate (PBPDC) as an effective organocatalyst for efficient addition of trimethylsilyl cyanide to a wide variety of aldehydes and ketones [23]. This addition has afforded corresponding cyanohydrin trimethylsilyl ethers in high to quantitative yields. The reaction was carried out with 2 mol% catalyst at room temperature. The conversion of cinnamaldehyde into (*E*)-2-hydroxy-4-phenylbut-3-enenitrile trimethysilyl ether was 98% in 25 min [23].

Six different cyanohydrins i.e. 2-(anthracen-9-yl)-2-hydroxyacetonitrile, 2-hydroxy-3-phenylpropanitrile, 2-hydroxy-3-phenylbutanenitrile, 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile, 2-hydroxy-2-(3-benzyloxyphenyl)acetonitrile and 2-hydroxy-2-(pyridine-3-yl)acetonitrile were synthesized by TMSCN and LiCl as catalyst. All these cyanohydrins were synthesized according to the mentioned protocol in section **3A.4.2.2**. We attained 23.87% yield in the synthesis of cyanohydrin of 9-anthraldehyde. Surya Prakash *et al* reported 90% yield of racemic 2-hydroxy-2-(anthracen-9-yl)acetonitrile trimethylsilyl ether within 5 min without any catalyst while in presence of 1 mol% K_2CO_3, the yield was 95% [21].

Our synthesis yielded 2-hydroxy-3-phenylpropanitrile in 40% while in case of 2-hydroxy-3-phenylbutanenitrile, the yield was 45%. Danieli *et al* reported the synthesis of racemic 2-hydroxy-3-phenylbutanenitrile using NaCN that resulted in 78% yield [3].

We reported the conversion of 3-phenoxybenzaldehyde into 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile in 46% yield. The conversion of 3-benzyloxybenzaldehyde and 3-pyridinecarboxaldehyde into corresponding cyanohydrins resulted in 68% and 24% yield.

Apart from KCN and TMSCN, we also used acetone cyanohydrin as cyanide source and synthesized six different racemic cyanohydrins. We converted six different aldehydes i.e. 3,5-dimethoxybenzaldehyde, 2,5-dimethoxybenzaldehyde, 4-benzyloxybenzaldehyde, 4-bromobenzaldehyde, 3-hydroxybenzaldehyde, and 4-hydroxybenzaldehyde into corresponding racemic cyanohydrins using acetone cyanohydrins. After purification 2-hydroxy-2-(3,5-dimethoxyphenyl)acetonitrile was obtained in 41.5% yield. 2,5-Dimethoxybenzaldehyde was converted into 2-hydroxy-2-(2,5-

dimethoxyphenyl)acetonitrile with 50.82% yield while the yield of 2-hydroxy-2-(4-benzyloxyphenyl)acetonitrile was 20%.

Racemic 2-hydroxy-2-(4-bromophenyl)acetonitrile was obtained in 76.35% yield after purification. North *et al* described the synthesis of 2-hydroxy-2-(4-bromophenyl)acetonitrile trimethylsilyl ether in presence of 5 mol% $Al(OTf)_3$ or Bu_4NNCS. In the presence of $Al(OTf)_3$, their conversion of racemic 2-hydroxy-2-(4-bromophenyl)acetonitrile trimethylsilyl ether was 68% while it was 100% in presence of Bu_4NNCS [39].

Surya Prakash *et al* reported cyanosilylation of aldehydes in DMF, without using a catalyst [21]. They converted 4-bromobenzaldehyde into 2-hydroxy-2-(4-bromophenyl)acetonitrile trimethylsilyl ether within 150 min and the yield was 80% without any catalyst while Dekamin *et al* reported 100% conversion of 4-bromobenzaldehyde into racemic 2-hydroxy-2-(4-bromophenyl)acetonitrile trimethylsilyl ether in presence of PBPDC (catalyst) within 15 min [23].

We prepared racemic cyanohydrins of 3-hydroxybenzaldehyd and 4-hydroxybenzaldehyde in 58 and 68% yield respectively. Buck *et al* reported the synthesis of racemic 2-hydroxy-2-(3-hydroxyphenyl)acetonitrile and 2-hydroxy-2-(4-hydroxyphenyl)acetonitrile using excess amount of absolute HCN and 1 mol% of catalyst (CaO and $FeCl_3$) [40]. They obtained racemic 2-hydroxy-2-(3-hydroxyphenyl)acetonitrile in 66% yield in 20 h at 20 °C while racemic 2-hydroxy-2-(4-hydroxyphenyl)acetonitrile was obtained in 38% yield in 5 h. Gerrits *et al* synthesized racemic 2-hydroxy-2-(4-hydroxyphenyl)acetonitrile and observed 91% conversion of the product within 24 h [4]

while Ladenburg *et al* reported 90% yield of racemic 2-hydroxy-2-(4-hydroxyphenyl)acetonitrile [41].

3A.7. Conclusion

- We have successfully synthesized 18 racemic cyanohydrins using three different methods and characterized them by ^{1}H and ^{13}C-NMR.

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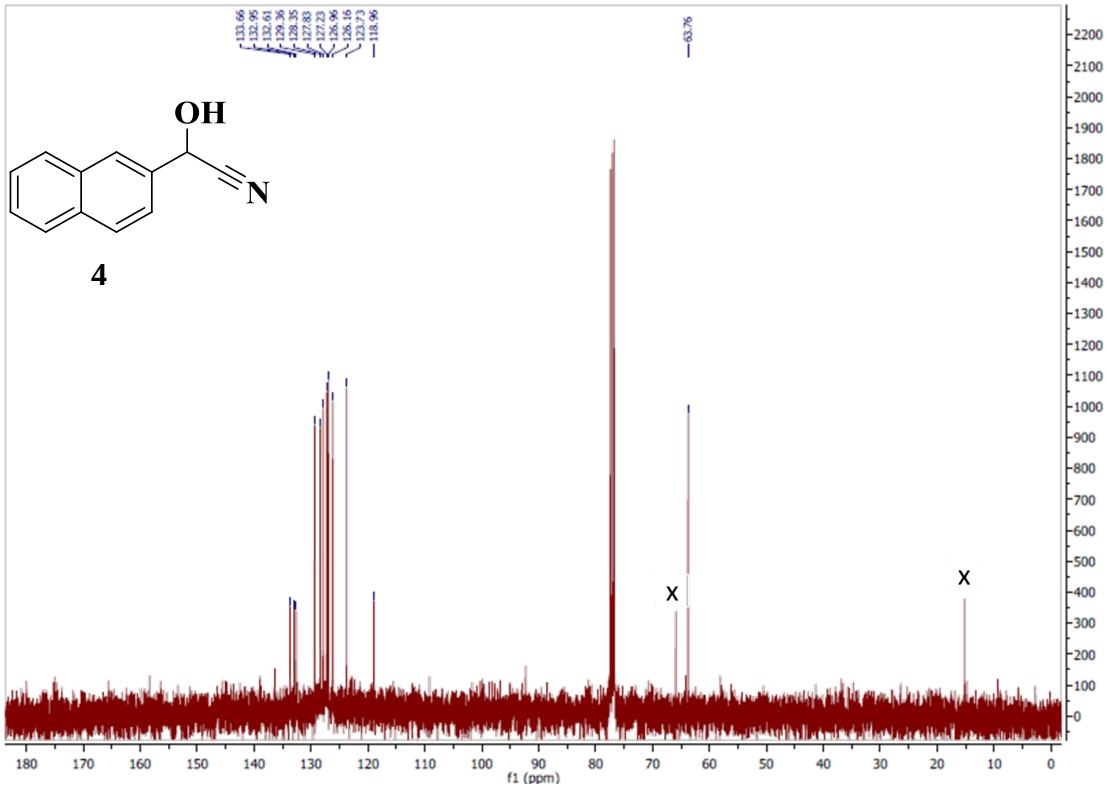


Figure 3A.2: ^{13}C NMR spectrum of 2-hydroxy-2-(naphthalen-2-yl)acetonitrile (**4**) in $CDCl_3$

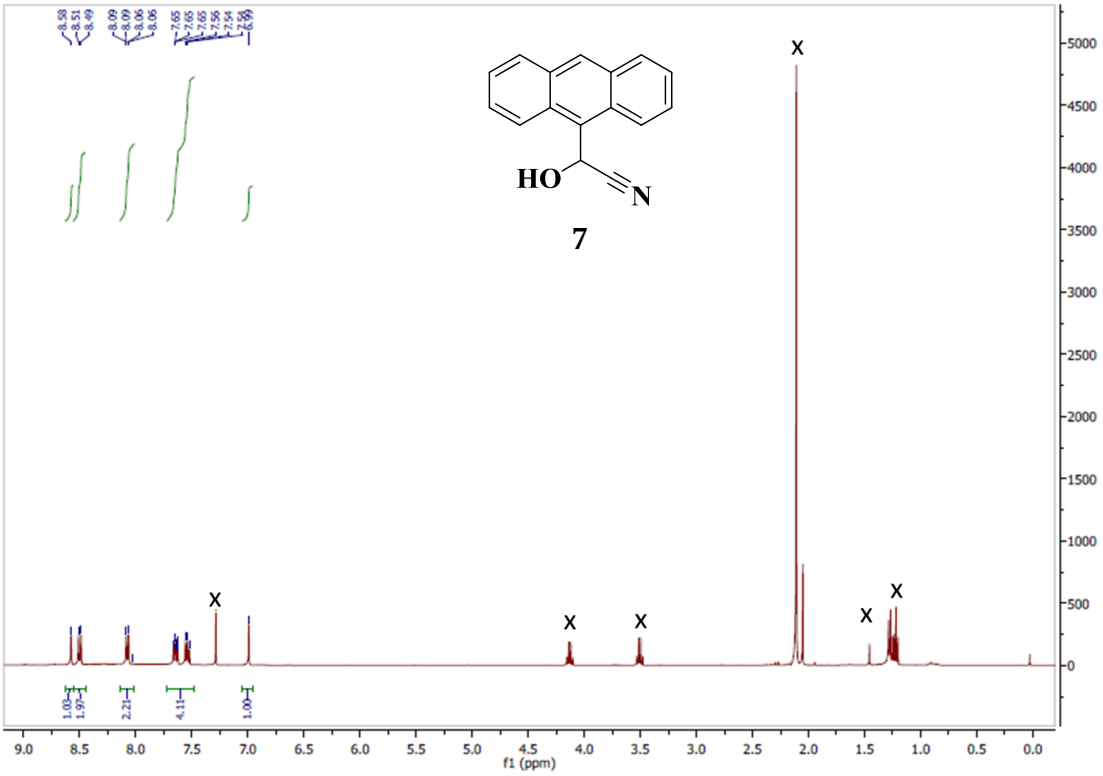


Figure 3A.3: ^{1}H NMR spectrum of 2-(anthracen-9-yl)-2-hydroxyacetonitrile (**7**) in $CDCl_3$

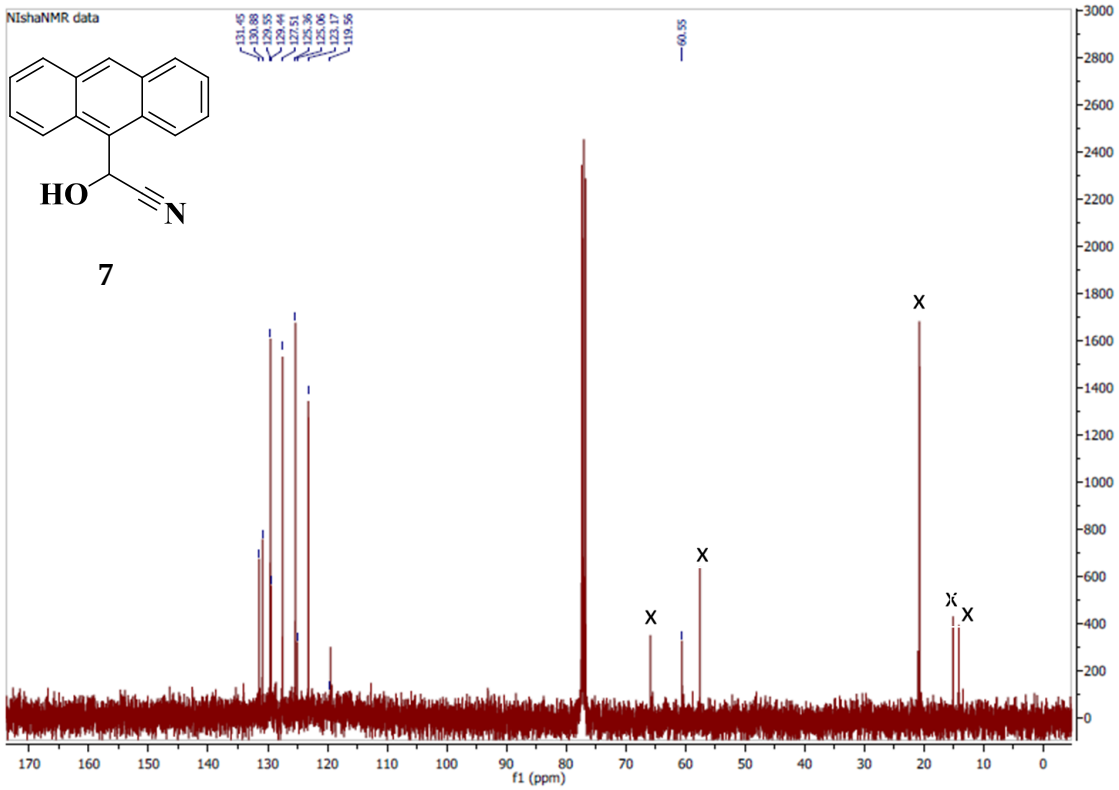


Figure 3A.4: ^{13}C NMR spectrum of 2-(anthracen-9-yl)-2-hydroxyacetonitrile (**7**) in $CDCl_3$

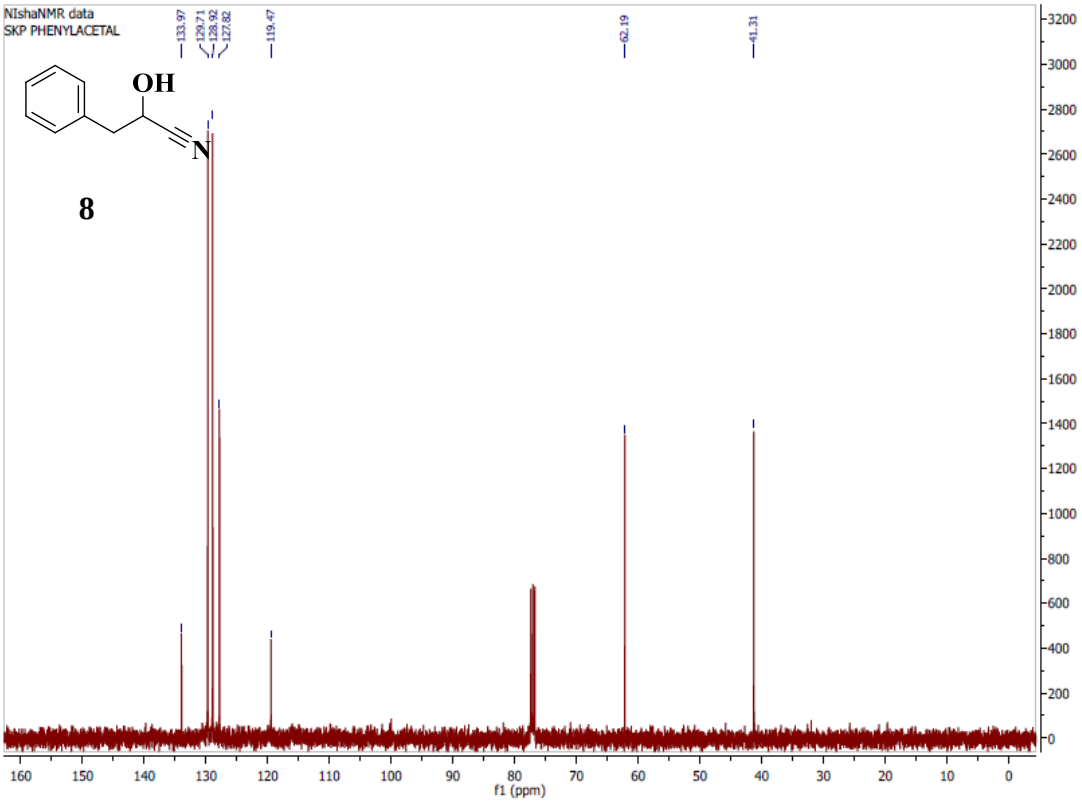


Figure 3A.5: ^{13}C NMR spectrum of 2-hydroxy-3-phenylpropanenitrile (**8**) in $CDCl_3$

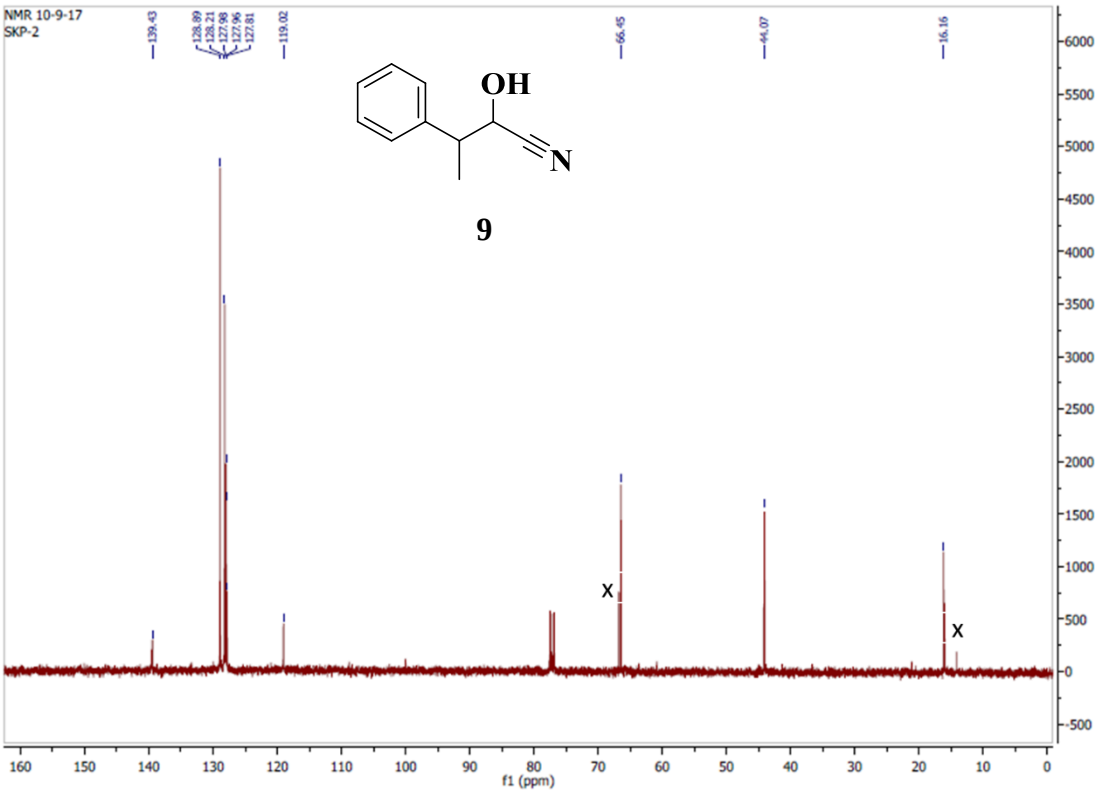


Figure 3A.6: ^{13}C NMR spectrum of 2-hydroxy-3-phenylbutanenitrile (**9**) in $CDCl_3$

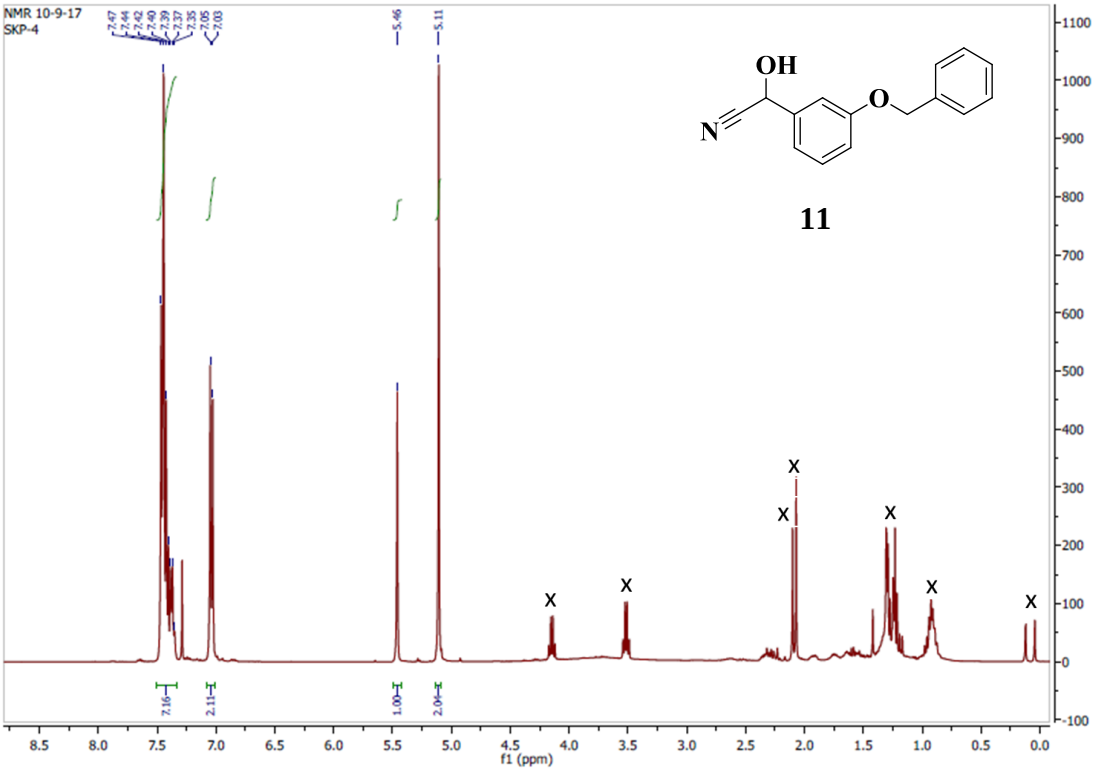


Figure 3A.7: ^{1}H NMR spectrum of 2-hydroxy-2-(3-benzyloxyphenyl)acetonitrile (**11**) in $CDCl_3$

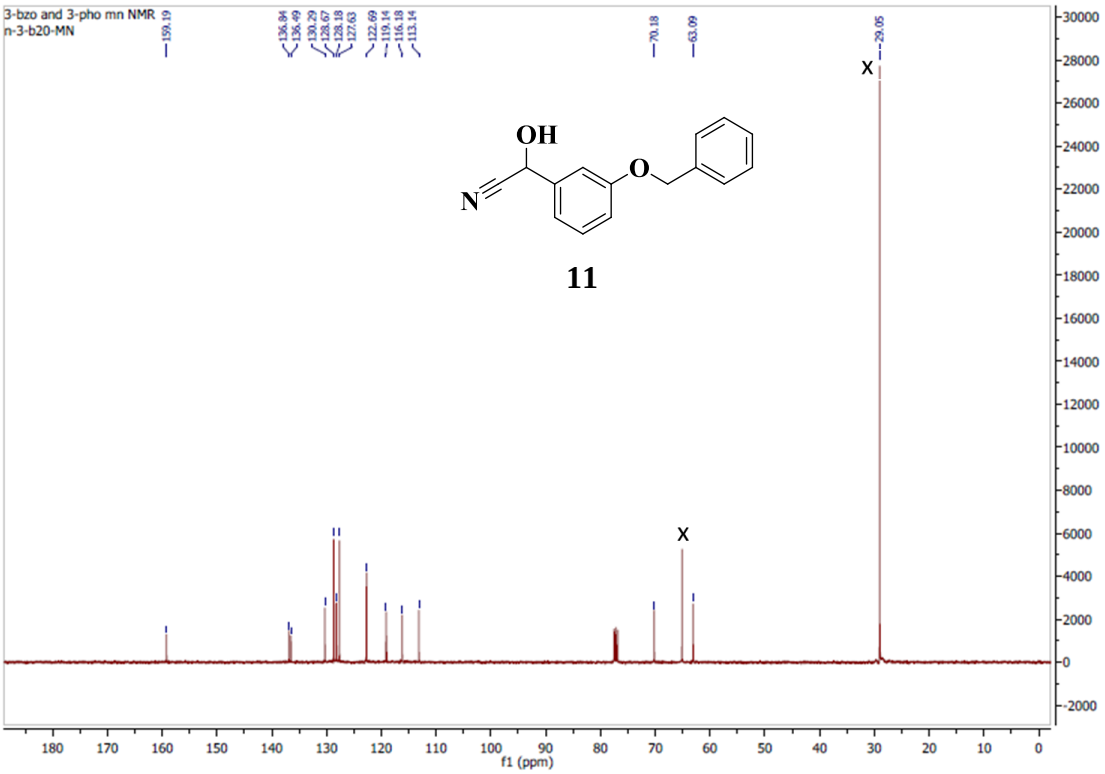


Figure 3A.8: ^{13}C NMR spectrum of 2-hydroxy-2-(3-benzyloxyphenyl)acetonitrile (**11**) in $CDCl_3$

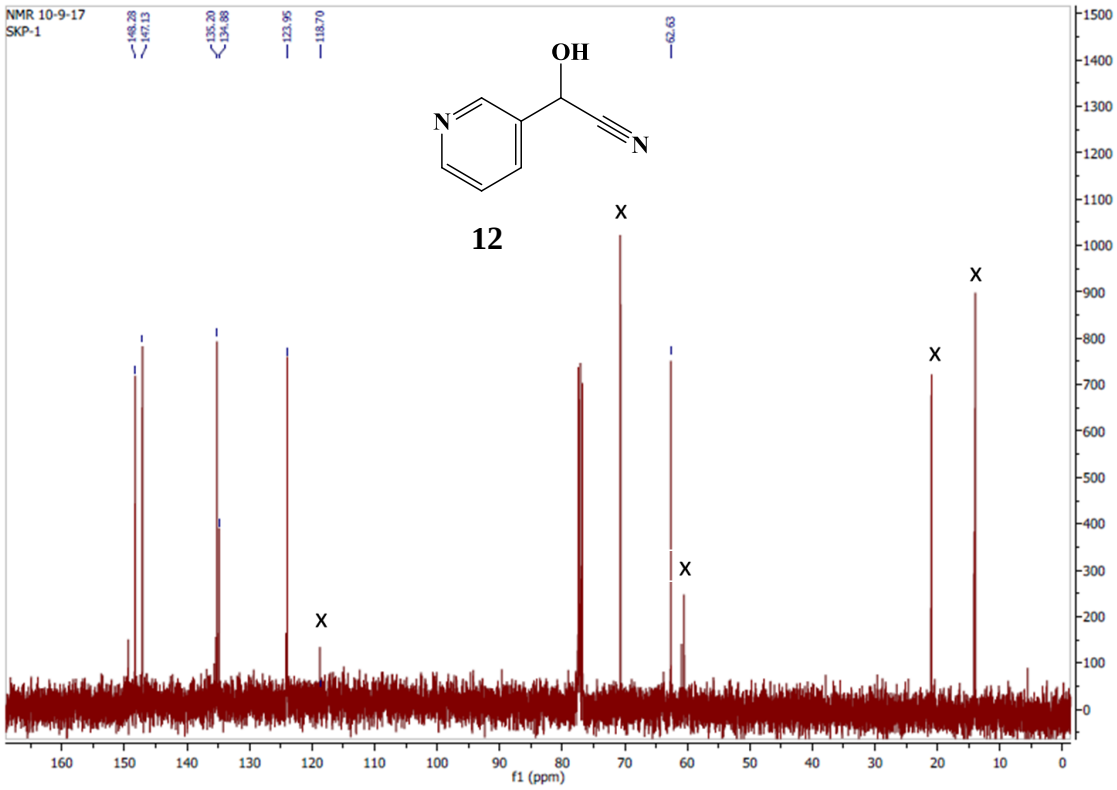


Figure 3A.9: ^{13}C NMR spectrum of 2-hydroxy-2-(pyridin-3-yl)acetonitrile (**12**) in $CDCl_3$

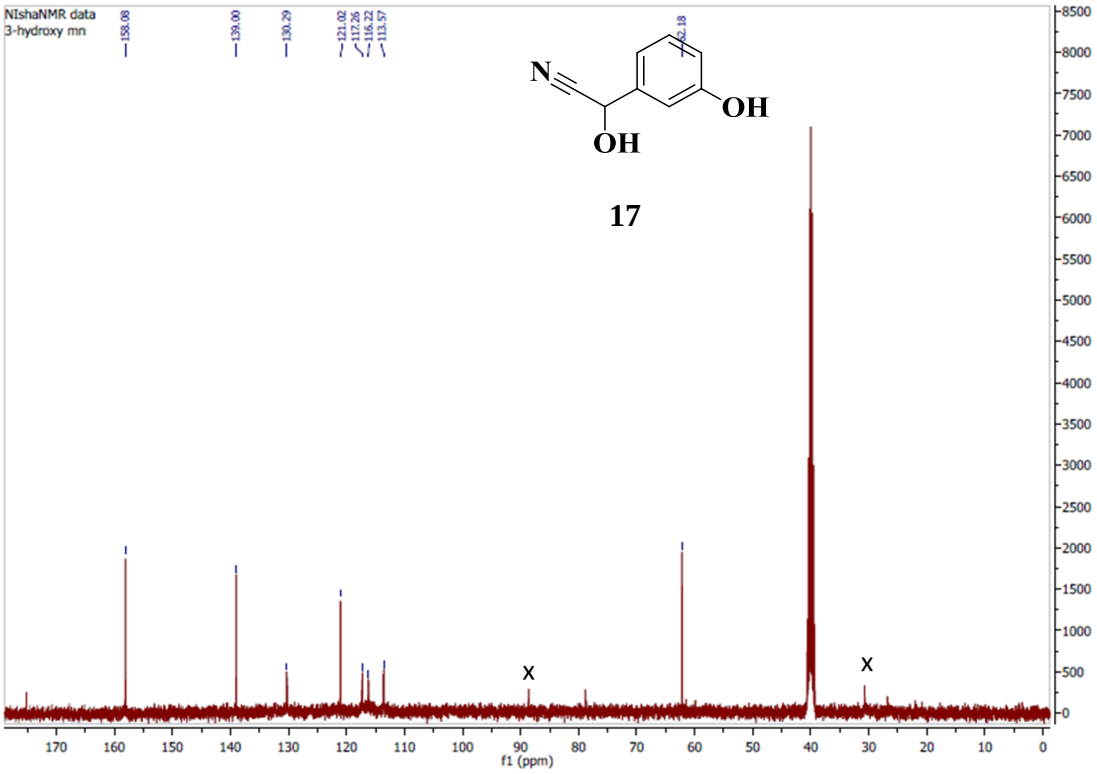


Figure 3A.10: ^{13}C NMR of 2-hydroxy-2-(3-hydroxyphenyl)acetonitrile (**17**) DMSO

Chapter 3B

Chiral resolution of racemic cyanohydrins

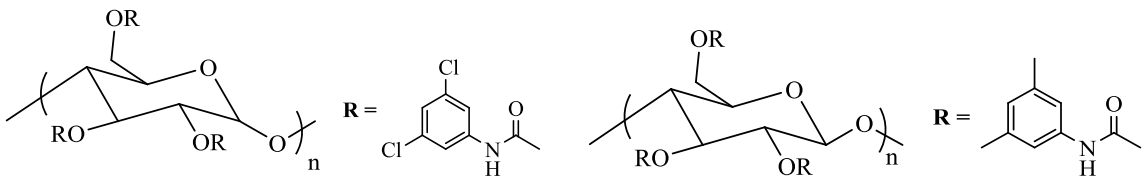
3B.1. Introduction

Importance of chiral cyanohydrins in pharmaceuticals, agrochemicals and fine chemical industry has been discussed earlier. With the significance of chiral cyanohydrins, the method of measurement of their optical purity becomes equally important. The common methods known to determine enantiomeric excess (ee) or optical purity of any chiral molecule are polarimetry, NMR and chromatographic methods. High-performance liquid chromatography (HPLC) and gas chromatography (GC) are the major chromatographic methods widely used for such study. Polarimetry and NMR based determination of optical purity requires more amount of compound and also in purified form, while in case of GC and HPLC small amount of compounds can be analyzed. The later chromatographic methods do not require purification of compounds from the reaction mixture, for their analysis. For analysis by GC, the compounds must be volatile and thermostable. In contrary to GC, HPLC is a better and efficient analytical method to determine ee of product because a broad range of compounds (volatile, non-volatile, low and high molecular weight substrates) can be analyzed in HPLC. In the present thesis, the main focus is to prepare enantiopure cyanohydrins using *Bm*HNL based biocatalysis. This involves measuring the % ee of the cyanohydrin enantiomers resulted from *Bm*HNL biocatalysis. Prior to determine the % ee of biocatalytic product, it is essential to resolve both cyanohydrin enantiomers of a racemic compound by chiral HPLC and also identify the two chromatograms of the racemic cyanohydrins which one is (*R*) and which one is (*S*). The

enantiomeric separation of a racemic cyanohydrin by chiral HPLC needs analytical method development and its optimization. To pursue this, one must consider three important components e.g. stationary phase i.e. chiral column in HPLC, composition of mobile phase and ratio of mobile phase. The racemic cyanohydrins whose chiral resolution is to be carried out, they all have common cyanohydrin functional part but yet they have different other functional groups which are responsible for their differential interaction with the chiral stationary phase. Due to the differential interaction, the retention times of enantiomers in HPLC chromatogram would differ accordingly. Not only that but because of the different nature of compounds their chiral separation may not be possible in a single stationary phase or column.

In this chapter, we discussed the chiral resolution of synthesized 18 racemic cyanohydrins (**Figure 3A.1** and **Table 3A.1**, **Chapter 3A**) in chiral columns by using HPLC. The developed method has been used further to determine % ee of *Bm*HNL catalyzed chiral cyanohydrins.

Two chiral columns Chiralpak IB and IE (**Figure 3B.1**) were used in the present study for resolution of racemic cyanohydrins. Chiralpak IE contains Amylose tris-(3,5-dichlorophenylcarbamate) immobilized on silica gel while Chiralpak IB contains Cellulose tris-(3,5-dimethylphenylcarbamate) immobilized on silica gel.



(i) (ii)

Figure 3B.1: (i) Chiral stationary phase of Chiralpak IE i.e. Amylose tris-(3,5-dichlorophenylcarbamate); (ii) Chiral stationary phase of Chiralpak IB i.e. Cellulose tris-(3,5-dimethylphenylcarbamate)

3B.2. Objectives of the present study

The main objective of the present work is:

To perform chiral resolution of the racemic cyanohydrins using chiral HPLC.

3B.3. Materials and methods

3B.3.1. Chemicals

Racemic cyanohydrins (**1-18**) (**Table 3A.1**) were synthesized as discussed in **Chapter 3A**. HPLC grade solvents hexane and isopropanol were purchased from Rankem Pvt. Ltd., India.

3B.3.2. Apparatus and sample preparation

Chiral resolution of racemic cyanohydrins was performed using Shimadzu HPLC with autosampler SIL-20AC. Chiral columns Chiralpak 1E and IB (250x4.6 mm, Diacel Corporation, Tokyo, Japan) were used for the present study. UV-Visible detector SPD-20A was used for detection and the data was processed by LC solution software. The analysis was performed at wavelength of 210 nm using different ratio of hexane and 2-propanol at flow rate of 1 mL/min. Retention factors were calculated using formula $k'1 = t1 - t0/t0$ and $k'2 = t2 - t0/t0$. Here t_0, t_1 and t_2 were retention time of first eluted peak, first enantiomer and second enantiomer respectively. Separation factor (α) was calculated as

ratio of retention factors i.e. $k'2/k'1$. Resolution factor *(R_s)* of racemic cyanohydrins was calculated using formula $Rs = \{2(t2 - t1)\}/(w1 + w2)$ where w1 and w2 are peak widths of first enantiomer and second enantiomer respectively. For sample preparation, the racemic cyanohydrin was dissolved in the mobile phase (hexane and 2-propanol) with identical composition that was used for chiral analysis.

3B.4. Results

Optimization of chiral resolution of the racemic cyanohydrins was performed using the materials described above. Efforts were made to produce the best chiral resolution of the two enantiomers of racemic cyanohydrins. Compound **6**, **17** and **18** were resolved in Chiralpak IE while compounds **1-5**, **7**, **8**, **10**, **11** and **13-16** were separated in Chiralpak IB column (**Table 3B.1**). The numbers referred in this chapter belong to **Table 3A.1** of **Chapter 3A**. For the chiral resolution of different racemic cyanohydrins, the mobile phase i.e. hexane and isopropanol composition was varied from 97:03 to 75:25.

(*R*/*S*)-2,4-dimethoxymandelonitrile (**1**) was resolved in chiral column Chiralpak IB using hexane: 2-propanol with 90:10 ratio at 210 nm and 1 mL/min flow rate. The (*R*)- and (*S*)-enantiomers of **1** were eluted at 13.871 and 16.266 min while its corresponding aldehyde was eluted at 8.348 min. respectively. Retention times of aldehyde of **2**, (*R*)-**2** and (*S*)-**2** were 9.651, 30.276 and 32.091 min respectively in Chiralpak IB using 97:03 ratio of hexane: 2-propanol. Chiral resolution of compound **3** was carried out in Chiralpak IB with 85:15 ratio of hexane: 2-propanol in which the retention time of the corresponding aldehyde, (*R*)- and (*S*)-enantiomer was 7.253, 9.928, and 11.251 min respectively. Compound **4** was resolved in chiral column IB and 92:08 ratio of mobile phase. 2-

Naphthaldehye was eluted at 6.128 min while (*R*)- and (*S*)-enantiomer of **4** were eluted at 13.466 and 15.018 min respectively.

In case of racemic (*E*)-2-hydroxy-4-phenylbut-3-enenitrile (**5**), retention times were 7.273, 14.836 and 16.135 min for its aldehyde, (*R*)- and (*S*)-enantiomer respectively in 92:08 ratio of hexane:2-propanol. Retention times of (*R*)- and (*S*)-enantiomers of compound **6** were 21.462 and 19.960 min respectively in Chiralpak IE using 96:04 ratio of hexane: isopropanol while the corresponding aldehyde of **6** was eluted at 10.883 min. With 90:10 ratio of hexane: isopropanol in Chiralpak IE column the retention times of (*R*)-**17**, (*S*)-**17** and aldehyde of **17** were 14.41, 16.088 and 6.594 min respectively while compound **18** analyzed on the same column and solvent system showed the retention time of 12.262 min for (*R*)-**18** and 11.209 min for (*S*)-**18**.

Chiral resolution of **7** was performed using 75:25 ratio of hexane: isopropanol in Chiralpak IB column. The retention times were 6.360, 8.379 and 11.511 min for 9-anthraldehyde, (*R*)- and (*S*)-enantiomer of **7** respectively.Similarly (R/S)-2-hydroxy-3-phenylpropanitrile (**8**) was resolved in the same chiral column using 95:05 of hexane: isopropanol. Its aldehyde and (*R*)- and (*S*)-enantiomer appeared at 7.385, 12.252 and 14.944 min respectively. Compound **9** and **12** did not resolve in both chiral columns.

Racemic 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile (**10**) was resolved in column IB, 90:10 ratio of hexane: isopropanol which resulted in 5.652, 9.115 and 9.796 min retention time for 3-phenoxybenzaldehyde, (*R*)-enantiomer and (*S*)-enantiomer respectively. Retention times of 3,5-dimethoxybenzaldehyde, (*R*)-**13** and (*S*)-**13** were 5.773, 14.457 and 15.62 min respectively in Chiralpak IB column and hexane: isopropanol of 92:08 while in case of compound **14**, aldehyde was eluted at 6.351 min and (*R*)- & (*S*)-enantiomers were

eluted at 12.071 and 12.92 min respectively using 92:08 ratio of hexane: isopropanol. Compounds **11**, **15** and **16** were eluted in retention times as mentioned in **Table 3B.1**.

It is clear from **Table 3B.1** that both columns gave good separation for all the cyanohydrins as *Rs*>1 was observed.

The R of the table below belong to the structure shown in **Figure 3A.1** of **Chapter 3A.**

Table 3B.1: Separation factor (α) and resolutions (R_s) of racemic cyanohydrins

S. No.	R	$RT_{aldehyde}$	RT_R	RT_S	$k'1$	$k'2$	α	R_s	Column Chiralpak	Mobile phase Hex/IPA
1	2,4-di $MeOC_6H_3$	8.348	13.871	16.266	3.72	4.54	1.22	6.93	IB	90/10
2	2,3,4-tri $MeOC_6H_2$	9.651	30.276	32.091	9.30	9.92	1.07	2.55	IB	97/03
3	3,4,5-tri $MeOC_6H_2$	7.253	9.928	11.251	2.40	2.86	1.19	3.69	IB	85/15
4	2-Naphthyl	6.128	13.466	15.018	3.58	4.11	1.09	6.11	IB	92/08
5	*trans*-PhCH=CH	7.273	14.836	16.135	3.96	4.40	1.11	2.2	IB	92/08
6	4-CH_2=CH-CH_2O-C_6H_4	10.883	21.462	19.960	5.40	5.88	1.10	1.81	IE	96/04
7	9-Anthranyl	6.360	8.379	11.511	1.79	2.83	1.58	14.01	IB	75/25
8	Ph-CH_2	7.385	12.252	14.944	3.16	4.07	1.29	10.31	IB	95/05
9	PhCH(CH_3)	5.783	nr	nr	NA	NA	NA	NA	IB	96/04
10	3-PhO-C_6H_4	5.652	9.115	9.796	1.88	2.10	1.11	2.89	IB	90/10
11	3-$PhCH_2O$-C_6H_4	6.758	13.11	10.94	2.63	3.36	1.27	16.44	IB	90/10
12	3-(NC_5H_4)	7.188	nr	nr	NA	NA	NA	NA	IB	97/03
13	3,5-di $MeOC_6H_3$	5.773	14.457	15.62	3.94	4.34	1.10	7.16	IB	92/08
14	2,5-di $MeOC_6H_3$	6.351	12.071	12.92	3.13	3.42	1.09	3.30	IB	92/08
15	4-$PhCH_2O$-C_6H_4	9.653	25.863	27.261	7.76	8.23	1.06	2.31	IB	95/05
16	4-BrC_6H_4	5.775	21.260	22.316	6.16	6.51	1.06	2.58	IB	95/05
17	3-OHC_6H_4	6.594	14.41	16.088	3.59	4.13	1.15	4.45	IE	90/10
18	4-OHC_6H_4	6.689	12.262	11.209	1.92	2.27	1.18	4.52	IE	90/10

nr: not resolved; NA: not applicable

3B.5. Discussion

We have attempted to carry out the chiral resolution of eighteen racemic cyanohydrins (**Table 3B.1**) using Diacel Chiralpak IB and IE chiral columns at 210 nm and 1 mL/min flow-rate. However, we were successful in achieving the chiral resolution of sixteen substrates as given in **Table 3B.1**. The selectivity factor α which represents the separation of the two components, in the present case the two enantiomers is always greater than one. We have observed $\alpha>1$ in the chiral resolution of all the sixteen racemic cyanohydrins (**Table 3B.1**). Similarly, a resolution factor (R_s) of 1.5 or more is recommended for baseline separation of two chromatographic peaks in HPLC. We have observed better R_s values ($R_s>1.5$) for all sixteen cyanohydrins in their chiral resolution.

Enantiomers of compound **4** were resolved in chiral column IB and 92:08 ratio of mobile phase at 25 ^{o}C.

Resolution of the same compound was reported by Zheng *et al* in column OD-H which is equivalent of Chiralpak IB. They used 90:10 ratio of hexane:2-propanol for elution and observed the retention time of 2-naphthaldehyde, (*R*)- and (*S*)-enantiomer of 2-hydroxy-2-(naphthalene-2-yl)acetonitrile as 9.60, 23.19 and 27.25 min respectively [1]. Although these retention times were different but the order of elution of aldehyde, (*R*)- and (*S*)-enantiomers of **4** were the same as we observed with Chiralpak IB column.

Chiral resolution of (R/S)-**5** was carried out in Chiralpak IB with 92:08 ratio of hexane:2-propanol (**Table 3B.1**). Gerrits *et al* reported its chiral resolution in OD-H column at 40 ^{o}C and 261 nm in hexane: 2-propanol: acetic acid in ratio of 87:13:0.1%. This column has the same chiral stationary phase as Chiralpak IB. The retention time of the corresponding aldehyde, (*R*)- and (*S*)-of **5** was 7.78, 14.42 and 17.40 min respectively. They also reported

the chiral separation of compound **18** but in Chiralcel OJ column at 40 °C and 230 nm in hexane: 2-propanol: acetic acid in 87:13:0.1% ratio. The retention time of 4-hydroxybenzaldehyde, (*R*)-**18** and (*S*)-**18** was 6.65, 25.3 and 28.3 min respectively [2]. In the present study, compound **18** was resolved in Chiralpak IE column at 25 ^{o}C and 210 nm in 90:10 ratio of mobile phase.

Enantiomeric separation of racemic-**8** was carried out in chiral column IB using 95:05 of mobile phase that showed the retention times of 7.385, 12.252 and 14.944 min for 2-phenyl acetaldehyde, (*R*)- and (*S*)-enantiomer of **8** respectively. Zheng *et al* have performed the chiral resolution of **8** in Chiralcel OD-H column, using 90:10 ratio of hexane: 2-propanol at 0.7 mL/min flow rate and 254 nm. They observed the retention time of aldehyde, (*R*)- and (*S*)-**8** as 9.36, 13.40 and 17.40 min respectively. Chiral resolution of racemic **10** was also reported by the same group in similar conditions as mentioned above [1]. In the present study, compound **10** was resolved in chiral column IB. Other than the racemic cyanohydrins **4**, **5**, **8** and **18**, chiral separation of other racemic cyanohydrins of Table 3B.1 has not been reported in chiral column IB and IE or their equivalent chiral columns. Cyanohydrins **9** and **12** did not resolve in both columns used in the present study.

Alagoz *et al* have reported the chiral resolution of racemic cyanohydrins **16** and **18** using chiral column Nucleocel Delta. This column contains a chiral stationary phase as cellulose tris-(3,5-dimethylphenylcarbamate). They observed retention time of (*R*)- and (*S*)-**18** as 14.8 and 14.1 min respectively while in case of compound **16**, their retention time of (*R*)- and (*S*)-enantiomer was 13.1 and 15.2 min respectively [3]. In case of **18**, the order of elution is (*S*)-**18** first and then (*R*)-**18**. We also observed a similar trend (Table 3B.1). The

order of elution of enantiomers of **16** was also found match between their report and our results.

M. Dadashipour *et al* have performed chiral resolution of a number of racemic cyanohydrins using chiral HPLC with chiral OJ-H at 254 nm. They used hexane and 2-propanol as a solvent system and 1 mL/min flow rate for their chiral analysis. With 39:1 ratio of hexane: isopropanol, the retention times of 4-bromobenzaldehyde, (*R*)-**16** and (*S*)-**16** were 8.1, 45.4 and 47.5 min respectively. When 85:15 of hexane: 2-propanol was used in the chiral analysis they observed 3-phenoxybenzaldehyde at 7.3 min, (*R*)-**10** at 16.7 min and (*S*)-**10** at 21.3 min. They resolved racemic 2-hydroxy-2-(4-hydroxyphenyl)acetonitrile in 85:15 of hexane: 2-propanol. The corresponding aldehyde was eluted at 6.8 min while (*R*) and (*S*)-**18** were eluted at 58.1 and 65.7 min respectively. Their chiral analysis showed retention time of 3-pyridinecarboxaldehyde, (*R*)- and (*S*)-**12** as 12.2, 13.4 and 14.9 min respectively. They found the retention time of 2,4-dimethoxybenzaldehyde and two enantiomers of **1** as 9, 19.8 and 17.2 min respectively in 85:15 of hexane:2-propanol. In case of 3,5-dimethoxybenzaldehyde, (*R*)- and (*S*)-**13**, they have seen their retention times as 7.5, 15.4 and 17.0 min respectively. Their chiral analysis showed the aldehyde of **2** with retention time of 9.5 min while 12.7 min for (*R*)-**2** and 16.1 min for (*S*)-**2**. Two cyanohydrins i.e. **3** and **7** could not be resolved by them in chiral HPLC [4].

Nanda *et al* synthesized a number of racemic cyanohydrins using chiral OJ-H, 1 mL/min at 25 ^{o}C, 254 nm and 1 mL/min flow-rate. They observed retention times of 4-bromobenzaldehyde, (*R*)- and (*S*)-**16** as 6.5, 32.0 and 33.2 min respectively in 19:1 ratio of hexane: isopropanol while in 90:10 ratio the retention times for 4-hydroxybenzaldehyde, (*R*)- and (*S*)-**18** were 9.9, 58.1 and 65.7 min respectively. They found the retention time of

4-benzyloxybenzaldehyde, (*R*)- and (*S*)-**15** as 12.9, 28.9 and 34.0 min when 80:20 hexane: 2-propanol was used. Their analysis in hexane: 2-propanol of 90:10 ratio showed the retention time for the aldehyde of **10** as 9.1 min, (*R*)- **10** at 25.4 min and (*S*)-**10** at 33.0 min. Retention time of 3-pyridinecarboxaldehyde, (*R*)- and (*S*)-**12** were 9.1, 13.2 and 14.8 min respectively when 90:10 ratio of hexane:2-propanol was used while in case of cyanohydrin **7** that was also analyzed in the same solvent ratio, the retention times were 11.9 min for 9-anthraldehyde, 25.5 min for (*R*)-enantiomer and 31.4 min for the (*S*)-enantiomer. With 90:10 ratio of hexane: 2-propanol, in the HPLC analysis the retention time of aldehyde of **14** was 17.8 min, (*R*)-enantiomer was 43.0 min and the (*S*)-enantiomer was 48.7 min while in case of compound **1**, the retention times were 12.0, 31.5 and 36.9 min for aldehyde, (*R*)-enantiomers and (*S*)-enantiomer respectively in the same composition of mobile phase. Similarly, the retention time of aldehyde of **13** was 8.2 min, its (*R*)-enantiomer was 23.5 min and the (*S*)-enantiomer was 25.9 min. Retention time of 2,3,4-trimethoxybenzaldehyde, (*R*)- and (*S*)-**2** were 11.3, 31.2 and 34.4 min respectively in 90:10 ratio of hexane: 2-propanol with 0.7 mL/min flow rate while in case of compound **3**, the retention times were 14.4, 29.5 and 35.5 min for its aldehyde, (*R*)-enantiomer and (*S*)-enantiomer respectively in 90:10 ratio of hexane: 2-propanol with 0.7 mL/min [5]

M. Dadashipour *et al* synthesized a few racemic cyanohydrins, characterized them by NMR and used them as standards to analyze biocatalytic products. They also resolved the racemic cyanohydrins using chiral HPLC with Chiralcel OJ-H column, at 254 nm and 1 mL/min flow-rate. Chiral resolution of **14** was performed using 95:5 ratio of hexane: isopropanol. The retention times were 7.9, 30.5 and 31.8 min for 4-bromobenzaldehyde, (*R*)- and (*S*)-enantiomer respectively while they did the chiral separation of **1** in hexane: 2-

propanol of 85:15 ratio where the retention time was 5 min for its aldehyde, 8.1 min for (*R*)-enantiomers and 10.2 min for (*S*)-enantiomer. Similarly, in case of compound **13**, the retention times were 10.5, 51.0 and 55.8 min for its aldehyde, (*R*)- and (*S*)-enantiomers respectively using 85:15 hexane: 2-propanol. In case of chiral resolution of racemic cyanohydrin **12** in 85:15 ratio of hexane: 2-propanol, they found the retention times as 9.2 min for aldehyde, 10.3 min for (*R*)-enantiomer and 11.5 min for (*S*)-enantiomer. [6].

3B.6. Conclusions

- Chiral resolution of synthesized racemic cyanohydrins (**1**-**18**, **Table 3B.1**) were performed in chiral HPLC using Chiralpak IB and IE chiral columns. Different percentages of hexane and isopropanol were used in the chiral separation of the cyanohydrins. Two racemic cyanohydrins **9** and **12** did not resolve in both columns. Three compounds (**6**, **17**, and **18**) were separated in Chiralpak IE and all the other cyanohydrins were resolved in the second column i.e. Chiralpak IB.
- Separation factor (α) and resolution factor (R_s) of the HPLC chromatograms of the chiral resolution of racemic cyanohydrins were calculated.

References:

[1] Y.C. Zheng, J.H. Xu, H. Wang, G.Q. Lin, R. Hong, H.L. Yu, Hydroxynitrile Lyase Isozymes from *Prunus communis*: Identification, characterization and synthetic applications, Adv. Synth. Catal. 359 (2017) 1185–1193.

[2] P. Jan Gerrits, F. Zumbrägel, J. Marcus, Analyzing the hydrocyanation reaction: Chiral HPLC and the synthesis of racemic cyanohydrins, Tetrahedron. 57 (2001) 8691–8698.

[3] D. Alagöz, S.S. Tükel, D. Yildirim, Enantioselective synthesis of various cyanohydrins using covalently immobilized preparations of hydroxynitrile lyase from *Prunus dulcis*, Appl. Biochem. Biotechnol. 177 (2015) 1348–1363.

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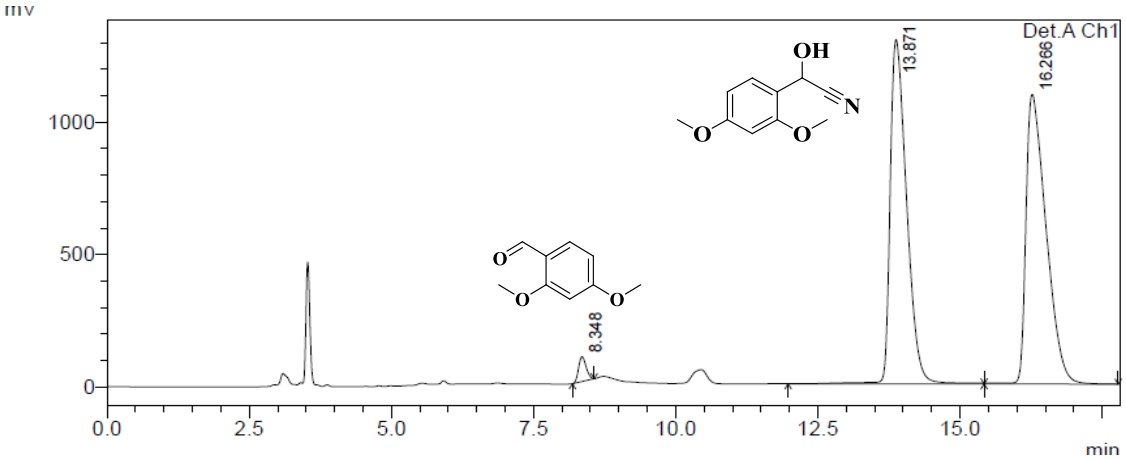
[5] S. Nanda, Y. Kato, Y. Asano, A new (*R*)-hydroxynitrile lyase from *Prunus mume*: Asymmetric synthesis of cyanohydrins, Tetrahedron. 61 (2005) 10908–10916.

[6] M. Dadashipour, Y. Ishida, K. Yamamoto, Y. Asano, Discovery and molecular and biocatalytic properties of hydroxynitrile lyase from an invasive millipede, *Chamberlinius hualienensis*, Proc. Natl. Acad. Sci. 112 (2015) 10605–10610.

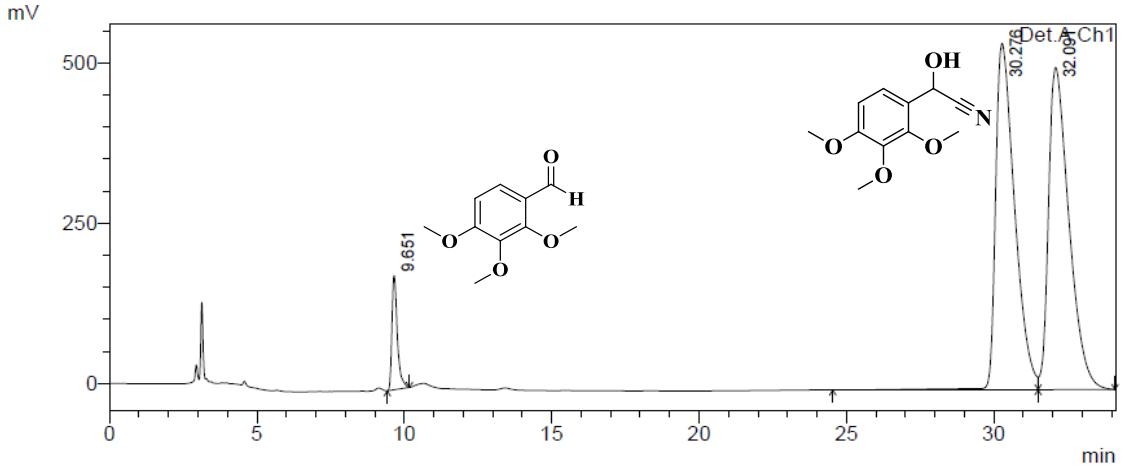
HPLC chromatograms of chiral resolution of the racemic cyanohydrins 1-18

1. 2-hydroxy-2-(2,4-dimethoxyphenyl)acetonitrile



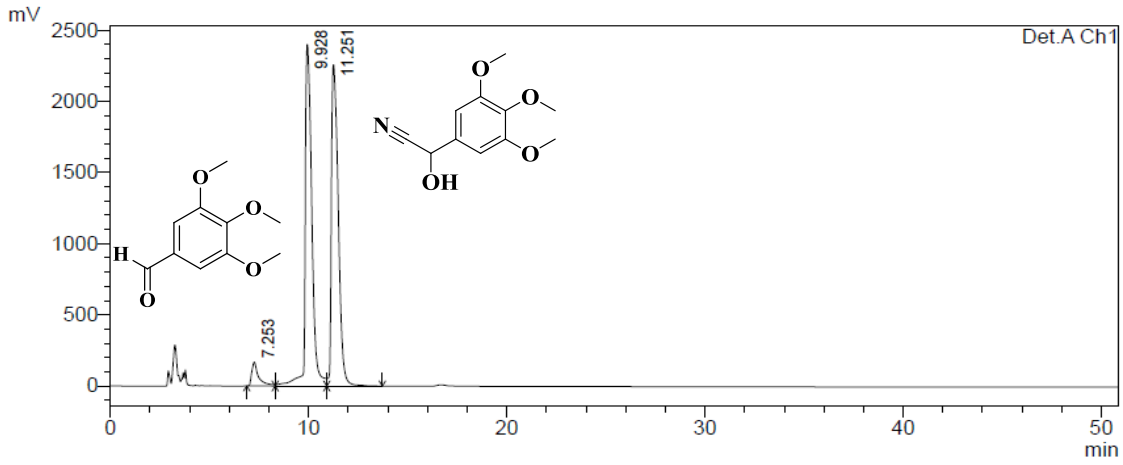
Peak	Ret. Time	Area	Height	Area %
1	8.348	875419	93994	1.619
2	13.871	26259330	1301590	48.578
3	16.266	26921407	1095092	49.803
Total		54056156	2490677	100.000

2. 2-hydroxy-2-(2,3,4-trimethoxyphenyl)acetonitrile



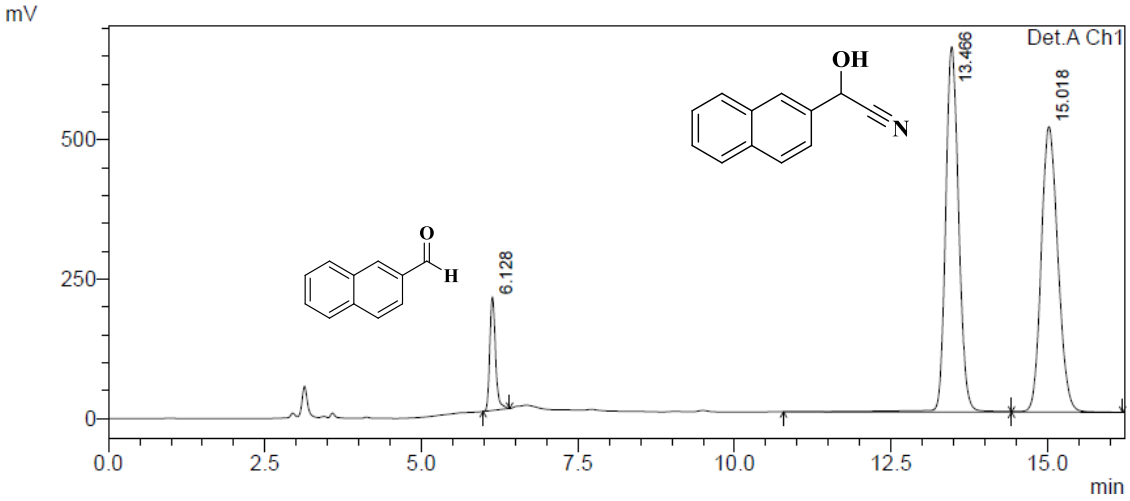
Peak	Ret. Time	Area	Height	Area %
1	9.651	2282679	178227	4.491
2	30.276	24271494	540039	47.749
3	32.091	24277393	502680	47.760
Total		50831566	1220946	100.000

3. 2-hydroxy-2-(3,4,5-trimethoxyphenyl)acetonitrile



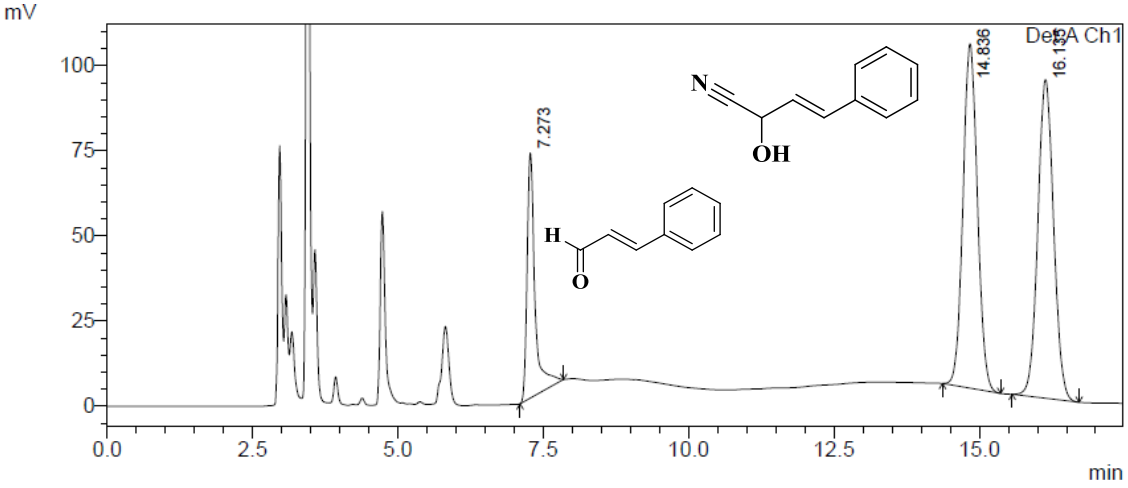
Peak	Ret. Time	Area	Height	Area %
1	7.253	4058419	167594	3.537
2	9.928	56114258	2402076	48.911
3	11.251	54554282	2258695	47.551
Total		114726959	4828365	100.000

4. 2-hydroxy-2-(naphthalen-6-yl)acetonitrile



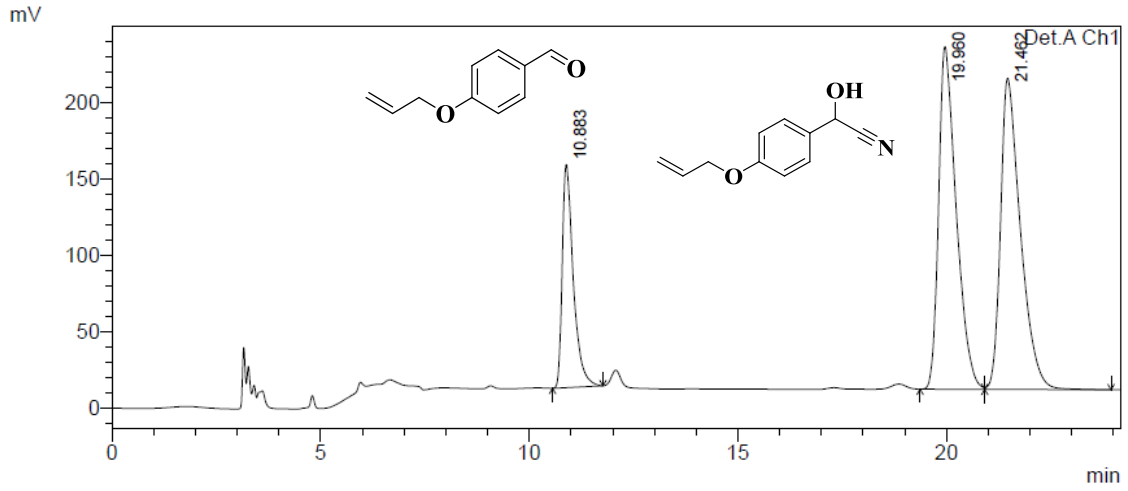
Peak	Ret. Time	Area	Height	Area %
1	6.128	1300321	204352	6.375
2	13.466	9583970	655591	46.986
3	15.018	9513233	512428	46.639
Total		20397524	1372371	100.000

5. (*E*)-2-hydroxy-4-phenylbut-3-enenitrile [2](Jan Gerrits et al., 2001)



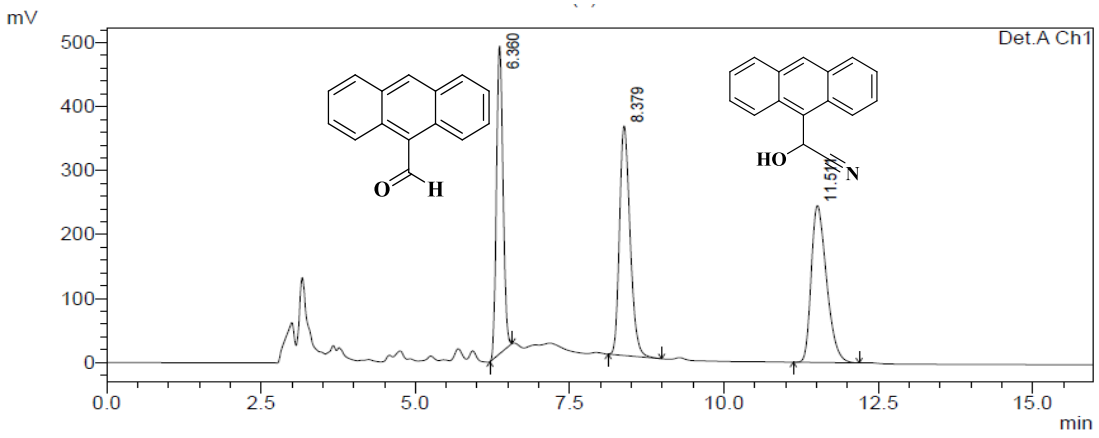
Peak	Ret. Time	Area	Height	Area %	Height %
1	7.273	678060	72002	16.140	26.995
2	14.836	1748890	101148	41.630	37.922
3	16.135	1774072	93578	42.230	35.084

6. 2-(4-(allyloxy)phenyl)-2-hydroxyacetonitrile



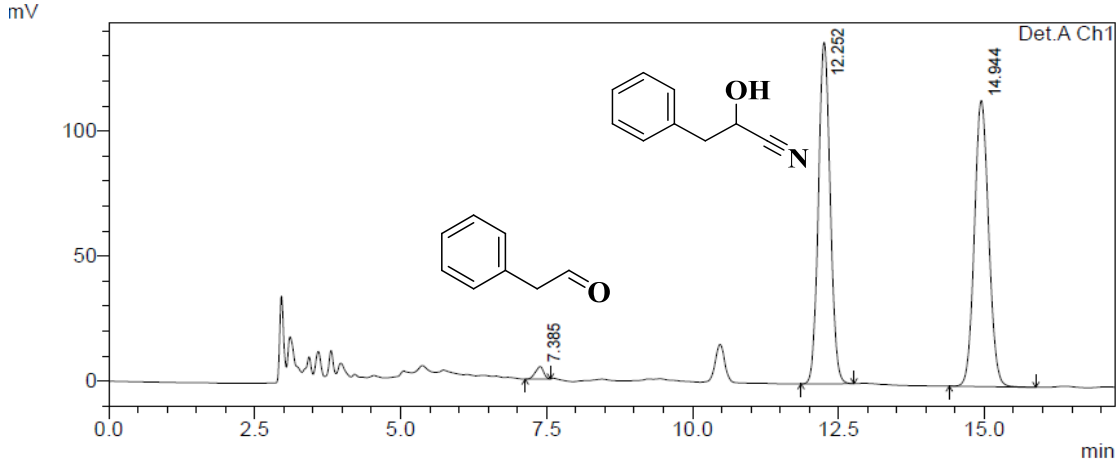
Peak	Ret. Time	Area	Height	Area %
1	10.883	2681465	145856	16.742
2	19.960	6585892	224036	41.120
3	21.462	6749078	203415	42.138
Total		16016434	573307	100.000

7. 2-(anthracen-9-yl)-2-hydroxyacetonitrile



Peak	Ret. Time	Area	Height	Area %
1	6.360	3566527	481640	29.473
2	8.379	4291369	359231	35.464
3	11.511	4242897	245326	35.063
Total		12100793	1086197	100.000

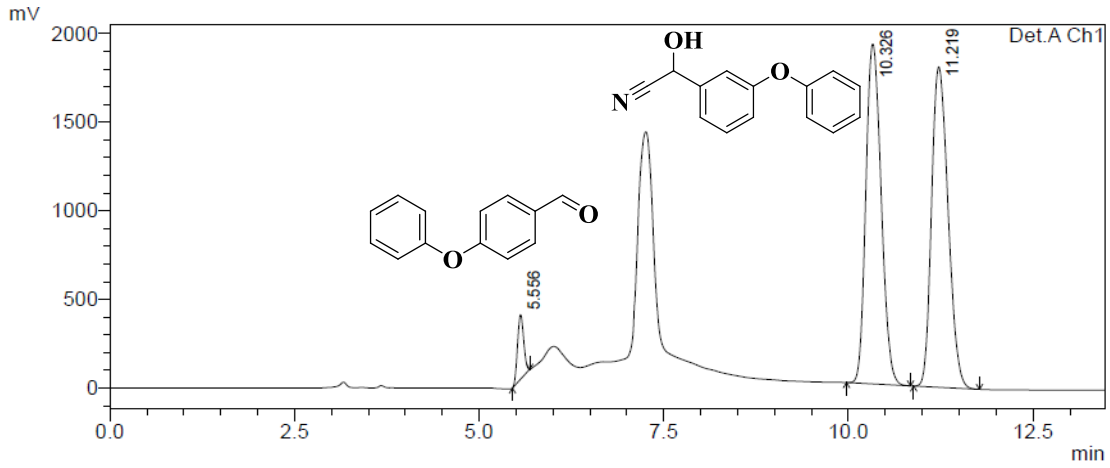
8. 2-hydroxy-3-phenylpropanenitrile



Peak	Ret. Time	Area	Height	Area %
1	7.385	48646	4979	1.243
2	12.252	1920973	136482	49.079
3	14.944	1944394	114328	49.678
Total		3914013	255790	100.000

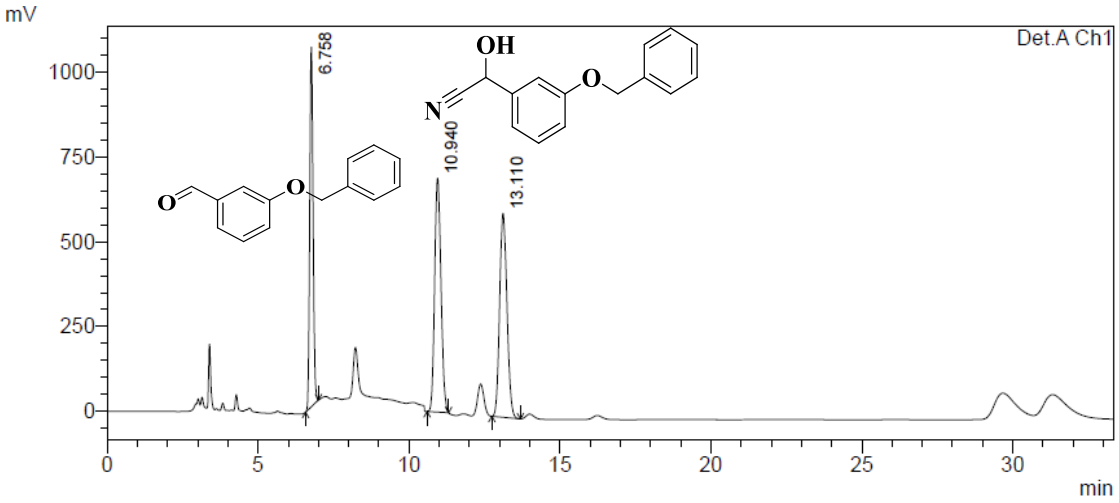
9. 2-hydroxy-3-phenylbutanenitrile: did not resolve

10. 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile



Peak	Ret. Time	Area	Height	Area %
1	5.556	2138292	366618	3.688
2	10.326	27666779	1918734	47.718
3	11.219	28174114	1808616	48.594
Total		57978185	4093969	100.000

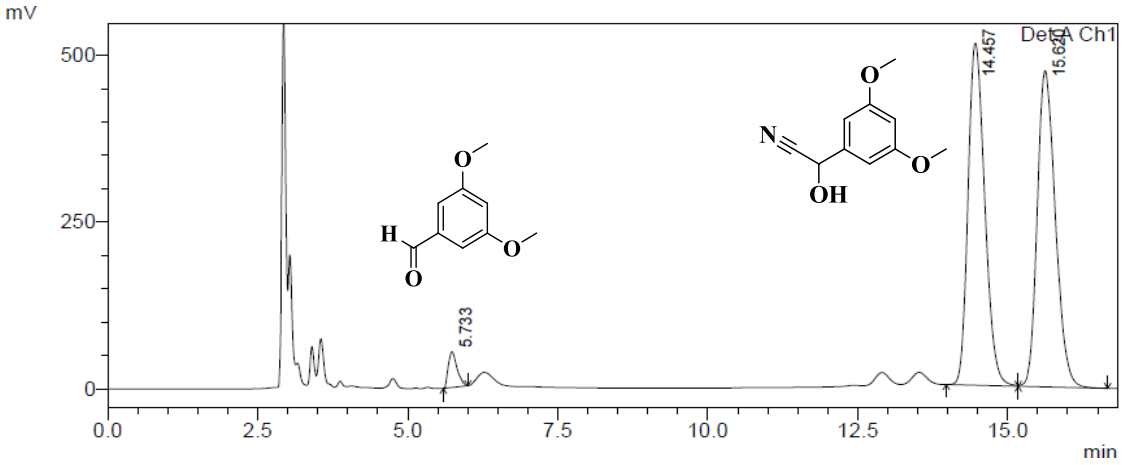
11. 2-hydroxy-2-(3-benzyloxyphenyl)acetonitrile



Peak	Ret. Time	Area	Height	Area %
1	6.758	8187279	1059898	28.790
2	10.940	10069908	689804	35.410
3	13.110	10181118	600715	35.801
Total		28438305	2350417	100.000

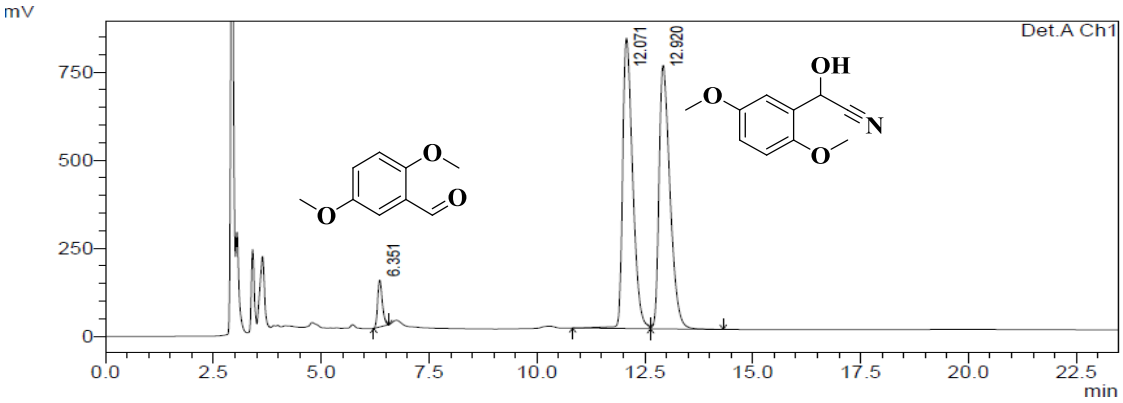
12. 2-Hydroxy-2-(pyridin-3-yl)acetonitrile: did not resolve

13. 2-hydroxy-2-(3,5-dimethoxyphenyl)acetonitrile



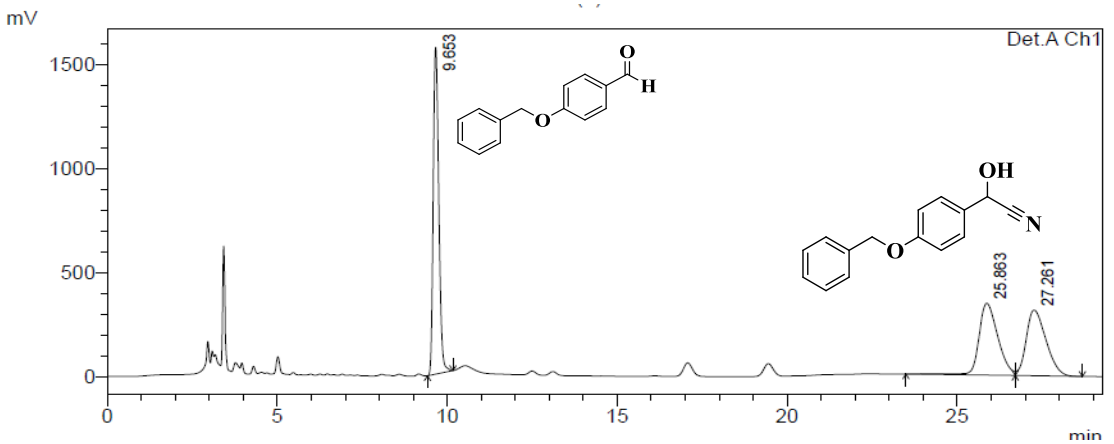
Peak	Ret. Time	Area	Height	Area %
1	5.773	546925	53552	2.676
2	14.457	9929781	512701	48.590
3	15.620	9959307	473781	48.734
Total		20436013	1040034	100.000

14. 2-hydroxy-2-(2,5-dimethoxyphenyl)acetonitrile



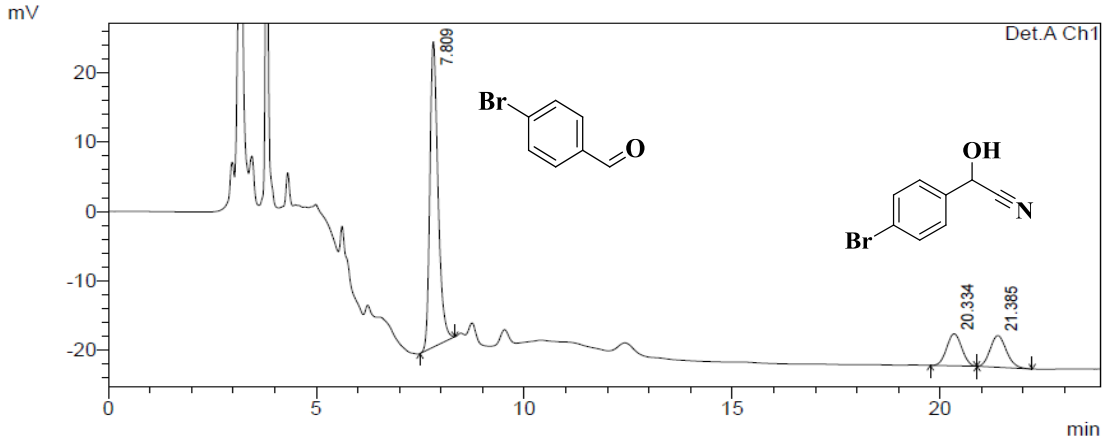
Peak	Ret. Time	Area	Height	Area %	Height %
1	6.351	998741	132985	3.632	7.795
2	12.071	13299180	824768	48.366	48.343
3	12.920	13199128	748306	48.002	43.862
Total		27497049	1706059	100.000	100.000

15. 2-hydroxy-2-(4-benzyloxyphenyl)acetonitrile



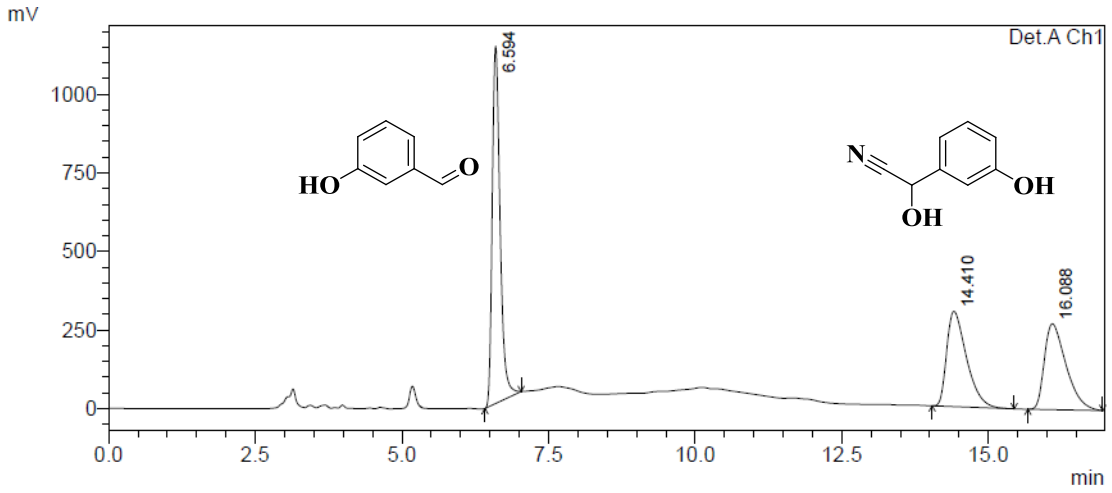
Peak	Ret. Time	Area	Height	Area %
1	9.653	17875939	1574420	40.676
2	25.863	13312804	346307	30.293
3	27.261	12757888	316552	29.030
Total		43946631	2237279	100.000

16. 2-hydroxy-2-(4-bromophenyl)acetonitrile



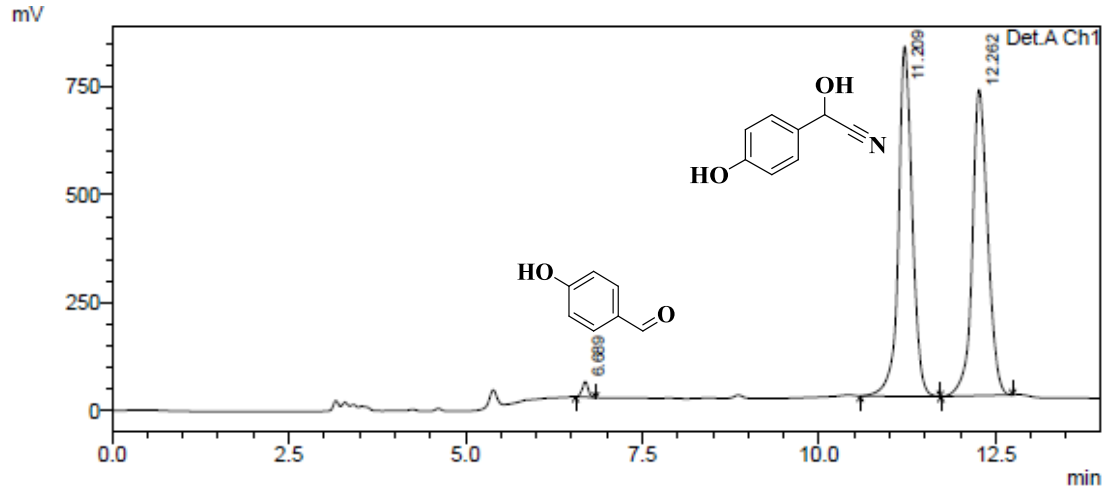
Peak	Ret. Time	Area	Height	Area %
1	7.809	604433	44079	71.551
2	20.334	116433	4612	13.783
3	21.385	123890	4569	14.666
Total		844757	53260	100.000

17. 2-hydroxy-2-(3-hydroxyphenyl)acetonitrile



Peak	Ret. Time	Area	Height	Area %
1	6.594	10580087	1138658	42.743
2	14.410	7132180	303220	28.828
3	16.088	7032841	273357	28.419

18. 2-hydroxy-2-(4-hydroxyphenyl)acetonitrile



Peak	Ret. Time	Area	Height	Area %
1	6.689	200013	35553	0.911
2	11.209	10997867	809374	50.104
3	12.262	10752039	707981	48.984
Total		21949920	1552908	100.000

Chapter 4

Baliospermum montanum hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins in a biphasic solvent

4.1. Introduction

In nature, HNLs catalyze cyanohydrin cleavage that releases toxic cyanide and a carbonyl compound. In addition to this, they catalyze the synthesis of chiral cyanohydrins by addition of nucleophilic cyanide into a carbonyl center. Optically pure cyanohydrins are important chiral molecules because they contain two different functional groups, a hydroxyl and a nitrile. A large variety of chiral compounds can be prepared from them by accelerating chemistry on these functional groups [1–3]. Chiral cyanohydrins are useful chiral intermediates in the preparation of a variety of pharmaceuticals, agrochemicals and bioactive molecules [4–7]. As there is a huge application of chiral cyanohydrins, hence the demand for biocatalytic synthesis of these molecules especially by HNL has increased significantly [8–11]. However, the HNL catalyzed cyanohydrin synthesis in aqueous system is constrained by mainly two issues. First, racemic cyanohydrin synthesis occurs spontaneously in the reaction condition which brings down the enantiomeric excess of the enzyme synthesized product. Second, often the substrates used are poorly soluble in aqueous medium [12,13]. Because of these two major reasons HNL catalysis are usually carried out in aqueous-organic solvent biphasic systems. The advantages of using organic solvent in HNL biocatalysis are (i) solubility of product that helps in easy isolation and (ii)

substrate partition to organic solvent that minimizes enzyme inhibition due to substrate. Note that benzaldehyde is known to inhibit a number of α/β hydrolase fold HNLs. A number of HNLs e.g. *Pa*HNL (with FAD), *Hevea brasiliensis* HNL (*Hb*HNL), *Manihot esculenta* HNL (*Me*HNL), and *Arabidopsis thaliana* HNL (*At*HNL), [14] of α/β hydrolase fold, *Granulicella tundricola* (*Gt*HNL) and *Acidobacterium capsulatum* (*Ac*HNL) from cupin family and the recently discovered *Davallia tyermannii* (*Dt*HNL) [15], fern HNL have been reported to use a biphasic system in their biocatalytic synthesis of chiral cyanohydrins irrespective of their different source, structure, catalytic mechanism and substrate preference.

Analysis of the *Bm*HNL biocatalytic synthesis of cyanohydrins reported by Asano group indicates that their biocatalysis was mainly carried out in an aqueous system [8,16,17]. They reported only 54% ee of (*S*)-mandelonitrile in the purified *Bm*HNL biocatalysis in an aqueous system [8]. Based on the poor ee of above biocatalysis, we aimed to investigate the effect of an aqueous-organic solvent system in the *Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins using crude enzyme extract. The advantages of this method are (i) it avoids tedious protein purification, and (ii) it is economic, less laborious and required less resources to prepare crude enzyme.

4.2. Objectives

- To study and optimize biocatalytic parameters of *Bm*HNL biocatalysis in a biphasic system.
- To synthesize chiral cyanohydrins by *Bm*HNL biocatalysis in optimized biocatalytic conditions.

4.3. Materials and Methods

Material related to *Bm*HNL cloning and expression are given in the Materials and Methods section of **Chapter 2**. Aldehydes were purchased from Sigma Aldrich, AVRA, SRL and local vendors. Mandelonitrile (catalog no. 116025) was purchased from Sigma Aldrich. Organic solvents such as toluene, hexane, isopropyl alcohol, *tert*-butyl methyl ether (TBME), *di*-isopropyl ether (DIPE), dimethyl sulphoxide (DMSO) and *n*-butyl acetate were purchased from Finar Limited.

4.3.1. Preparation of crude enzyme extract

*Bm*HNL crude extract was prepared as per section **2.4.13.1** of **Chapter 2**. The resulting supernatant was used as fresh crude enzyme for all biotransformation. The protein content was measured by a Nanodrop.

4.3.2. HNL assay

HNL activity was measured using the method described in section **2.4.14** of **Chapter 2**. The only exception was here 5 µL of crude enzyme (supernatant) in 20 mM KPB pH 7.0 was used instead of the purified enzyme reported in **2.4.13** of **Chapter 2**.

4.3.3. Synthesis of racemic cyanohydrins

Synthesis of racemic cyanohydrins was performed in order to use them as analytical standards. Three different methods with KCN, TMSCN and acetone cyanohydrin as a cyanide source were used to synthesize a number of racemic cyanohydrins as narrated in section **3A.4.2** of **Chapter 3**. All racemic cyanohydrins synthesized were characterized by ^{1}H and ^{13}C NMR spectroscopy (BRUKER 400 MHz NMR). The racemic cyanohydrins were used as analytical HPLC standards.

4.3.4. Reaction time of *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in aqueous medium

Crude *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was carried out for different time periods. In reaction mixture 160 µL of crude *Bm*HNL (6 U), 40 µL of 20 mM benzaldehyde in DMSO and 100 µL of 1 M KCN (prepared in double distilled water) were added into 700 µL of 300 mM citrate- buffer pH 4.2. The reaction was carried out at 22 ºC and 800 rpm in thermomixer. 100 µL aliquots were taken at different time points and reaction was stopped by adding 400 µL of hexane: isopropanol in ratio of 90:10. The reaction was analyzed using chiral HPLC using Chiralpak IE column with an appropriate ratio of hexane: isopropanol and flow rate.

The % of ee of a product was calculated using formula

$$\%\ ee = \frac{[\%\ \mathrm{Area}(S) - \mathrm{MN}] - [\%\ \mathrm{Area}(R) - \mathrm{MN}]}{[\%\ \mathrm{Area}(S) - \mathrm{MN}] + [\%\ \mathrm{Area}(R) - \mathrm{MN}]} 100$$

First, the % ee of control was subtracted from the % ee of reaction and the corresponding values were taken to calculate the % ee of the product using the above formula. [% $\mathrm{Area}_{(S)\text{-}\mathrm{MN}}$] is % area of (*S*)-mandelonitrile and [% $\mathrm{Area}_{(R)\text{-}\mathrm{MN}}$] is % area of (*R*)-mandelonitrile.

The % conversion of (*S*)-mandelonitrile was calculated by

$$\%\ conversion = \frac{\%\ \mathrm{Area}(S) - \mathrm{MN}]\mathrm{rxn} - [\%\ \mathrm{Area}(S) - \mathrm{MN}]\mathrm{cont}}{[\%\ \mathrm{AreaBenzaldehyde}]\mathrm{rxn}}\ 100$$

[% $\mathrm{Area}_{\mathrm{Benzaldehyde}}]_{\mathrm{rxn}}$: % area of benzaldehyde in the reaction.

4.3.5. Effect of organic solvents in the enantioselective synthesis of (*S*)-mandelonitrile

Influence of different organic solvents in the biocatalytic synthesis of chiral mandelonitrile was investigated. We have selected six different organic solvents for this study based on

their common use in several HNL catalyzed reactions. The solvents selected were hexane, toluene, acetonitrile, *tert*-butyl methyl ether (TBME), acetonitrile, *di*-isopropyl ether (DIPE), and *n*-butyl acetate. The reaction mixture contained 160 µL of crude *Bm*HNL (6 U), 40 µL of 20 mM benzaldehyde in DMSO, 100 µL of 1 M KCN, dissolved in double distilled water, 350 µL of an organic solvent (35% v/v) and 350 µL of 300 mM citrate buffer pH 4.2. The reaction was carried out at 22 ºC by shaking at 800 rpm for 10 minutes.

4.3.6. Effect of % volume of organic solvent in the enantioselective synthesis of (*S*)-mandelonitrile

The effect of the amount of organic solvent in the *Bm*HNL catalyzed enantioselective synthesis of cyanohydrin was studied. Toward this, three best organic solvents i.e. *n*-butyl acetate, DIPE, and toluene were selected. The biocatalysis conditions were kept the same to the previous experiment, except the % v/v of each of these organic solvents was varied from 10 to 70. Therefore, the volume of buffer was adjusted accordingly to get a net volume of 1 mL.

4.3.7. Optimization of substrate concentration

To determine the maximum substrate concentration that can be used in the biocatalysis, we have optimized the process by varying benzaldehyde concentration in the enantioselective synthesis of mandelonitrile. The reaction mixture consisted of 6 U of crude *Bm*HNL (160 µL), 40 µL of substrate (5 mM to 55 mM stock solution) pre-dissolved in DMSO equivalent to 0.2 mM to 2.2 mM, 100 µL of 1 M KCN in double distilled water, 100 µL of *n*-butyl acetate (10% v/v) and 600 µL of 300 mM citrate buffer pH 4.2. Biocatalysis was carried out at 22 ºC by shaking at 800 rpm for 10 minutes.

4.3.8. Effect of time of biotransformation in biphasic media

Effect of time of *Bm*HNL biocatalysis in a biphasic system in the enantioselective synthesis of mandelonitrile was studied. This study was carried out using two best benzaldehyde concentrations i.e. 1.4 and 0.8 mM. Two different reactions each containing a mixture of 6 U of crude *Bm*HNL, 100 µL of 1 M KCN in double distilled water, 100 µL of *n*-butyl acetate (10% v/v), 600 µL of 300 mM citrate buffer pH 4.2 and 1.4 or 0.8 mM substrate were carried out at 22 ºC by shaking at 800 rpm up to 30 minutes.

4.3.9. Effect of pH

The optimum pH of *Bm*HNL catalyzed enantioselective synthesis of cyanohydrin in a biphasic system was investigated. The reaction mixture contained 6 U of crude *Bm*HNL, 0.8 mM benzaldehyde in DMSO, 100 µL of 1 M KCN in double distilled water, 100 µL of *n*-butyl acetate and 600 µL of 300 mM citrate buffer of different pH i.e. 3, 3.5, 4.2, 5, 5.5 and 6. Biocatalysis was performed at 22 ºC by shaking at 800 rpm for 10 minutes.

4.3.10. Effect of temperature

Effect of temperature in the *Bm*HNL catalyzed enantioselective synthesis of (*S*)-mandelonitrile was studied. The reaction mixture composition was kept identical to the previous experiment except the buffer pH was fixed at 4.2. Biocatalysis was carried out at different temperatures i.e. 5, 10, 15, 18, 20 and 22 ºC by shaking the reaction mixture at 800 rpm for 10 minutes.

4.3.11. Effect of KCN concentration

In order to find out the effect of amount of KCN in the enantioselective synthesis of mandelonitrile, different molar ratios of KCN to aldehyde were tested in biocatalysis. The reaction mixture contained 6 U of crude *Bm*HNL, 0.8 mM benzaldehyde, 100 µL of *n*-butyl acetate and 600 µL of 300 mM citrate buffer pH 4.2. Ratio of concentration of benzaldehyde (mM) vs KCN (mM) was varied as 1:5, 1:10, 1:25, 1:50, 1:100, 1:125 and 1:150. The biocatalysis was performed at 22 ºC by shaking at 800 rpm for 10 minutes.

4.3.12. Synthesis of different (*S*)-cyanohydrins

Biocatalysis of crude *Bm*HNL in a biphasic system under optimized reaction conditions was carried out for the enantioselective synthesis of different cyanohydrins. The optimized reaction conditions were: 6 U of crude *Bm*HNL, 40 µL of 20 mM substrate (0.8 mM), 100 µL of 1 M KCN in double distilled water, 100 µL of *n*-butyl acetate (10% v/v) and 600 µL of 300 mM citrate buffer pH 4.2. However considering the fact that the reaction time may vary for different substrates, we have studied the biocatalysis of different substrates from 10 to 60 minutes. The best results of % ee and conversion of the product were determined by chiral HPLC and are summarized in **Table 4.1**.

4.4. Results

4.4.1. Reaction time of *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in aqueous medium

Crude *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was carried out in 300 mM citrate buffer pH 4.2 as reported by Dadashipour *et al* [13] and monitored at different time

intervals (**Figure 4.1**). Highest % ee of product was observed in 10 minutes i.e. 39%. The % conversion at 10 minutes was found to be 40.6%. The % ee of product was decreased with increase in time.

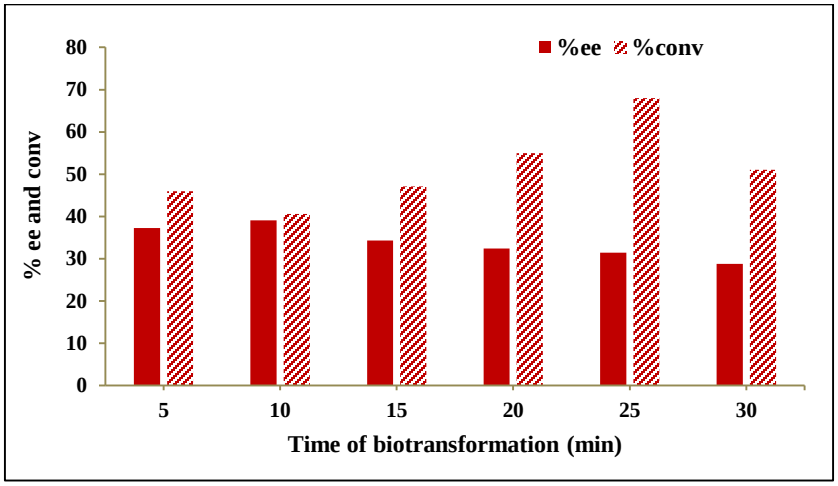


Figure 4.1: Time of biotransformation of *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in aqueous medium

4.4.2. Effect of different organic solvents in synthesis of (*S*)-mandelonitrile

The effect of different organic solvents e.g. hexane, toluene, TBME, acetonitrile, DIPE, and *n*-butyl acetate in the synthesis of (*S*)-mandelonitrile was investigated. Among the selected organic solvents, *Bm*HNL biocatalysis produced comparable % ee i.e. 37, 33 and 31 of (*S*)-mandelonitrile in three solvents which were *n*-butyl acetate, DIPE, and toluene respectively (**Figure 4.2**), as compared to 41% ee in presence of citrate buffer pH 4.2 only. The enzyme showed 34.35% conversion in buffer only while in case of *n*-butyl acetate the conversion was 33.1%. The highest conversion i.e. 57.55% was observed with DIPE. The enzyme showed 24.21% conversion of product in toluene-buffer system. In biphasic system containing hexane and acetonitrile, very low % ee (5-7%) was observed while 23.5% ee and ~43% conversion was observed in TBME-buffer system.

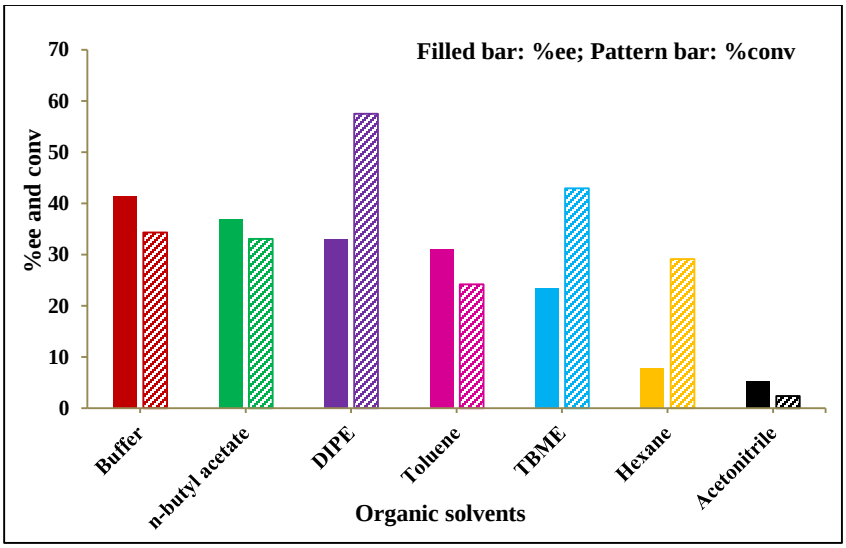


Figure 4.2: Effect of different organic solvents in synthesis of (*S*)-mandelonitrile

4.4.3. Effect of different % volume of *n*-butyl acetate, DIPE and toluene in synthesis of (*S*)-mandelonitrile

Out of the six organic solvents tested in **4.4.2**, three best solvents i.e. *n*-butyl acetate, DIPE, and toluene were selected for the current experiment. Among the different % volume of organic solvents tested, with 10% v/v *n*-butyl acetate highest % ee and conv (47.13% ee and 41.75% conv) of (*S*)-mandelonitrile was produced while in case of 10% DIPE, the enzyme showed 36% ee and 58% conv (**Figure 4.3**). The % conv was drastically low in case of 10% v/v of toluene (19%) whereas % ee was 44. A decrease in the % of ee and conversion was observed with increased % of organic solvents. This decrease in activity could be due to the instability of enzyme with increased content of organic solvent. 10% v/v of *n*-butyl acetate was used for further studies.

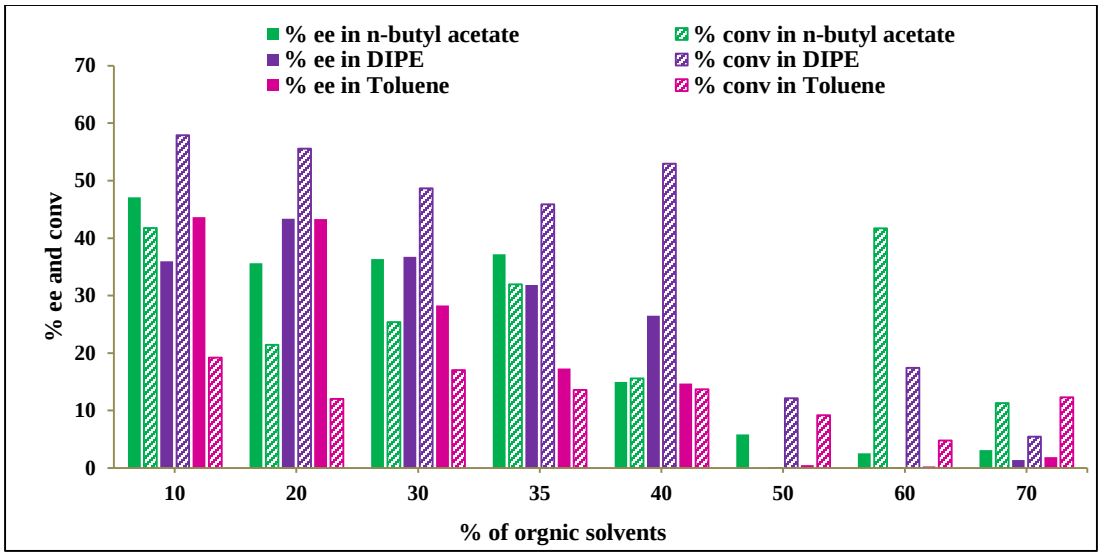


Figure 4.3: Effect of % v/v of three organic solvents in synthesis of (*S*)-mandelonitrile

4.4.4. Optimization of substrate concentration

The effect of different benzaldehyde concentration toward the synthesis of (*S*)-mandelonitrile using 10% v/v of *n*-butyl acetate was studied. Benzaldehyde concentrations were varied from 0.4 to 2.2 mM. The highest % ee i.e. 47% of product was produced in 0.6 mM benzaldehyde while highest % conversion of 64.35 was observed in 0.8 mM benzaldehyde (**Figure 4.4**). Further increase in benzaldehyde concentration from 0.8 to 1.4 mM showed almost constant % ee of ~45. On further increase in substrate concentration, the % ee of product decreased. In case of 1.4 mM benzaldehyde, 52.6% conversion of product was observed. As 1.4 mM was found to be the highest benzaldehyde concentration that showed almost comparable % ee to the optimal value, thus this concentration was selected for further optimization reactions. Along with it 0.8 mM substrate concentration was also selected for further optimization studies.

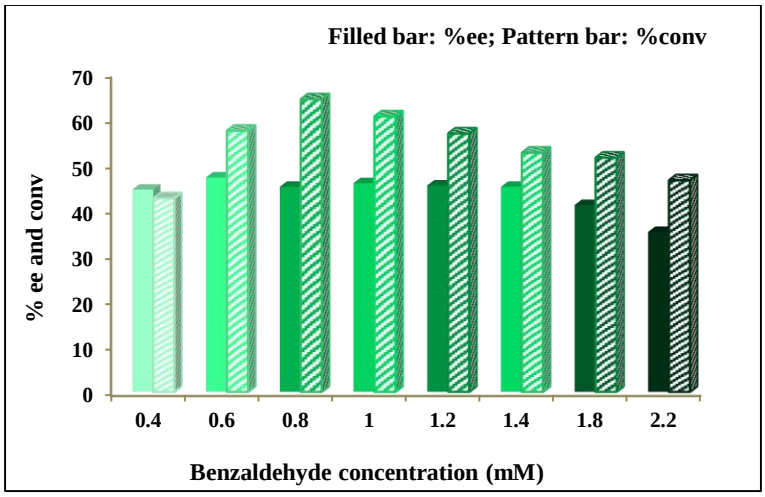


Figure 4.4: Effect of different benzaldehyde concentration in synthesis of (*S*)-mandelonitrile

4.4.5. Effect of time of biotransformation

Based on the above result, the two benzaldehyde concentration i.e. 0.8 and 1.4 mM were selected to perform *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile. The reaction was carried out till 30 min. In case of 0.8 mM benzaldehyde, the highest % ee observed was 48.2% in 10 min (**Figure 4.5**), which started decreasing with increase in time i.e. 38.4% in 30 min. Similarly, in 10 min 65.5% conversion of product was observed with 0.8 mM benzaldehyde. The % conversion increased from 65.5 to 72.5% in 30 min. With 1.4 mM benzaldehyde, *Bm*HNL showed highest % ee and conversion in 10 min i.e. 43.23 and 45.3 respectively. Similar to 0.8 mM benzaldehyde % ee of product decreased to 35.7% and % conversion increased to 58.2 with an increase in time.

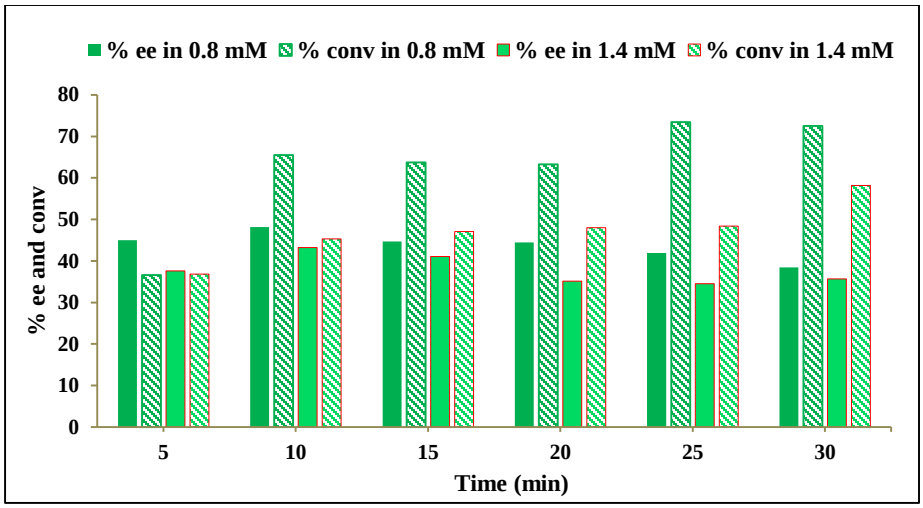


Figure 4.5: Time of biotransformation using 0.8 and 1.4 mM benzaldehyde.

4.4.6. Effect of pH

In HNL catalyzed chiral cyanohydrin synthesis, pH is known to play a significant role. At pH higher than 5, due to a background reaction, racemic cyanohydrins are produced [12,14,18,19]. This brings down the % ee of the product. In order to eliminate the background reaction, pH less than or equal to 5 is preferentially used in the HNL catalyzed transformations. We investigated the effect of pH in the *Bm*HNL catalyzed stereoselective synthesis of cyanohydrins by using buffers of pH 3.0 to 6.0. Highest ee of 47% was observed at pH 3.5 (**Figure 4.6**) with comparable ee of 44% at pH 3 and 46% ee at pH 4.2. The highest % conversion i.e. 53% was observed at pH 4.2.

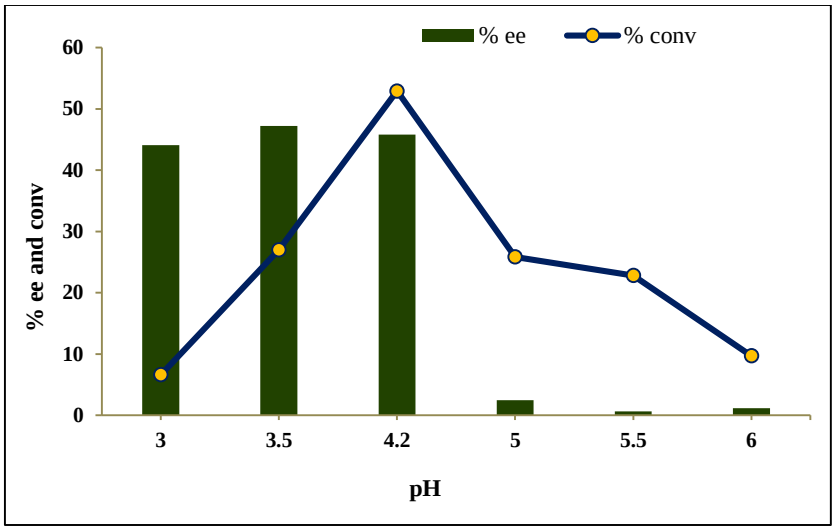


Figure 4.6: Effect of different pH in synthesis of (*S*)-mandelonitrile

4.4.7. Effect of temperature

The effect of temperature on *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was studied. To pursue this, biotransformation was performed at six temperatures from 5 to 22 ºC. High temperature usually leads to enzyme inactivation, instability of cyanohydrin and increases side reaction, hence temperature range from 5 to 22 ºC were selected. Enzyme activity at low temperature was found to be low, with 23.7% ee at 5 ºC and 23.9 at 10 ºC while the % conversion was found to be 31.6 at 5 ºC and 27.3 at 10 ºC (**Figure 4.7**). This result shows the similarity in both % ee as well as % conversion at low temperature i.e. 5 and 10 ºC. In comparison to low temperature, a good increase in both % ee and conversion were observed with temperature beyond 15 ºC i.e. 15, 18, 20 and 22 ºC. Highest % ee of 44 and % conversion of 65 was observed at 22 ºC.

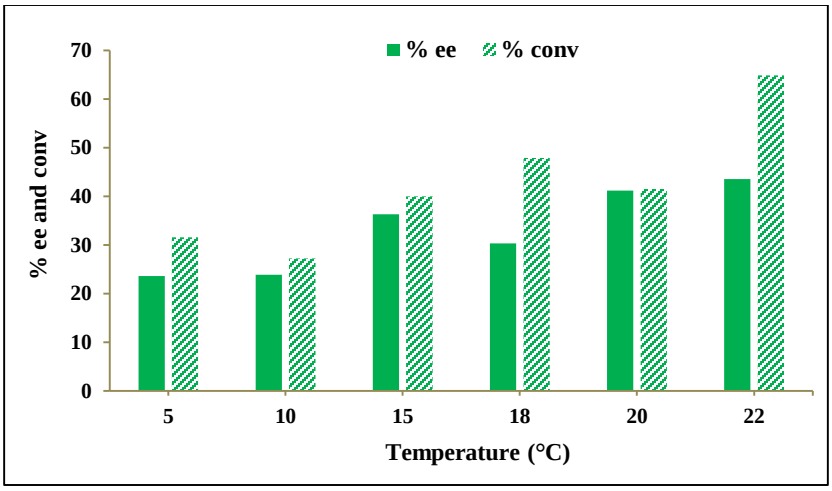


Figure 4.7: Effect of different temperature in synthesis of (*S*)-mandelonitrile

4.4.8. Effect of KCN concentration

To investigate the effect of KCN concentration in the enantioselective mandelonitrile synthesis, *Bm*HNL biocatalysis in the biphasic system was performed with different benzaldehyde to KCN molar ratio ranging from 1:5 to 1:150 mM separately. Highest % ee of 51.5 was obtained with 5 equivalent KCN, but the conversion was found to be very low i.e. 1.5% (**Figure 4.8**). Among the various selected ratios, with 1:125 equivalent of KCN it showed best % ee of 48.58 and good conversion i.e. 50%. Increased % conversion to 79.44 was observed with increase in benzaldehyde vs KCN ratio i.e. 1:150 however, the lowest % ee of ~30% was observed in this case.

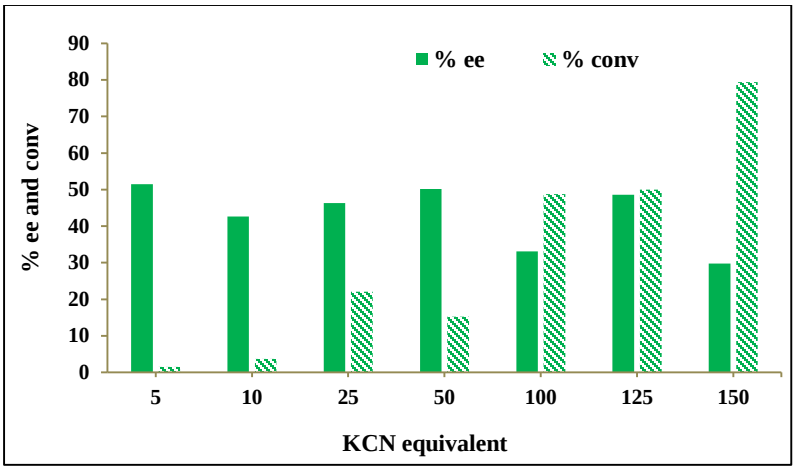
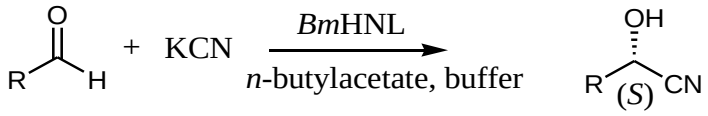


Figure 4.8: Effect of different benzaldehyde: KCN molar ratio in synthesis of (*S*)-mandelonitrile

4.4.9. Synthesis of (*S*)-cyanohydrins

Using crude *Bm*HNL in the optimized reaction conditions in a biphasic system, several aromatic aldehydes were converted to their corresponding (*S*)-cyanohydrins. **Table 4.1** summarizes the % ee and conversion of the synthesized products from each biotransformation.

Seventeen aldehydes (**1-17**) were converted into their corresponding cyanohydrins in a biphasic system under optimal biocatalytic conditions, using very less amount of the crude *Bm*HNL (6 U). The % conversion and ee of products of these reactions were analyzed by chiral HPLC. The biocatalysis has produced (*S*)-mandelonitrile of 48.5% ee and 65.5% conversion (**Figure 4.9a** and **4.9b**). In case of 4-bromobenzaldehyde, 75.2 % ee of product was observed with only 8.44% conversion. The enzyme showed good % ee of product with a few aromatic aldehydes. For 2-phenyl acetaldehyde (**Figure 4.9c** and **4.9d**), 4-benzyloxybenzaldehyde (**Figure 4.9e** and **4.9f**), 3-benzyloxybenzaldehyde and 4-hydroxybenzaldehyde, the % ee of the corresponding cyanohydrin was 58.8, 68.5, 38.5, and 53.2% respectively.



Scheme 4.1: CLEA *Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins

Table 4.1: Crude *Bm*HNL catalyzed synthesis of different chiral cyanohydrins

Substrate no	R	Reaction time (min)	% ee	% conv
1	Ph	10	48.5	65.5
2	3,5-di $MeOC_6H_3$	60	28	9.8
3	2,4-di $MeOC_6H_3$	30	23.6	72.8
4	2,5-di $MeOC_6H_3$	50	27.8	3.2
5	2,3,4-tri $MeOC_6H_2$	60	8.2	5.8
6	3,4,5-tri $MeOC_6H_2$	60	0.1	1.6
7	2-Naphthyl	30	1.7	1.8
8	Ph-CH_2	30	58.8	25.9
9	4-$PhCH_2O$-C_6H_4	60	68.5	2.2
10	3-PhO-C_6H_4	30	7.4	24.4
11	4-BrC_6H_4	30	75.2	8.4
12	*trans*-PhCH=CH	30	23.4	<1
13	9-Anthranyl	20	1.4	1.5
14	3-OHC_6H_4	60	12.5	4.4
15	3-$PhCH_2O$-C_6H_4	20	38.5	8
16	4-OHC_6H_4	30	53.2	<1

Moderate % ee was observed with another few substrates. The cyanohydrins of 3,5-dimethoxybenzaldehyde , 2,5-dimethoxybenzaldehyde and 2,4-dimethoxybenzaldehyde were synthesized in 28, 27.82 and 23.62% ee respectively. Crude *Bm*HNL catalyzed

synthesis of (*S*)-**5** (**Chapter 3.A**, No. **12** in **Table 4.1**) resulted in 23.43% ee. *Bm*HNL catalyzed synthesis of (*S*)-**2** (**Chapter 3.A**, No. **5** in **Table 4.1**) was produced in 8.2% ee while in case of 3-phenoxy benzaldehyde, the corresponding cyanohydrin i.e. (*S*)-**10** (Chapter 3.A) was produced in 7.4% ee. Crude *Bm*HNL produced 12.5% ee of (*S*)-**17** (**Chapter 3.A**, No. **14** in **Table 4.1**).

4.5. Discussion

4.5.1. Reaction time of *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in aqueous medium

*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in aqueous system has produced a maximum of 39% ee of (*S*)-mandelonitrile and 40.6% conversion in 10 minutes (**Figure 4.1**). Dadashipour *et al* reported the purified *Bm*HNL biocatalysis for 5 min and found 54% ee of (*S*)-mandelonitrile [8,16]. Kawahara *et al* carried out the biocatalysis with *Bm*HNL H103C/N156G for 10 minutes that produced (*S*)-mandelonitrile in 93% ee [17]. This indicates that the time of crude enzyme catalyzed biocatalysis is comparable with that of using purified and engineered *Bm*HNL. The % ee of products clearly increased with pure enzyme compared to crude while engineered enzyme showed higher % ee of product.

4.5.2. Effect of different organic solvents in synthesis of (*S*)-mandelonitrile

Enzymatic transformation is usually carried out in aqueous medium. Nevertheless, the aqueous medium biocatalysis is mostly accompanied by several limitations such as low organic substrate solubility, strenuous product recovery and undesired background reactions [20]. It has been observed that HNL catalyzed cyanohydrin synthesis in aqueous medium has higher content of racemic cyanohydrin formation due to the undesired background reaction, compared to that of in a biphasic or organic solvent system [12]. This

spontaneous racemic product formation in biotransformation is a major barrier in accomplishing high enantioselective cyanohydrin synthesis as it brings down enantiomeric excess of product which can be overcome moderately by employing organic solvent along with aqueous or biphasic system.

There exist many evidences that support the role of organic solvents in improved enantioselectivity of enzymes in biotransformation [12,13,21–26] as well as on their selectivity and activity [12,19,26,27]. As the *Bm*HNL catalyzed syntheses of (*S*)-cyanohydrins were limited to the use of aqueous system [8,16,17], we explored the effect of various organic solvents with log *P* from –0.33 to 3.5 (e.g. hexane=3.5, toluene=2.5, TBME=1.4, acetonitrile= –0.33, DIPE= 1.9, and *n*-butyl acetate= 1.7) [19,28] in *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile. Log *P* determines the polarity of the solvent. Mostly, solvents with log $P < 2$ (hydrophilic solvents) are not suitable for biotransformation. The probable reason for this is that these solvents may denature the enzyme by distorting the essential water layer that stabilizes the enzyme [29]. Among the four hydrophobic solvents e.g. hexane, toluene, DIPE and *n*-butyl acetate that were used in this study, the later three have shown a reasonable % ee of product i.e. 31-37%. Hexane although having higher log *P* value did not follow the same trend as observed by the other three. The exact reason for the same has not been further investigated. Acetonitrile, a polar organic solvent appears to follow the rule as it gave very low % ee and % conversion. Among the six organic solvents tested in the *Bm*HNL biphasic system biocatalysis, three of them i.e. *n*-butyl acetate, DIPE and toluene showed comparable % ee i.e. 37, 33 and 31 of (*S*)-mandelonitrile respectively (**Figure 4.2**), vs. 41% ee in presence of only citrate buffer pH 4.2. Both DIPE and *n*-butylacetate were already known to have superior role in

HNL catalyzed biotransformation. DIPE has been used mostly in HNL mediated stereoselective synthesis of cyanohydrins [21,30–37] while *n*-butyl acetate worked optimally in *At*HNL catalyzed synthesis of (*R*)-*β*-nitroalcohols [38]. Since *At*HNL and *Bm*HNL both belong to α/β hydrolase fold family, it may be possible to expect almost similar effect of DIPE and *n*-butylacetate on both the enzyme. Furthermore, this investigation revealed low % ee in case of hexane and acetonitrile while TBME didn't show any promising result compared to the best three mentioned above. A similar trend has been noticed in case of *At*HNL catalyzed synthesis of (*R*)-*β*-nitroalcohol for both TBME and hexane. In the present study, we successfully ascertained the role of organic solvent in *Bm*HNL biocatalysis. Use of *n*-butyl acetate reduced the spontaneous racemic mandelonitrile formation. While in case of buffer ~9% of (*R*) and (*S*)-mandelonitrile was observed, ~6.5% of each (*R*) and (*S*)-mandelonitrile appeared in the presence of *n*-butyl acetate in control reaction.

Griengl *et al* reported an increase in yield from 67 to 96% in case of *Hb*HNL catalyzed synthesis of (*S*)-mandelonitrile [13] while the yield increased from 50 to 77% for *Hb*HNL catalyzed hydrocyanation of *trans*-cinnamaldehyde in DIPE [13]. Further, their report also confirmed an increased % conversion from 67 to 97 with unchanged % ee of 99 in *Hb*HNL catalyzed synthesis of (*S*)-mandelonitrile using biphasic system (buffer and MTBE in 1:1 ratio) instead of aqueous medium [22]. In DIPE with 14% v/v 100 mM tartrate buffer pH 3.75, *Sb*HNL showed 87% conversion and 98% ee of (*S*)-mandelonitrile in 23 h at 5 ^{o}C in comparison to 96% ee and 80% conversion of product in 50 mM sodium citrate buffer pH 3.25 over 45 min [39,40].

4.5.3. Effect of different % of *n*-butyl acetate, DIPE and toluene in synthesis of (*S*)-mandelonitrile

In HNL biocatalysis, the use of organic solvents for enhancing stereoselectivity of the enzyme, conversion of product and minimizing side reaction is already reported. In order to find the effect of different % v/v of organic solvents in the *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile, varied % v/v of selected organic solvents i.e. *n*-butyl acetate, DIPE, and toluene were used in biocatalysis. 10% v/v *n*-butyl acetate showed highest i.e. 47.13% ee of (*S*)-mandelonitrile (**Figure 4.3**). Analysis of the effect of organic solvent and their ratio in HNL biocatalysis provides a broad spectrum of data with minimum correlation. For example, *At*HNL catalyzed (*R*)-cyanohydrin synthesis showed maximum % ee in 50% DIPE [14] while 50-100% DIPE gave best results in *Me*HNL catalyzed synthesis of (*S*)-cyanohydrins [21,25,35–37,41]. Other organic solvents such as 50% diethyl ether, DIPE, MTBE, and combination of MTBE: hexane in 40:60 ratio were reported in case of *Hb*HNL catalyzed (*S*)-cyanohydrin syntheses [13,19,22]. Impact of varied % of v/v of organic solvents has also been studied with HNLs of other than α/β hydrolase fold. *Dt*HNL catalyzed synthesis of (*R*)-cyanohydrins has been reported in 67% MTBE [15], while *Gt*HNL catalyzed synthesis of (*R*)-cyanohydrins has been carried out in 2:1 ratio of MTBE: buffer (66.67%) [42]. In case of *Ej*HNL and *Pe*HNL catalyzed synthesis of (*R*)-cyanohydrins, 80% diethyl ether and DIPE were used respectively [43,44]. The varied % ee of products obtained in HNL catalyzed transformation using different % v/v of organic solvents depended on both the enzyme and substrate being used. Studies to find out the improvement in % ee of product in HNL biocatalysis due to the addition of organic solvent could not be performed due to the lack of sufficient data to compare between experiments

involving the synthesis of chiral cyanohydrin with and without organic solvent. Our further optimization studies have been performed using 10% *n*-butyl acetate.

4.5.4. Optimization of substrate concentration

To elucidate the effect of different benzaldehyde concentration in the synthesis of (*S*)-mandelonitrile, *Bm*HNL biocatalysis was carried out with varied benzaldehyde concentrations (0.4 to 2.2 mM) in 10% v/v of *n*-butyl acetate. Among the different benzaldehyde concentrations, the highest % ee of (*S*)-mandelonitrile was observed with 0.6 mM benzaldehyde and highest % conversion was achieved with 0.8 mM. The % ee remained almost the same in case of benzaldehyde concentrations of 0.8 to 1.4 mM (**Figure 4.4**). Beyond 1.4 mM, decreased % ee, as well as % conversion, was observed which could be due to substrate inhibition of enzyme [8]. A similar trend was reported with *Hb*HNL catalyzed cyanohydrin synthesis. Hanefield *et al* reported *Hb*HNL inactivation and subsequently decreased ee with an increase in benzaldehyde concentrations from 5 to 16 mM [2]. They reported a rapid decrease in % ee even at 5 mM benzaldehyde concentration.

4.5.5. Effect of time of biotransformation

The time of *Bm*HNL biocatalysis of synthesis of (*S*)-mandelonitrile was studied. The highest % ee of (*S*)-mandelonitrile was observed in 10 min with both 0.8 and 1.4 mM benzaldehyde which were 48.2 and 43.23 respectively. The % ee decreased with increase in time. This decrease in enantioselectivity with increasing time after 10 min could be accounted to spontaneous non- enzymatic racemization of product. A similar trend of increase in % conv and a decrease in % ee was reported with *Hb*HNL catalyzed (*S*)-mandelonitrile synthesis in dibutyl ether (DBE) [19].

4.5.6. Effect of pH

To elucidate the effect of pH of buffers in the *Bm*HNL catalyzed stereoselective synthesis, biocatalysis was performed using buffers of pH ranging from 3.0 to 6.0 separately. The highest % conversion of 53 and comparable % ee of 46 was observed at pH 4.2. Though *Bm*HNL has been reported to be stable up to pH 7 and has optimum activity at pH 5.0, but in the present study utilizing organic solvent, we observed a drastic decrease in both % ee and conversion of (*S*)-mandelonitrile at and above pH 5. The reason for decreased % ee and conversion was the formation of racemic cyanohydrin at pH 5 and higher. However, certain HNLs that are stable at pH 5 or above, would produce high % ee of cyanohydrins which eventually counterbalance the decreased % ee caused by the non-enzymatic reaction. Hence, the impact of non-enzymatic reaction would vary for different HNLs. In order to eliminate the background reaction, pH less than or equal to 5 is preferentially used in the HNL catalyzed transformations. These observations were in agreement with *Chua*HNL catalyzed enantioselective synthesis of cyanohydrins [18] where increased % ee was observed at low pH. The most probable reason for such observation of increased % ee at lower pH could be due to suppression of non-enzymatic racemization of mandelonitrile. Considering the best results achieved for % ee and conversion, pH 4.2 was selected as the optimum pH for all further optimization experiments.

4.5.7. Effect of temperature

Temperature is also a key factor in the enantioselective synthesis of cyanohydrins alike pH [12,13,23,45]. To investigate the effect of temperature, *Bm*HNL biocatalysis was carried out at different temperatures between 5 to 22 ^{o}C. Among the tested temperatures, the

highest % ee of 44 and conversion of 65% were observed at 22 ^{o}C. Most of the HNL catalyzed enantioselective cyanohydrin syntheses were reported to be carried out between 0 to 25 ^{o}C. Report for *Bm*HNL catalyzed (*S*)-cyanohydrins synthesis at 22 ^{o}C already exists [8,16,17]. *Chua*HNL catalyzed synthesis of (*S*)-cyanohydrins were performed at 22 ^{o}C [18]. Forster *et al* reported *Me*HNL catalyzed synthesis of (*S*)-cyanohydrins at 25 ^{o}C [32]. *Hb*HNL catalyzed synthesis of (*S*)-cyanohydrins was performed at 15°C and synthesis of (*S*)-ketone cyanohydrins was reported at 0 ^{o}C [22].

4.5.8. Effect of KCN concentration

Aldehyde to cyanide molar ratio is another crucial factor in HNL catalyzed cyanohydrin syntheses. To elucidate the effect of ratio of benzaldehyde: KCN, *Bm*HNL catalyzed (*S*)-mandelonitrile synthesis was carried with different molar ratios of KCN. Optimum KCN molar ratio was found to be 125 equivalent in the present study where the % ee and conversion were 48.58 and 50% respectively. Decreased % ee of (*S*)-mandelonitrile was observed with increased KCN molar ratio. The exact reason for this trend is unclear however, the possible reason could be inhibition of enzyme at high KCN concentration. It has been reported that HCN being insoluble at high concentration results in its accumulation in the aqueous phase and hence enhances enzyme's inactivation [12,19]. Other HNLs are also known to be inhibited at higher HCN concentration e.g. *Hb*HNL inactivated beyond 1.5 M HCN that resulted in a decrease in % ee of product [12].

4.5.9. Synthesis of (*S*)-cyanohydrins

In the presence of organic solvent, 17 aldehydes were converted into their corresponding (*S*)-cyanohydrins. Among the seventeen, three substrates benzaldehyde, 3,5-

dimethoxybenzaldehyde and 4-bromobenzaldehyde (No. **1**, **2** and **11**, **Table 4.1**) have been reported to be used in the synthesis of the corresponding (*S*)-cyanohydrins using purified *Bm*HNL [8]. A comparison of % ee and conversion of products was carried out for these three substrates resulted from our study where 6 U of crude *Bm*HNL per reaction in biphasic media was used vs. the one reported by Asano and coworkers with purified *Bm*HNL in aqueous media. Our study produced 48.5, 28 and 75% ee of the (*S*)-cyanohydrins of benzaldehyde (**Figure 4.9a** and **4.9b**), 3,5-dimethoxybenzaldehyde and 4-bromobenzaldehyde, respectively while purified *Bm*HNL catalyzed transformation showed 54, 85 and 79% ee respectively. Although comparable % ee of the product was observed with two substrates, the poor enantioselectivity in case of synthesis of (*S*)-cyanohydrin of 3,5-dimethoxybenzaldehyde is not clear. Further, the % conversion of products in case of benzaldehyde, 3,5-dimethoxybenzaldehyde and 4-bromobenzaldehyde were reported to be 68, 28 and 47 with purified *Bm*HNL, however, our experiments produced 65, 10 and 8% conversion respectively. While comparable % conversion was observed in case of benzaldehyde as a substrate, a decrease in % conversion was noticed with the other two substrates. One probable reason for such low % ee and conversion could be due to the use of crude cell lysates however, it is unclear why such effect is applicable to specific substrates. *Bm*HNL is the only HNL known to synthesize (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile i.e. (*S*)-**13** (**Chapter 3.A**) either using both purified and crude enzyme extract. Crude *Pm*HNL catalyzed synthesis of (*R*)-**13** (**Chapter 3.A**) showed 97% ee and 17% yield in aqueous system [46]. The same cyanohydrin synthesized by *Pm*HNL in preparative scale using biphasic system showed 93% ee and 48% yield in 50 h. Other than *Bm*HNL, *Sb*HNL catalyzed synthesis of (*S*)-2-(4-bromophenyl)-2-

hydroxyacetonitrile i.e. (*S*)-**16** (**Chapter 3.A**) was reported in 2% v/v of 100 mM tartrate buffer pH 3.75 that resulted in 44% ee and 87% conv in 134 h at 5 °C while almond meal showed 98% ee and 97% conversion in 48 h [40]. *Me*HNL synthesized the same in biphasic medium in >99.5% ee and 90% conv in 22 h [37].

The rest fourteen substrates were converted into their respective (*S*)-cyanohydrins for the first time by *Bm*HNL and are reported here. In case of 2-phenyl acetaldehyde (**Figure 4.9c** and **4.9d**), 4-benzyloxybenzaldehyde (**Figure 4.9e** and **4.9f**), 3-benzyloxybenzaldehyde and 4-hydroxybenzaldehyde (No. **8**, **9, 15** and **16**, **Table 4.1**), *Bm*HNL showed relatively high % ee of their (*S*)-cyanohydrins i.e. 59, 68.5, 38.5 and 53.2% respectively. Synthesis of (*S*)-2-hydroxy-2-(4-hydroxyphenyl) acetonitrile [(*S*)-**18**, **Chapter 3.A**] by *Sorghum vulgare* HNL in DIPE produced it in 94% ee and 84% yield [30]. *Sb*HNL catalyzed synthesis of the same in aqueous medium showed 99% ee and 87% yield [47]. Kiljunen and Kanerva reported the synthesis of the same cyanohydrin using crude *Sb*HNL in 14% v/v tartrate buffer pH 3.75 in 68 h with 26% conversion and 98% ee [40]. *Me*HNL in a biphasic system synthesized this product from its corresponding aldehyde in 94% ee and 51% conversion [21].

Aromatic aldehydes with two substitutions in the aromatic ring i.e. 2,4-dimethoxybenzaldehyde, and 2,5-dimethoxybenzaldehyde (No. **3** and **4, Table 4.1**) produced poor % ee of their corresponding (*S*)-cyanohydrins i.e. 23.6 and 27.8% respectively. When *trans*-cinnamaldehyde was used, *Bm*HNL resulted in 23.4% ee of its (*S*)-cyanohydrin (No. **12**, **Table 4.1**). With bulky aromatic aldehydes like 2,3,4-trimethoxybenzaldehyde and 3-phenoxy benzaldehyde as substrates (No. **5** and **10**, **Table 4.1**), *Bm*HNL produced very low i.e. 8.2 and 7.4% ee of their (*S*)-cyanohydrins

respectively. This drastic decrease in enantioselectivity could be due to the bulkiness of the substrates especially multi substituted aromatic or due to the presence of another phenyl ring in case of phenoxy benzaldehyde. However, it also produced such low % ee in the synthesis of (*S*)-cyanohydrin of 3-hydroxybenzaldehyde [(*S*)-**17**, **Chapter 3.A**] i.e. 12.5%. The % conversion from aldehyde to chiral cyanohydrins by *Bm*HNL varied from substrate to substrate. While it was 65.5 and 72.8 in case of benzaldehyde and 2,4-dimethoxybenzaldehyde respectively, for other substrates % conversion decreased. Dadashipour *et al* have reported similar poor yield in case of *Bm*HNL catalyzed synthesis of cyanohydrins with few aldehydes [8]. The decrease in % conversion to cyanohydrins reported by us could be due to the use of organic solvents. Fushuku *et al* reported a decrease in yield with increasing % of organic solvent in the stereoselective synthesis of (*R*)-*β*-nitroalcohols by *At*HNL [38].

Effenberger *et al* reported *Sorghum vulgare* HNL catalyzed synthesis of (*S*)-**17** (Chapter 3.A) in DIPE in 91% ee and 97% yield [30]. Uwe *et al* reported the synthesis of this molecule by *Sb*HNL in 50 mM sodium citrate buffer pH 3.20 at 20 °C that gave 98% ee and 90% yield [47]. *Me*HNL synthesis of the same cyanohydrin in DIPE produced in high % ee i.e. 97% with 88% conv [21]. *Pa*HNL catalyzed kinetic resolution of the corresponding racemic cyanohydrin in 50 mM citrate buffer pH 3.5 synthesized it in 36% ee [48]. Buhler *et al* reported *Me*HNL catalyzed synthesis of (*S*)-2-hydroxy-3-phenylpropanenitrile i.e (*S*)-**8** (**Chapter 3.A**) in 98% ee and 99% conversion in a biphasic system [21]. Crude *Hb*HNL in 100 mM sodium citrate buffer pH 4.0 has been reported to synthesize the same cyanohydrin in 99% ee and 44% conversion [49]. (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile synthesis has been reported with HNLs other than *Bm*HNL.

In 0.1 M sodium citrate buffer pH 4.5 *Hb*HNL synthesized it in 99% ee and 9% conversion while in buffer/MTBE medium the % ee and % conversion both were found to be 99% [13,22,49]. *Sorghum vulgare* HNL catalyzed synthesis of this chiral molecule in biphasic medium has been reported to have 96% ee and 93% conversion [30]. *Me*HNL has also been reported to synthesize the same in 96% ee and 47% conv in biphasic medium [21] while another study revealed *Me*HNL catalyzed synthesis of it in 97% ee and 85% conv at high pH in biphasic system [36]. Synthesis of (*S*)-*trans*-cinnamaldehyde cyanohydrin has been reported with HNLs other than *Bm*HNL. *Hb*HNL in aqueous system produced (*S*)-*trans*-cinnamaldehyde cyanohydrin in 50% yield and 95% ee [49], however in biphasic medium it increased to 93% conv and 98% ee [22]. CLEA-*Me*HNL catalyzed synthesis of the same cyanohydrin in 2% v/v citrate buffer pH 5.5 and DIPE in 6.2 h showed 90% conv and 92% ee [50]. In a biphasic system (citrate buffer 5.5 and DIPE) guanabana seed meal carrying crude HNL showed 11% conv and 82% ee of (*S*)-*trans*-cinnamaldehyde cyanohydrin [51].

To the best of our knowledge eight (*S*)-cyanohydrins e.g. (*S*)-2-hydroxy-2-(2,4-dimethoxyphenyl) acetonitrile, (*S*)-2-hydroxy-2-(2,5-dimethoxyphenyl)acetonitrile, (*S*)-2-hydroxy-2-(2,3,4,-trimethoxyphenyl)acetonitrile, (*S*)-2-hydroxy-2-(3,4,5-trimethoxyphenyl)acetonitrile, (*S*)-2-(4-(benzyloxy)phenyl)-2-hydroxyacetonitrile, (*S*)-2-(anthracen-9-yl)-2-hydroxyacetonitrile, (*S*)-2-(3-(benzyloxy)phenyl)-2-hydroxyacetonitrile and (*S*)-2-(4-(allyloxy)phenyl)-2-hydroxyacetonitrile (Figure 4.9g and 4.9h) have not been synthesized by any other (*S*)-selective HNL except this report.

4.6. Conclusions

Asymmetric synthesis of cyanohydrins by *Baliospermum montanum* HNL in two-phase system was investigated. Crude *Bm*HNL was used in the enantioselective synthesis of different cyanohydrins under optimized conditions. Optimization of different biocatalytic parameters i.e. different organic solvents and their %, substrate concentration, pH, temperature, time of biotransformation, and ratio of aldehyde to KCN concentration were carried out with the standard reaction of biocatalytic transformation of benzaldehyde to (*S*)-mandelonitrile, to obtain high % ee and conversion of product. The optimized reaction conditions were: 6 U of crude *Bm*HNL, 0.8 mM substrate, 1:125 molar ratio of aldehyde: KCN, 10% v/v of *n*-butyl acetate, 60% of 300 mM citrate buffer pH 4.2 and 22 ^{o}C. Seventeen different chiral cyanohydrins were synthesized under optimal biocatalytic conditions. Although the % conversion and ee varied for different products, up to 75% of ee and 72% of conversion of product was achieved. Fourteen (*S*)-cyanohydrins were synthesized by *Bm*HNL for the first time and among them, eight have not been synthesized by any other (*S*)-selective HNL except this report.

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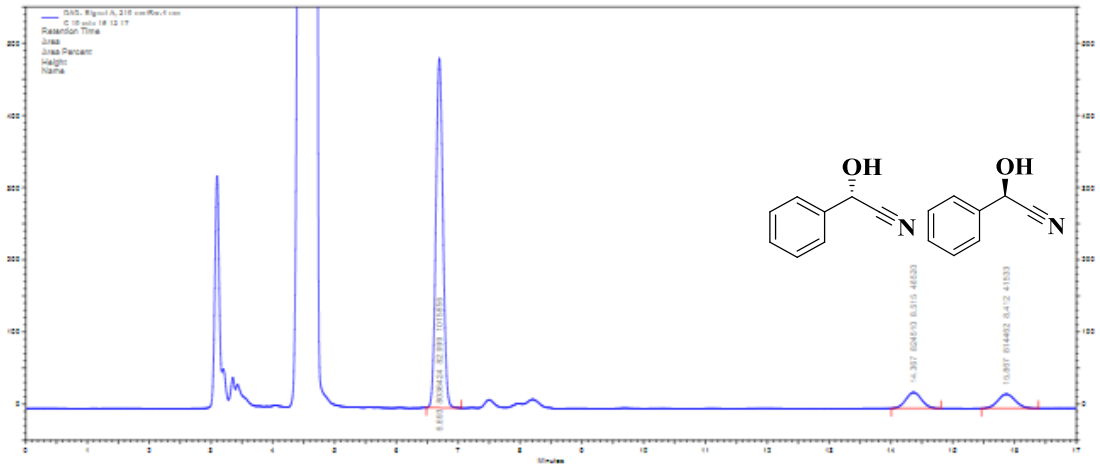
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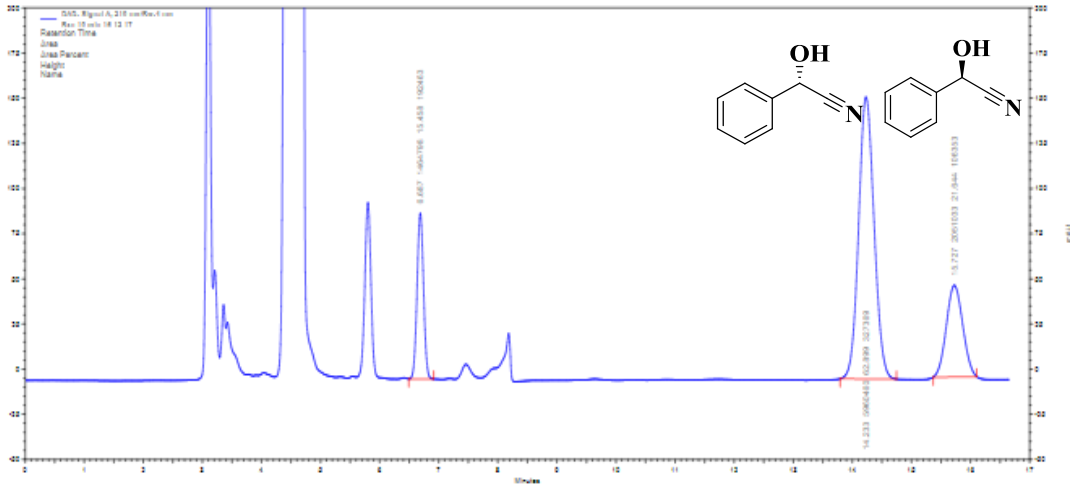
(a)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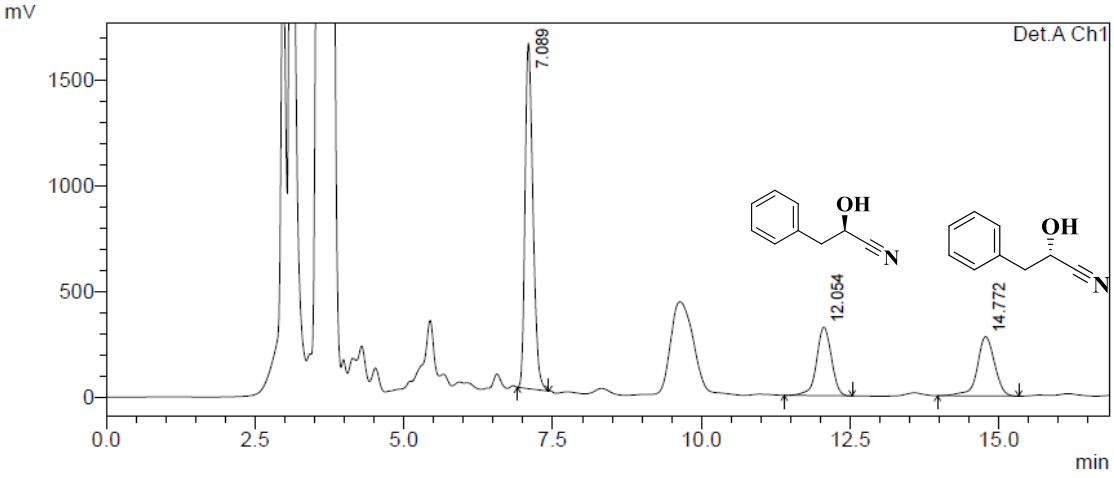
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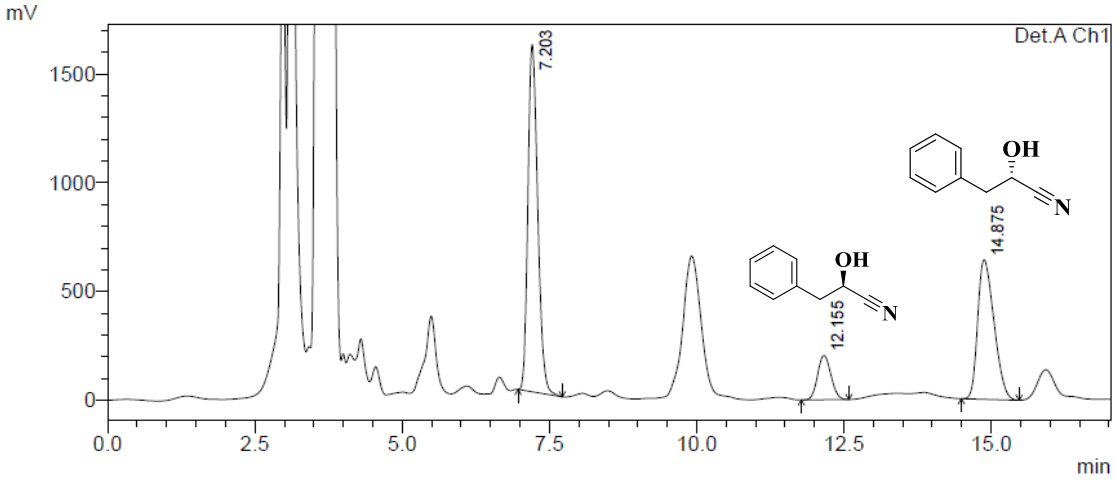
min

(c)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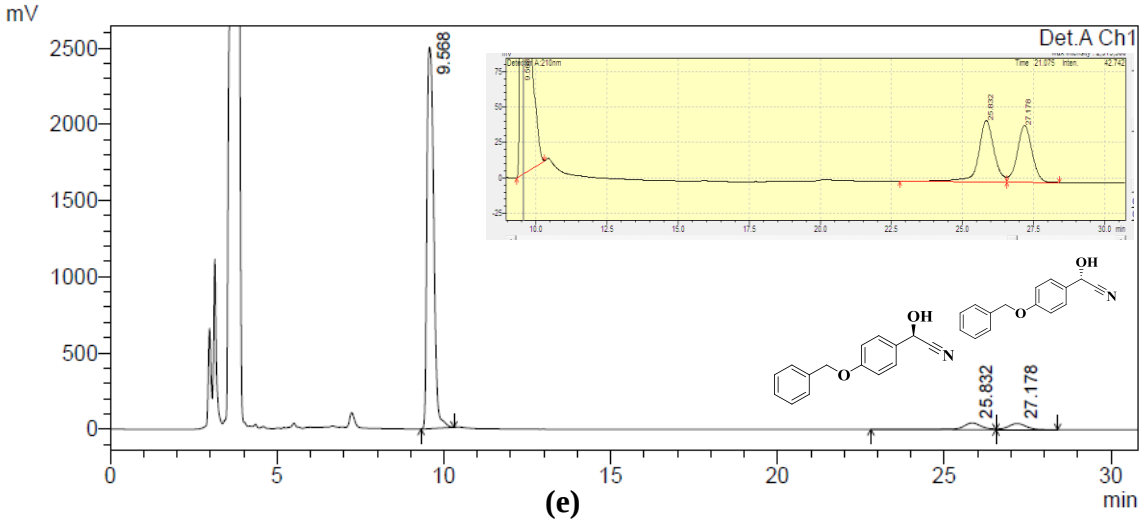
min

(d)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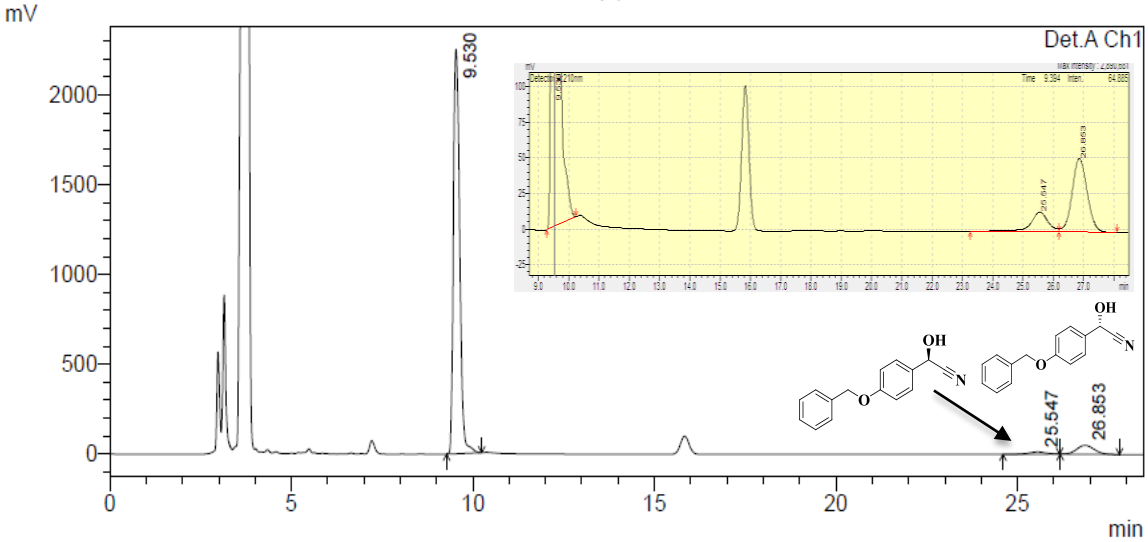
20
25
30
min

(e)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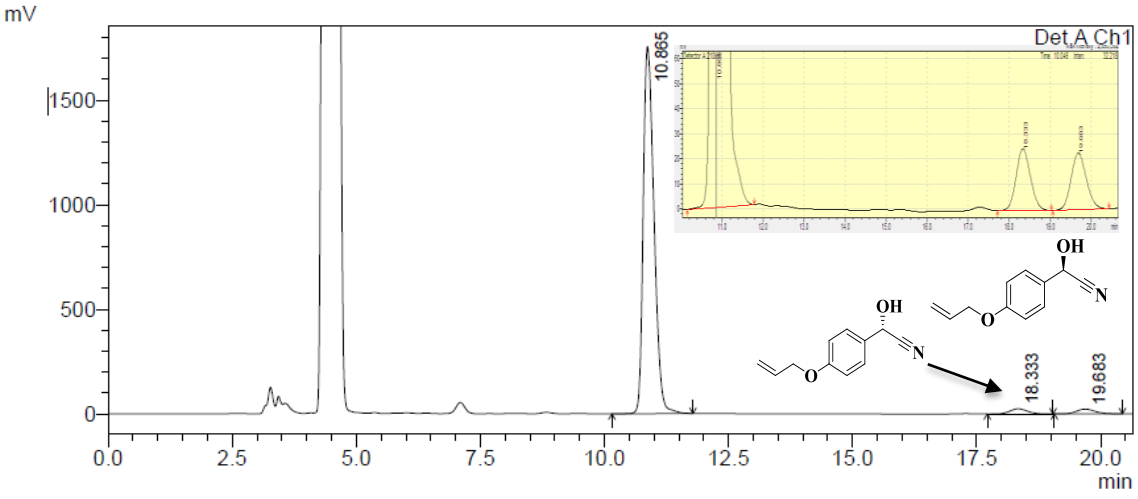
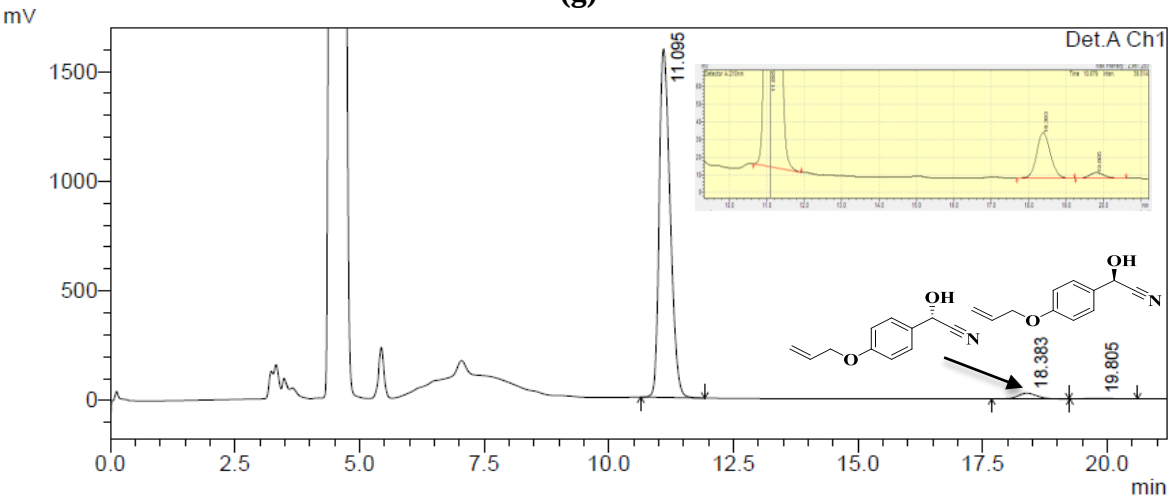
min

(f)



(g)



(h)

Figure 4.9: HPLC chromatogram of Crude *Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins. (**a**) and (**b**) represent control and reaction in the synthesis of (*S*)-2-hydroxy-2-phenylacetonitrile respectively; (**c**) and (**d**) represent control and reaction in the synthesis of (*S*)- 2-hydroxy-3-phenylpropanenitrile; (**e**) and (**f**) represent control and reaction in the synthesis of (*S*)-2-hydroxy-2-(4-benzyloxyphenyl)acetonitrile; (**g**) and (**h**) represent control and reaction in the synthesis of (*S*)-2-hydroxy-2-(4-(allyloxy)phenyl)acetonitrile.

Chapter 5

Immobilized Baliospermum montanum hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins

5.1. Introduction

HNLs catalyze cyanogenesis in vivo but also catalyze its reverse reaction i.e. synthesis of chiral cyanohydrins in the laboratory conditions [1–3]. Chiral cyanohydrins are important precursors in the synthesis of many pharmaceutical intermediates, agrochemicals and fine chemicals [1,2,4]. The synthesis of chiral cyanohydrins can be carried out using chemical catalyst and biocatalysts. Compared to the use of chemical catalysts biocatalytic synthesis of chiral cyanohydrin has several advantageous. Biocatalytic processes use eco-friendly catalysts, ambient reaction conditions, cost-effective and mostly follow green chemistry conditions. Heavy metals are commonly used as chemical catalyst in the asymmetric synthesis of cyanohydrins while lipases, esterases, and HNLs are the biocatalysts in chiral cyanohydrin synthesis. Wide application of these enantiopure cyanohydrins and the environment-friendly method that the HNL catalysis provides, both enhances the significance of HNL research. Therefore there is a growing demand for discovery of new HNLs, [3,5–9] enzymatic methods to synthesize chiral cyanohydrins as well as improve enzyme's biocatalytic properties. Among the several α/β hydrolase fold HNLs reported, *Bm*HNL has shown unique biocatalytic features in terms of its substrate preference [10,11]. *Bm*HNL accepts aromatic aldehydes because of the presence of hydrophobic residues in

its binding pocket. However, *Bm*HNL has not been explored in biocatalysis unlike its other HNL counterparts of α/β hydrolase fold family.

Non-enzymatic racemization of cyanohydrin at pH 5 or above is the major limitation in HNL biocatalysis which limits the enantiomeric excess of enzymatic product. To avoid this problem, HNL biocatalysis is usually carried out at (i) low pH, (ii) low temperature and (iii) in the presence of organic solvent. Use of organic solvent helps in lowering the substrate concentration in aqueous phase [12] which could be a reason for minimizing the racemic cyanohydrin formation. Further, it helps in product extraction of product and may minimize degradation of product. However, enzyme stability is a major issue in organic solvent and low pH. Stability of *Bm*HNL in organic solvent has not been investigated while Asano and coworkers have reported its pH stability for 1 h even at lower pH i.e. 3.5 [11]. Possible solutions to both these issues are (i) engineering *Bm*HNL to improve its pH stability and organic solvent tolerance, or (ii) immobilization of *Bm*HNL which may improve these two properties. Reusability of a biocatalyst is an important criteria for its industrial application, as it makes the process more economic. Considering all these important properties, we have studied immobilization of *Bm*HNL using cross-linking method.

Cross-linked enzyme aggregates (CLEA) is an important method of immobilizing enzymes which has several advantages e.g. high enzyme loading, less leaching, no requirement of purified protein and cost-effective etc [13]. CLEA involves cross-linking protein molecules with each other without the interference of carrier and hence the enzymes get better access to the substrate. CLEA of many HNLs e.g. *Prunus amygdalus* (*Pa*HNL), *Manihot esculenta* (*Me*HNL), *Hevea brasiliensis* (*Hb*HNL), *Linum usitatissimum* (*Lu*HNL), *Prunus*

dulcis (*Pd*HNL), and *Davallia tyermannii* (*Dt*HNL) has been reported [12,14–19]. CLEA-HNLs are known to improve several biocatalytic properties e.g. reusability, organic solvent tolerance, activity, and enantioselectivity in the chiral cyanohydrin synthesis. We describe here for the first time preparation and characterization of CLEA of *Bm*HNL and also its biocatalytic application in the synthesis of several (*S*)-cyanohydrins.

5.2. Objectives

- To optimize the process of preparation of immobilized *Bm*HNL.
- To prepare immobilized *Bm*HNL and characterize.
- To optimize the biocatalytic parameters of immobilized *Bm*HNL catalyzed synthesis of chiral cyanohydrins.
- To synthesize optically pure cyanohydrins using immobilized *Bm*HNL under optimal biocatalytic parameters and chiral analysis of the products.

5.3. Experimental

5.3.1. Chemicals and materials

*Bm*HNL (LOCUS: AB505969) synthetic gene was sub-cloned into pCold1 and expressed as per **Chapter 2**. Glutaraldehyde was obtained from Molychem, India. Mandelonitrile was purchased from Sigma Aldrich. Aldehydes were purchased from Sigma Aldrich, AVRA, SRL and Alfa-Aesar. Internal standards were synthesized as per **Chapter 3.A**. HPLC grade solvents were obtained from RANKEM, Molychem, FINAR, and SRL.

5.3.2. Preparation of crude enzyme extract

*Bm*HNL expression and cell-lysate preparation were carried out as per **Chapter 2**. The resulting cell lysate was used as crude enzyme and its protein content was measured by a Nanodrop.

5.3.3. Preparation of *Bm*HNL aggregates

*Bm*HNL aggregate was prepared by addition of 9 volume of precipitating agent into 1 volume of cell lysate. To pursue this, eight different precipitating agents were selected for the study. The precipitants used were ammonium sulfate (AS), *tert*-butyl methyl ether (TBME), acetone, acetonitrile (AcN), methanol, isopropyl alcohol (IPA), dimethylformamide (DMF) and 1,2-dimethoxyethane (DME). Each of the eight mixtures was incubated at 4 °C for 30 minutes on a rocker and then centrifuged at 12857*g*, 4 °C for 30 minutes. The supernatant and pellet were separated. Each pellet was re-suspended in 20 mM potassium phosphate buffer (pH 7.0). The protein content in supernatant and pellet was measured by Nanodrop followed by their HNL activity. A control experiment in a similar manner was also performed with 20 mM KPB pH 7.0 in place of crude *Bm*HNL.

5.3.4. Optimization of ratio of cross-linking agent

To optimize the ratio between enzyme and cross-linking agent, five best precipitating agents i.e. AS, TBME, AcN, IPA and DME of section 3.3 were selected. 25% solution of glutaraldehyde was used as cross-linking in this study. To the mixture of crude *Bm*HNL and precipitating agent (1:9 v/v), the cross-linker was added in 0 to 6 volumes. This resulted

in *Bm*HNL: precipitating agent: cross-linker in 1:9:0 to 1:9:6 v/v. Each of this mixture was kept on a rocker at low speed at 4 °C for 6 h and then centrifuged at 12857*g*, 4 °C for 30 minutes. The supernatant was removed and pellet was washed with 20 mM potassium phosphate buffer pH 7.0. HNL activity of the pellet i.e. CLEA-*Bm*HNL was measured.

5.3.4.1. Preparation of CLEA-*Bm*HNL under optimized conditions

CLEA-*Bm*HNL was prepared in a preparative scale by adding 1 volume of cell lysate of *Bm*HNL, 9 volumes of IPA and 4 volumes of 25% (v/v) solution of glutaraldehyde. The mixture was kept after shaking at 4 °C for 6 h over a rocker and centrifuged at 12857*g*, 4 °C for 30 minutes. The pellet was washed twice with 20 mM potassium phosphate buffer pH 7.0. The CLEA-*Bm*HNL thus obtained was re-suspended in 20 mM potassium phosphate buffer pH 7.0 and used for experiments.

5.3.5. Characterization of CLEA-*Bm*HNL

5.3.5.1. CLEA-*Bm*HNL characterization by SDS-PAGE

The CLEA-*Bm*HNL prepared and wash fraction of CLEA-*Bm*HNL were loaded on a 12% polyacrylamide gel in SDS-PAGE and stained by silver staining.

5.3.5.2. CLEA-*Bm*HNL characterization by scanning electron microscope

The CLEA-*Bm*HNL sample was air-dried over the slide and coated with Au metal using sputter for scanning electron microscopy (SEM) using ZEISS, Merlin compact 30 kVA microscope. Scanning electron microspore images were taken for CLEA-*Bm*HNL at various magnifications.

5.3.6. HNL assay

HNL activity was monitored using a Multiskan GO UV-Visible spectrophotometer at 25 °C. The assay was carried out in a cuvette with 1 mL of total reaction volume. The reaction mixture contained 850 µL of 50 mM citrate-phosphate buffer pH 5.0, 50 µL of diluted CLEA-*Bm*HNL (200 µg) and 100 µL of 70 mM of racemic mandelonitrile in 5 mM citrate buffer pH 3.15. The assay measured the formation of benzaldehyde resulted by enzymatic cleavage of mandelonitrile at 280 nm. A control experiment was carried out in an identical manner except the enzyme was replaced with 20 mM potassium phosphate buffer pH 7.

HNL activity of crude *Bm*HNL and *Bm*HNL aggregates was carried out as per section **2.4.14** of **Chapter 2**.

5.3.7. Synthesis of racemic cyanohydrins

Synthesis of racemic cyanohydrins was carried out using three different methods as per **Chapter 3** and they were used as analytical HPLC standards.

5.3.8. Effect of reaction time in the enantioselective synthesis of (*S*)-mandelonitrile

Effect of time of biotransformation in the enantioselective synthesis of mandelonitrile was studied. The reaction mixture contained 7 U of CLEA-*Bm*HNL (48 mg CLEA-*Bm*HNL), 100 µL of 1 M KCN in double distilled water (ddH_2O), 40 µL of 20 mM of benzaldehyde (0.8 mM final concentration) in dimethyl sulfoxide (DMSO) and 768 µL of 300 mM citrate buffer pH 4.2. The reaction was carried out in a thermomixer by incubating at 22 ºC, 1000 rpm up to 45 minutes.

The % of ee of a product was calculated using formula [10]:

$$\% \text{ of ee of a product} = \{\frac{[(S-MN)rxn-(S-MN)control]-[(R-MN)rxn-(R-MN)control]}{[(S-MN)rxn-(S-MN)control]+[(R-MN)rxn-(R-MN)control]}\}*100$$

$$\% \text{ conversion} = \{[\frac{(S-MN)+(R-MN)}{(BA)+(S-MN)+(R-MN)}]_{rxn} - [\frac{(S-MN)+(R-MN)}{(BA)+(S-MN)+(R-MN)}]_{control}\}*100.$$

(*S*-MN)$_{rxn}$ and (*S*-MN)$_{control}$ represent the area of (*S*)-mandelonitrile peak in the biotransformation and control respectively. Similarly, (*R*-MN)$_{rxn}$ and (*R*-MN)$_{control}$ represent area of (*R*)-mandelonitrile peak in the HPLC. BA: peak area of benzaldehyde.

5.3.9. Optimization of substrate concentration

To investigate the optimum substrate concentration for the enantioselective synthesis of mandelonitrile, varying concentrations of benzaldehyde was used in the biotransformation. The reaction mixture of 1 mL total contained 7 U of CLEA-*Bm*HNL, 40 µL of benzaldehyde of 10 to 55 mM stock solution in DMSO which is equivalent to a final concentration 0.4 to 2.2 mM, 100 µL of 1 M KCN in ddH_2O and 768 µL of 300 mM citrate buffer pH 4.2. Biocatalysis was carried out in a thermomixer by incubating the reaction mixture at 22 ºC, 1000 rpm. After 20 min, 1 mL of hexane: IPA (90:10) was added to it. The organic extract was analyzed by chiral HPLC in a Chiralpak IE column using hexane: IPA to find the % conversion and ee of mandelonitrile synthesized.

5.3.10. Optimization of amount of CLEA-*Bm*HNL

Optimum amount of CLEA-*Bm*HNL in the enantioselective synthesis of (*S*)-mandelonitrile was found out by varying enzyme units against two substrate concentrations i.e. 0.4 and

1.2 mM. The enzyme amount was varied from 5 to 15 U. Two different sets of reactions were carried out for different substrate concentrations. Total 1 mL reaction mixture contained CLEA-*Bm*HNL (5-15 U), 40 µL of 10 or 30 mM stock solution of benzaldehyde in DMSO, 100 µL of 1 M KCN in ddH_2O and rest amount of 300 mM citrate buffer pH 4.2. Biocatalysis conditions and HPLC analysis of products was as described earlier.

5.3.11. Effect of organic solvents in the biotransformation

Effect of different organic solvents in the enantioselective synthesis of mandelonitrile was studied. To a 368 µL of 300 mM citrate buffer pH 4.2, 7 U of CLEA-*Bm*HNL, 40 µL of 30 mM benzaldehyde in DMSO, 100 µL of 1 M KCN in ddH_2O, and 400 µL of an organic solvent (40% v/v) were added. Six different organic solvents e.g. hexane, toluene, *n*-butyl acetate, *di*-isopropyl ether (DIPE), TBME and AcN were used in this optimization study. Biocatalysis conditions and HPLC analysis of products were the same as described earlier.

5.3.12. Effect of ratio of organic solvent

The effect of ratio of organic solvent to buffer v/v in the biotransformation mixture was studied in the CLEA-*Bm*HNL catalyzed enantioselective synthesis of (*S*)-mandelonitrile. Among the organic solvents used in section **5.3.11**, the one showed the best result i.e. toluene was chosen for this study. The biocatalysis conditions were kept identical to the previous experiment, except the % volume of toluene in the biotransformation was varied from 30 to 78 of the total volume.

5.3.13. Effect of buffer pH

Optimum pH of the CLEA-*Bm*HNL catalyzed enantioselective synthesis of (*S*)-mandelonitrile was investigated by varying the pH of the buffer used in biocatalysis. The reaction mixture composed of 7 U of CLEA-*Bm*HNL, 1.2 mM benzaldehyde in DMSO, 100 µL of 1 M KCN in ddH_2O and 768 µL of 300 mM citrate buffer of different pH i.e. 3, 3.5, 4.2, 5, 5.5, and 6. Biocatalysis conditions and HPLC analysis of products was as described earlier.

5.3.14. Reusability of CLEA-*Bm*HNL

Reusability of CLEA-*Bm*HNL was determined by repeated use of the biocatalyst in optimized condition to synthesize (*S*)-mandelonitrile. The optimal biocatalytic condition includes 7 U of CLEA-*Bm*HNL, 1.2 mM benzaldehyde, 100 µL of 1 M KCN in ddH_2O (100 mM) and 768 µL of 300 mM citrate buffer pH 4.2 incubated in a thermomixer at 22 ºC, 1000 rpm for 20 minutes. After 20 minutes, the reaction mixture was centrifuged at 14,500*g*, 4 ºC for 1 min. The supernatant was extracted using 1 mL of hexane: isopropanol (90:10). The pellet thus remained was used for the subsequent cycle of biocatalysis that was performed keeping all other parameters identical to the optimal condition. Ten cycles of biocatalysis were performed. In each cycle, the reaction composition was kept the same while the CLEA-*Bm*HNL of the previous cycle was used for the successive round without wash.

5.3.15. Synthesis of (*S*)-cyanohydrins using CLEA-*Bm*HNL

The CLEA-*Bm*HNL was used in the enantioselective synthesis of different cyanohydrins under optimized conditions i.e. 7 U of CLEA-*Bm*HNL, 1.2 mM of an aldehyde, 100 µL of

1 M KCN in ddH_2O and 768 µL of 300 mM citrate buffer pH 4.2. Separate biocatalysis was performed using different aldehydes as substrates to prepare their corresponding chiral cyanohydrins. As the reaction time of different substrates would differ from benzaldehyde, the corresponding biocatalysis was carried out for 20-120 minutes. Percentage ee and conversion of each biocatalysis at different time was determined by chiral HPLC.

5.4. Results

5.4.1. CLEA-*Bm*HNL preparation

5.4.1.1. Preparation of *Bm*HNL aggregates

Enzyme aggregates with eight different precipitants were prepared and tested for their HNL activity. Highest activity of 1.51 U/mg was observed in case of the precipitant AS (**Figure 5.1**). *Bm*HNL aggregates resulted from AcN showed 1.43 U/mg specific activity while it was 1.41 U/mg in case of TBME. IPA and DME showed specific activity of 1.2 and 1.19 U/mg respectively.

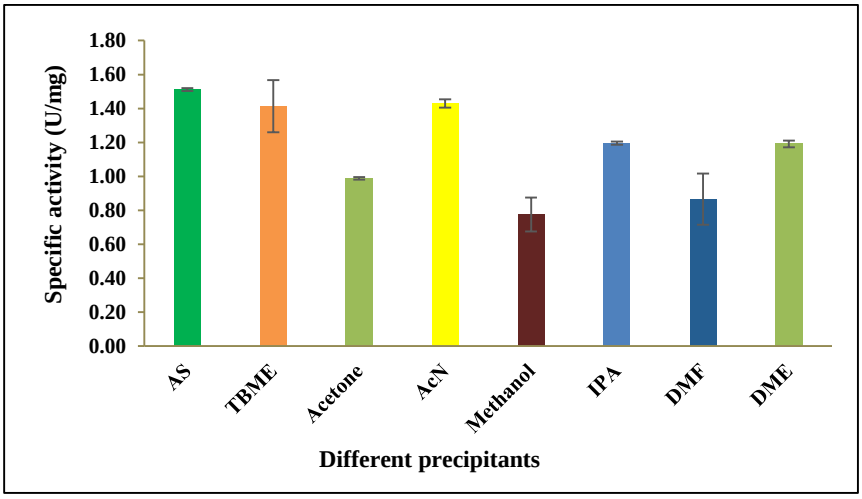


Figure 5.1: Effect of different precipitating agents

5.4.1.2. Preparation of cross-linked enzyme aggregate of *Bm*HNL

To find out optimal enzyme to cross-linker ratio, different glutaraldehyde ratio was used in preparation of CLEA-*Bm*HNL with five best precipitants i.e. AS, TBME, AcN, IPA and DME as per section **5.3.4**. The study revealed that 9 volume of IPA and 4 volume of glutaraldehyde along with 1 volume of cell lysate i.e. 1:9:4 showed highest specific activity (**Figure 5.2**) which was 1.52 U/mg. In absence of glutaraldehyde i.e. enzyme: IPA: glutaraldehyde ratio 1:9:0, it showed 1.1 U/mg. For ratios of 1:9:2 to 1:9:6 the specific activity was in the range of 1.11 to 1.31 U/mg respectively. AS without glutaraldehyde (1:9:0) showed the highest activity i.e. 1.3 U/mg which is higher as compared to the case of IPA without glutaraldehyde (1:9:0) but after addition of glutaraldehyde into AS, its specific activity decreased for all the tested ratios.

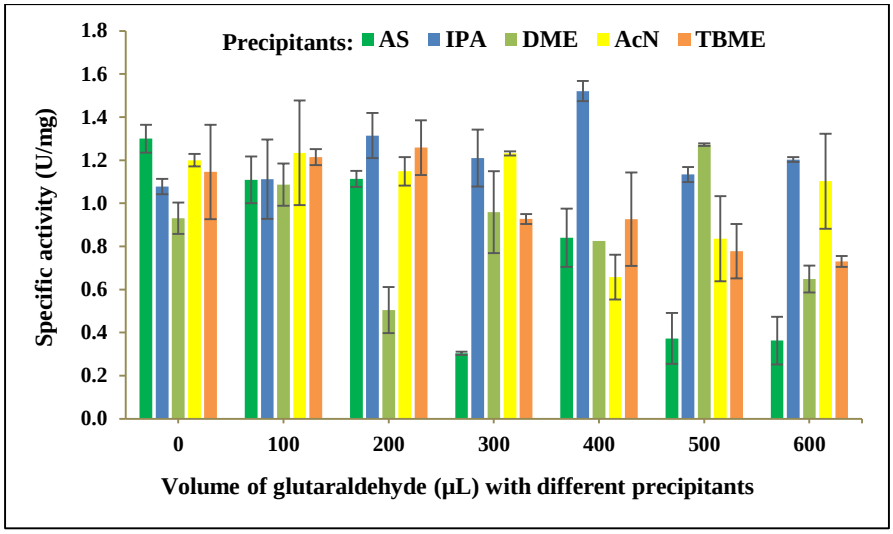


Figure 5.2: Effect of volume of glutaraldehyde along with different precipitants

Having optimized conditions for CLEA preparation i.e. 1:9:4 v/v of *Bm*HNL cell lysate: IPA: glutaraldehyde, cross-linking time: 6 h and at 4°C, we prepared CLEA-*Bm*HNL at preparative scale. CLEA-*Bm*HNL preparation was repeated several time in the laboratory

successfully. CLEA-*Bm*HNL with protein content 78.56 mg/mL and specific activity of 1.38 U/mg was used for successive experiments.

5.4.2. Characterization of CLEA-*Bm*HNL

5.4.2.1. SDS-PAGE analysis of CLEA-*Bm*HNL

SDS-PAGE analysis of CLEA-*Bm*HNL was carried out. Samples consisting of crude *Bm*HNL, partially purified *Bm*HNL, wash obtained during CLEA-*Bm*HNL preparation, CLEA-*Bm*HNL and purified *Bm*HNL were used (**Figure 5.3**). A band of ~29 kDa that appeared for pure *Bm*HNL was also seen in case of CLEA-*Bm*HNL. This confirmed the presence of enzyme in the CLEA-*Bm*HNL, while the band was not observed in case of wash.

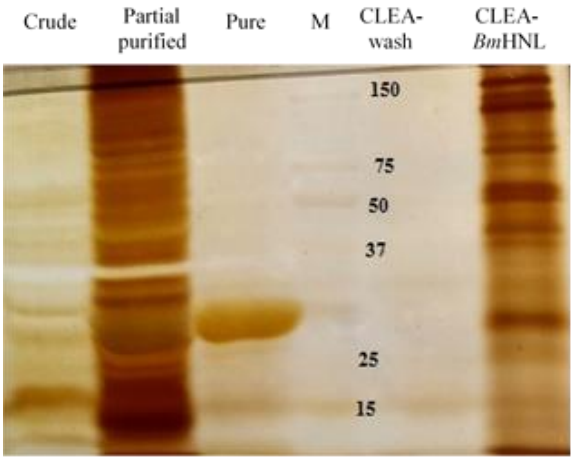


Figure 5.3: SDS-PAGE analysis 1: *Bm*HNL cell lysate, 2: Partial purified *Bm*HNL, 3: Pure *Bm*HNL, 4: Marker, 5: CLEA-*Bm*HNL wash, 6: CLEA-*Bm*HNL (silver stained)

5.4.2.2. Scanning electron microscope analysis of CLEA-*Bm*HNL

The FESEM images of CLEAs at different crosslinking times showed the aggregation of spherical CLEA particles (**Figure 5.4**). The porous structure with cavities was formed wherein few spherical particles were embedded. The amorphous structure with high surface area gradually increased as a consequence of extended crosslinking time.

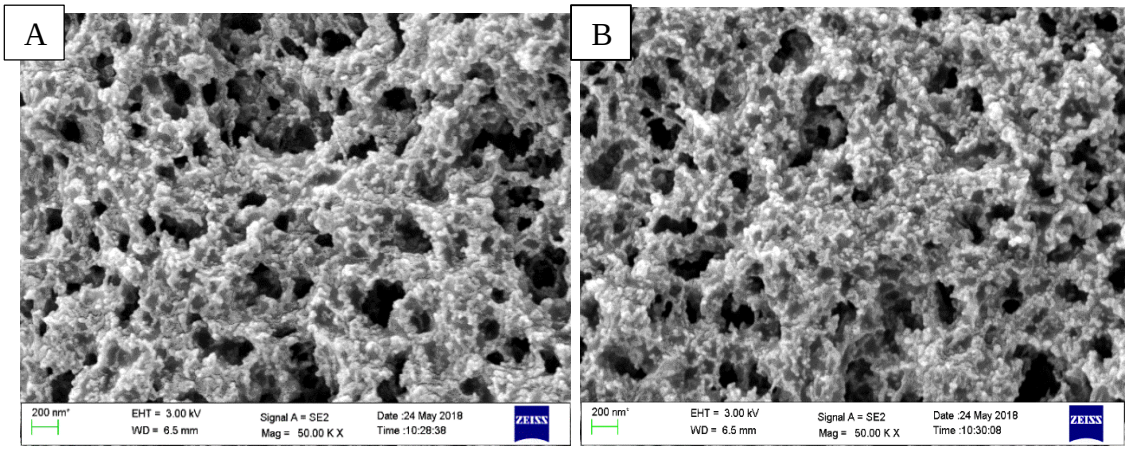


Figure 5.4 (A-B): SEM image of CLEA-*Bm*HNL prepared under optimized conditions

5.4.2.3. Yield, efficiency and activity recovery of CLEA-*Bm*HNL

Sheldon and Pelt reported that yield, efficiency and activity recovery are the terms used to describe the success of immobilization of an enzyme [13]. Yield, efficiency and activity recovery were calculated using the following three equations.

% yield = (immobilized activity/starting activity)*100

% efficiency = (observed activity/immobilized activity)*100

% activity recovery = (observed activity/starting activity) *100

Note: Immobilized activity = crude *Bm*HNL activity – activity of the supernatant resulted during CLEA preparation, starting activity = crude *Bm*HNL activity, and observed activity = CLEA-*Bm*HNL activity.

Table 5.1: Enzymatic activity of crude *Bm*HNL, CLEA-*Bm*HNL and supernatant of CLEA-*Bm*HNL

Enzyme or supernatant	Total volume (mL)	*Bm*HNL protein concentration (mg/mL)	Specific activity (U/mg)	Total activity (U)
Crude *Bm*HNL	12	21	1.36	342.72
CLEA-*Bm*HNL	6	23.33	1.02	142.78
Supernatant after CLEA prepared	98	0.0719	–ve	–ve

Yield = 100%, efficiency = 41.66% and activity recovery = 41.66%.

5.4.3. Optimization of biocatalytic parameters for CLEA-*Bm*HNL catalyzed enantioselective synthesis of (*S*)-mandelonitrile

5.4.3.1. Reaction time

CLEA-*Bm*HNL catalyzed enantioselective synthesis of mandelonitrile at different time intervals was investigated. Highest % ee 90 along with 46.68% conversion of product was observed in 20 min (**Figure 5.5**). Although highest % conversion i.e. ~51% was observed in 15 min, but the enzyme showed only 73% ee. In 25 min, the enzyme showed 41.57% conversion and 87.4% ee of (*S*)-mandelonitrile. The % ee and conversion both were decreased with time to 41 and 38% respectively in 45 min.

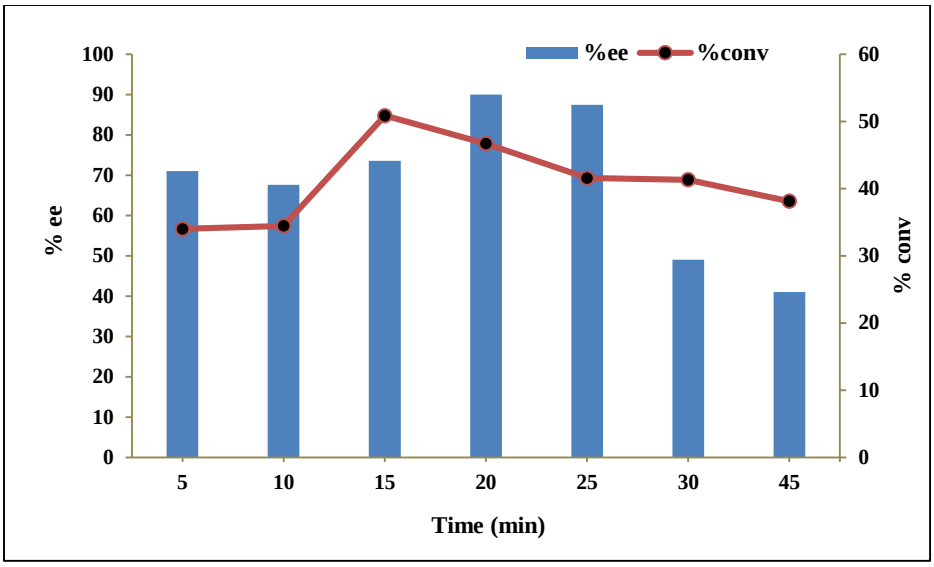


Figure 5.5: Time of biotransformation

5.4.3.2. Substrate concentration

To investigate the effect of different benzaldehyde concentrations, CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was carried out (**Figure 5.6**) for 20 min. Benzaldehyde concentration was varied from 0.4 to 2.2 mM in different biocatalysis.

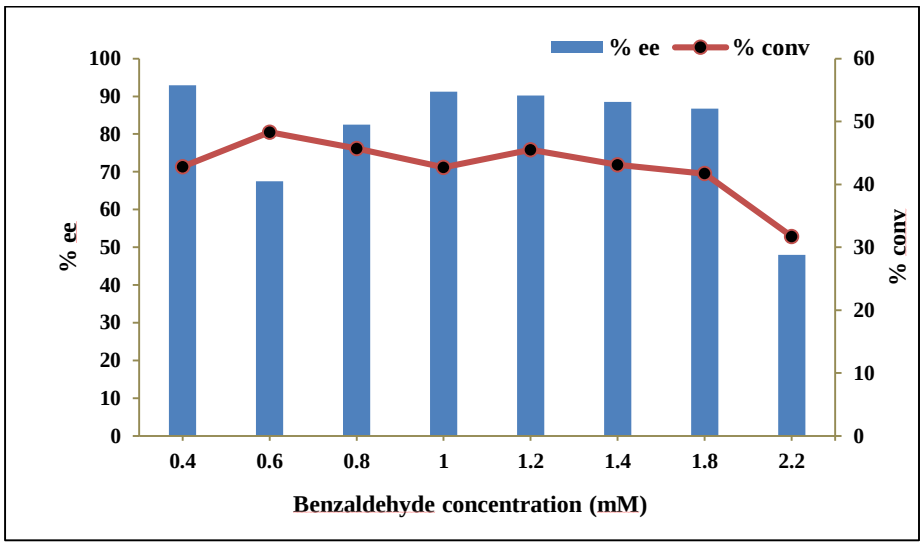


Figure 5.6: Effect of benzaldehyde concentration in the synthesis of (*S*)-mandelonitrile

The highest % ee was observed in case of 0.4 mM benzaldehyde. CLEA-*Bm*HNL showed highest ~93% ee and 42.8% conversion with 0.4 mM benzaldehyde. The enzyme also showed high % ee with 1 and 1.2 mM substrate i.e. 91.2 and 90.2% respectively while % conversion was 42.7 and 45.5 respectively. The % ee and conversion both decreased with further increase in substrate concentration.

5.4.3.3. Amount of enzyme

In order to know the optimum amount enzyme that can be used in the synthesis of (*S*)-mandelonitrile, different units of CLEA-*Bm*HNL was used in biotransformation. Based on the previous experiment (**section 5.4.3.2**) 0.4 and 1.2 mM benzaldehyde were selected for the current study. The amount of enzyme was varied from 5 to 15 U in the enantioselective synthesis of mandelonitrile (**Figure 5.7**).

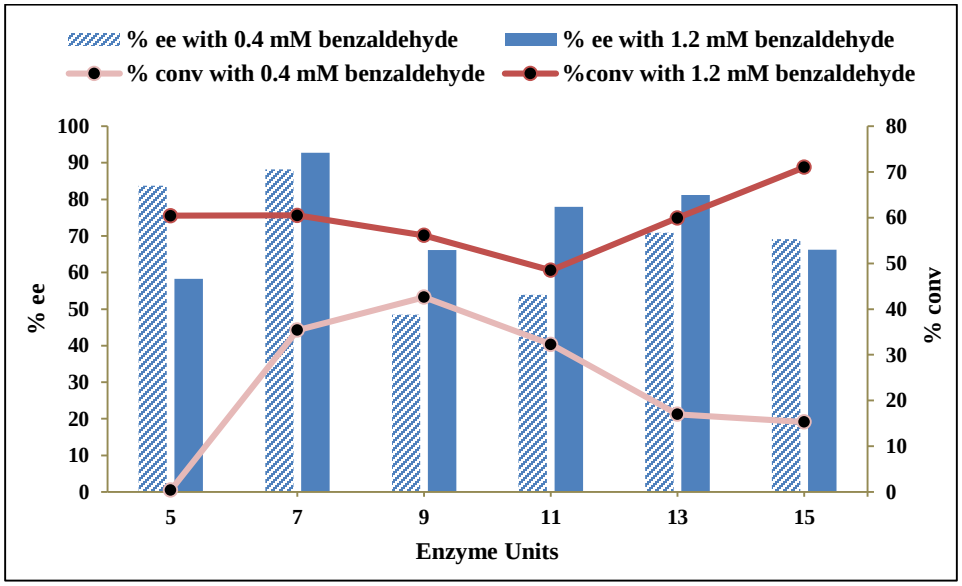


Figure 5.7: Effect of different CLEA-*Bm*HNL units in the synthesis of (*S*)-mandelonitrile

Highest % ee was obtained in the case of 7 U of CLEA-*Bm*HNL and 1.2 mM benzaldehyde while the conversion was 60.5%. With 15 U of CLEA-*Bm*HNL and 1.2 mM benzaldehyde, 66% ee of (*S*)-mandelonitrile was obtained while the conversion increased up to 71%. In case of 0.4 mM substrate concentration, highest % ee was found with 7 U of enzyme while the conversion was 35.4%. Decrease in % ee was observed with increased enzyme units in case of both the substrate concentrations.

5.4.3.4. Different organic solvents

To find out the effect of organic solvent in CLEA-*Bm*HNL catalyzed enantioselective synthesis of mandelonitrile, the biotransformation was carried out in six different solvents as per section **5.3.11**. The selected organic solvents were hexane, toluene, *n*-butyl acetate, DIPE, TBME and AcN which have been reported earlier for their use with other HNLs. The biocatalysis without the addition of organic solvent was used as a control. No improvement of % ee and conversion of product was observed in case of addition of organic solvents. Only toluene addition has shown 96.9% ee of (*S*)-mandelonitrile while it was 96.8% in aqueous medium (**Figure 5.8**). However, the % conversion was highest in the aqueous system i.e. ~38% compared to 9.3% in toluene. The enzyme showed 32% conversion with DIPE while the % ee was 74 only. In case of *n*-butyl acetate, the % ee and % conversion were 82 and 12 respectively. In case of acetonitrile both the % ee and conversion were less than 1.

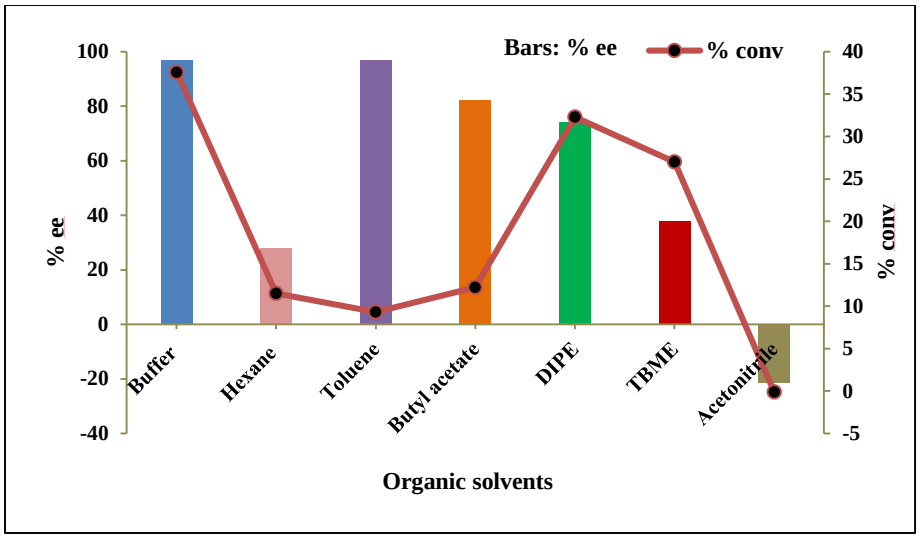


Figure 5.8: Effect of different organic solvents in the synthesis of (*S*)-mandelonitrile

5.4.3.5. Ratio of organic solvent to buffer

To find out the effect of % v/v of organic solvent in the biocatalysis, the volume of toluene was varied from 30 to 70%. Based on the previous experiment (**Figure 5.8**) toluene was selected for the current study. Chiral HPLC analysis of CLEA-*Bm*HNL catalyzed enantioselective synthesis of mandelonitrile in different toluene v/v ratio showed highest % ee i.e. 72.5 in 40% v/v of toluene while only 14.6% conversion was achieved (**Figure 5.9**). Highest % conversion i.e. 26.8 was obtained in 60% toluene but the % ee was decreased to 0.9. Decreased % ee and conversion were observed with increase in toluene v/v ratio.

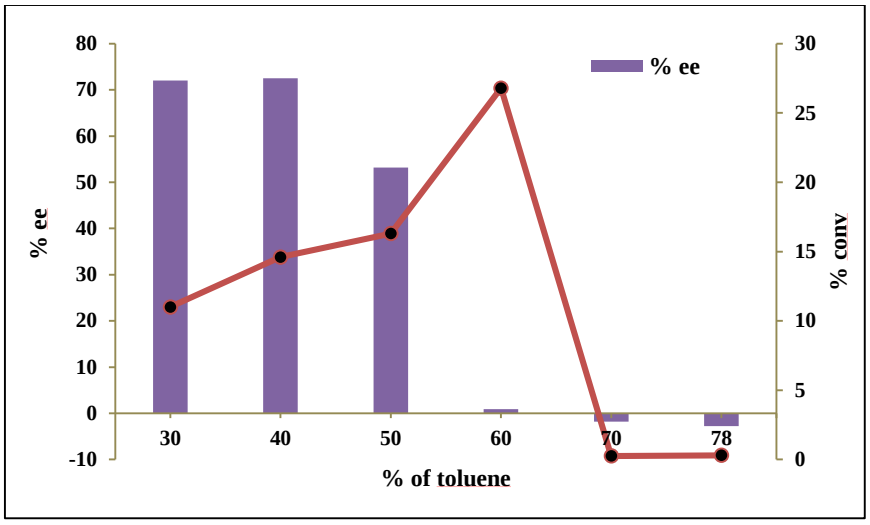


Figure 5.9: Effect of different ratio of toluene in the synthesis of (*S*)-mandelonitrile

5.4.3.6. Buffer pH

The effect of pH in the CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was studied using citrate buffer of varied pH of 3.0 to 6.0 (**Figure 5.10**). As organic solvent addition did not improve % ee and conversion, therefore, the biocatalysis was carried out in aqueous system only. Highest % ee of product was observed in pH 4.2 which was 98.76, with 50% conversion (**Figure 5.11**). In pH 3.0, CLEA-*Bm*HNL showed only 42.1% ee and 18.5% conversion. The study showed that both % ee and conversion decreased with increase in pH while at pH less than 4.0, decreased % ee was observed.

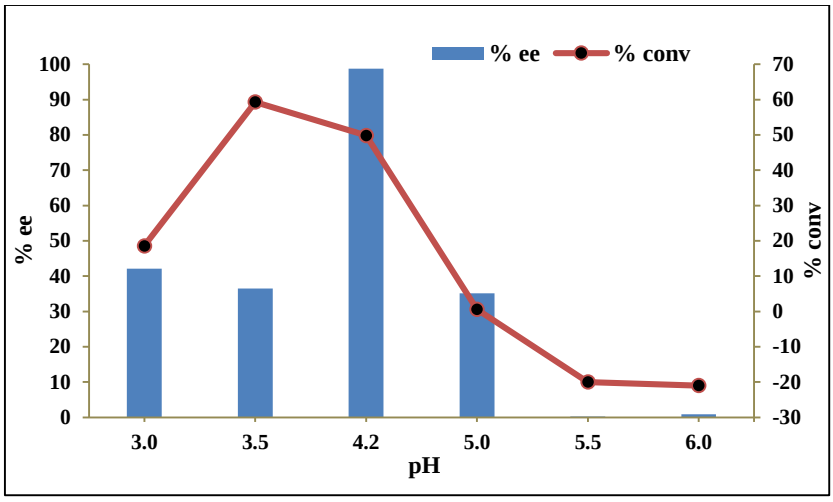


Figure 5.10: Effect of pH in the synthesis of (*S*)-mandelonitrile using CLEA-*Bm*HNL

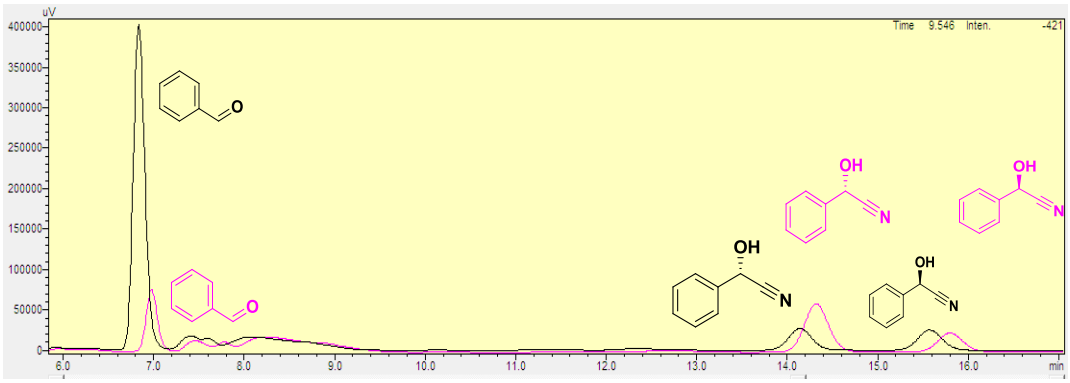


Figure 5.11: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in 300 mM citrate-phosphate buffer pH 4.2. (Black: control; Pink: Reaction)

5.4.4. Reusability of CLEA-*Bm*HNL

One of the important reason for doing enzyme immobilization is to reuse the biocatalyst. Reusability of CLEA-*Bm*HNL was tested by reusing the immobilized enzyme in the asymmetric synthesis of (*S*)-mandelonitrile for ten successive cycles (**Figure 5.12**) as per section **5.3.14**. Above 95% ee of product was observed in the first three cycles with 59 to 66% conversion. In 4th cycle the % ee was 88 while it decreased slightly to 81% in 5th

cycle. In subsequent cycles, CLEA-*Bm*HNL showed 66 to 68% ee i.e. in 5th to 8th cycle while in the last two cycles i.e. 9th and 10th, the % ee was almost 55. The % conversion of product was found between 58 and 72 for 4th to 8th cycles while it was ~44-45% in 9th and 10th cycles.

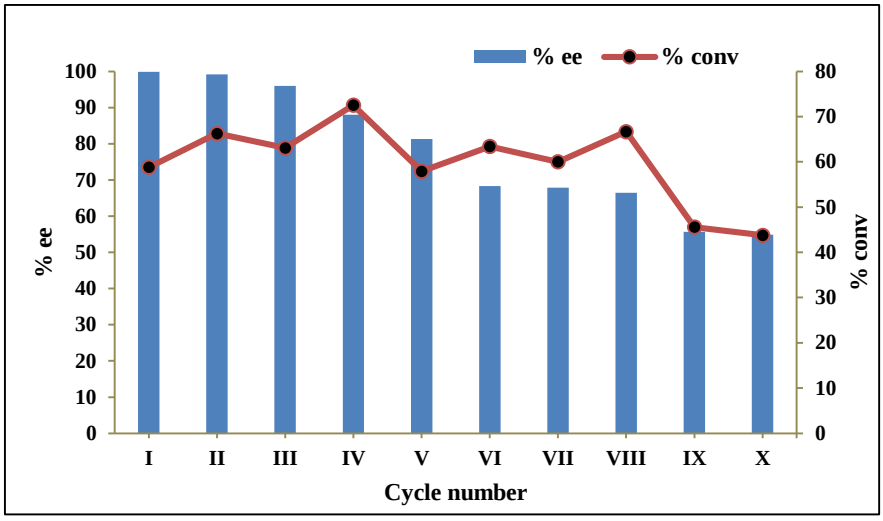
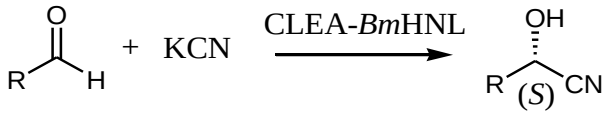


Figure 5.12: Recyclability of CLEA-*Bm*HNL towards the synthesis of (*S*)-mandelonitrile

5.4.5. Synthesis of (*S*)-cyanohydrins using CLEA-*Bm*HNL

CLEA-*Bm*HNL catalyzed asymmetric synthesis of cyanohydrins was carried out with a number of aldehydes as substrate (**Scheme 5.2**). Using standardized biotransformation conditions, eleven different aromatic aldehydes were converted into their corresponding (*S*)-cyanohydrins utilizing CLEA-*Bm*HNL under optimized reaction conditions as per section **5.3.15**. However, the reaction time was different with respect to different substrates. The % ee and conversion of the products are summarized in **Table 5.2**.



Scheme 5.1: CLEA *Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins

Table 5.2: CLEA-*Bm*HNL catalyzed synthesis of different chiral cyanohydrins

Substrate No.	R	Reaction time (min)	% ee	% conv
1	C_6H_5	20	99.8	59.8
2	3,5-di $MeOC_6H_3$	100	91.4	12.5
3	2,4-di $MeOC_6H_3$	60	96.3	1.3
4	2,5-di $MeOC_6H_3$	40	76.3	3.2
5	4-CH_2=CH-$CH_2OC_6H_4$	40	97.6	32.3
6	C_6H_5-CH_2	60	75.6	15
7	4-$PhCH_2OC_6H_4$	60	87.9	3.5
8	3-$PhOC_6H_4$	60	97.64	47.9
9	trans-PhCH=CH	100	98.6	3.5
10	3-$PhCH_2OC_6H_4$	60	92.8	48.7
11	4-OHC_6H_4	20	18.3	14.3

CLEA-*Bm*HNL produced (*S*)-mandelonitrile in 99.8% ee and 59.8% conversion in 20 minutes (**Figure 5.13**). The enzyme converted 3,5-dimethoxy benzaldehyde to its corresponding (*S*)-cyanohydrin i.e. (*S*)-**13** (**Chapter 3.A**) with 91.4% ee and 12.5% conversion in 100 min (**Figure 5.14**). Other nine substrates (No **3** to **11**, **Table 5.2**) used in the current study were not tested with *Bm*HNL earlier. CLEA-*Bm*HNL showed 96.3% ee in case of 2,4-dimethoxy benzaldehyde, however, conversion for this compound was only 1.3%. In case of 2,5-dimethoxybenzaldehyde, the enzyme showed 76.3% ee in 40 min

while less conversion of product i.e. 3.2% was observed similar to 2,4-dimethoxybenzaldehyde (**Figure 5.15**). Biocatalysis of CLEA-*Bm*HNL synthesized (*S*)-2-hydroxy-2-(4-benzyloxyphenyl) acetonitrile [(*S*)-**15, Chapter 3.A**] with 88% ee but the conversion was 3.5% only (**Figure 5.19**) while 76% ee and 15% conversion was found in case of 2-phenyl acetaldehyde (**Figure 5.18**). 4-allyloxybenzaldehyde was converted into corresponding (*S*)-cyanohydrin i.e. (*S*)-**6** in 40 min with 97.6% ee and 32.3% conversion (**Figure 5.17**) while CLEA-*Bm*HNL in 100 min converted *trans*-cinnamaldehyde into its (*S*)-cyanohydrin i.e. (*S*)-**5** with excellent % ee i.e. 98.6 (**Figure 5.21**). However, the % conversion was very poor i.e. 3.5 only. Biocatalysis of CLEA-*Bm*HNL catalyzed the synthesis of (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile [(*S*)-**10, Chapter 3.A**] (**Figure 5.20**) was produced in 97.6% ee and good % conversion i.e. 48% while in case of 3-benzyloxybenzaldehyde, the enzyme showed 92.7% ee and 49% conversion in 60 min (**Figure 5.22**). CLEA-*Bm*HNL catalyzed the synthesis of (*S*)-2-hydroxy-2-(4-hydroxyphenyl) acetonitrile [(*S*)-**18, Chapter 3.A**] was obtained with only 18.3% ee and 14.3% conversion (**Figure 5.23**).

5.5. Discussion

5.5.1. Preparation of *Bm*HNL aggregates

Preparation of enzyme aggregate is the first step CLEA preparation. To achieve an active enzyme after precipitation the selection of precipitant for enzyme aggregation is very crucial rather than getting just enzyme aggregates. The enzyme aggregates resulted from different precipitants do not show same amount of catalytic activity. Among the selected precipitants AS based enzyme aggregate has showed highest *Bm*HNL activity i.e. 1.51

U/mg (**Figure 5.1**). Ammonium sulfate is a common precipitant and frequently used during protein purification.

5.5.2. Preparation of cross-linked enzyme aggregate of *Bm*HNL

As it is well known that the preparation of CLEA is a two-step procedure that requires (i) preparation of enzyme aggregates and (ii) cross-linking of prepared enzyme aggregates. *Bm*HNL cross-linked enzyme aggregates have not been reported earlier, therefore, we optimized the preparation of *Bm*HNL-CLEA using the common cross-linker glutaraldehyde, a dialdehyde. The carbonyl groups of glutaraldehyde interact with amino group of a lysine residue present in the surface of an enzyme which results in linking one enzyme molecule to its neighbor, via Schiff base formation. Optimization of the ratio of glutaraldehyde to protein is essential in CLEA preparation to obtain CLEA of the HNL with high enzyme activity. Glutaraldehyde usage in high volume can result in enzyme inactivation. Among the selected five best precipitants of **section 5.3.3** those were used with 0 to 600 µL volume of glutaraldehyde, IPA was found as the best precipitant in *Bm*HNL-CLEA preparation with 400 µL of glutaraldehyde. Although IPA as a precipitant showed less activity compared to AS (**Figure 5.2**), however after cross-linking, the corresponding CLEA showed the highest activity. This could be due to the formation of some inactive CLEA or loss of some enzymatic activity in case of CLEA resulted from AS. CLEA-*Bm*HNL was prepared at preparative scale under optimized conditions as per mentioned in section **5.3.4.1** and used for biocatalysis after characterization.

5.5.3. Characterization of CLEA-*Bm*HNL

Characterization of CLEA-*Bm*HNL with SDS-PAGE analysis was carried out (**Figure 5.3**). In CLEA-*Bm*HNL wash desired band of protein was not observed which confirms that there is no leaching of enzyme after CLEA preparation of *Bm*HNL. Further, in case of CLEA-*Bm*HNL, the desired ~29 kDa band appeared corresponding to the pure *Bm*HNL. This confirmed presence of the enzyme in the prepared CLEA-*Bm*HNL. The morphology of the CLEA-*Bm*HNL, investigated by scanning electron microscopy (**Figure 5.4**), revealed the existence of amorphous structure and micropores.

5.5.4. Optimization of biocatalytic parameters for CLEA-*Bm*HNL catalyzed enantioselective synthesis of (*S*)-mandelonitrile

5.5.4.1. Reaction time

Study of CLEA-*Bm*HNL catalyzed enantioselective synthesis of mandelonitrile at different time intervals showed that the highest 90% ee of product was obtained in 20 min with 46.68% conversion (**Figure 5.5**). Beyond 25 min, % ee as well as conversion both were decreased. The probable reason for decrease in % ee could be the cleavage of (*S*)-mandelonitrile to benzaldehyde i.e. the reverse reaction, that might have occurred with longer reaction time. Such cleavage could also be a possible reason for decrease in % conversion, observed in **Figure 5.5**. A similar trend of decrease in % ee with increase in time has been reported with *Me*HNL-CLEA in a biphasic system [15]. There exist earlier reports of asymmetric synthesis of cyanohydrin using CLEA of other HNLs. The time of biotransformation in CLEA based enantioselective synthesis of cyanohydrins varies with the source of HNL. *Dt*HNL-CLEA and *Pd*HNL-CLEA have been known to synthesize (*R*)-

mandelonitrile. In case of *Dt*HNL-CLEA, (*R*)-mandelonitrile has been obtained with 98% ee and 99% conversion in 24 h [19] while *Pd*HNL-CLEA synthesized (*R*)-mandelonitrile in 99% yield and 99% ee in 72 h [20]. Cabirol *et al* reported the synthesis of (*R*)-mandelonitrile by *Pa*HNL-CLEA with 99% ee and 97% conversion [15]. *Me*HNL-CLEA catalyzed synthesis of (*S*)-mandelonitrile with 97% ee and 96% conversion in 2 h has been reported while *Hb*HNL-CLEA synthesized the same in 72 h with 67% ee and 55% conversion [15]. Comparison of the various biocatalysis time indicates that *Bm*HNL-CLEA biocatalysis required less time for its catalysis.

5.5.4.2. Substrate concentration

Study of the effect of different benzaldehyde concentrations in the CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile showed highest % ee with 0.4 mM benzaldehyde which was almost 93% (**Figure 5.6**). The % ee decreased slightly with 1 and 1.2 mM benzaldehyde i.e. 91.2 and 90.2% respectively while the % conversion remained almost the same for 0.4 to 1.2 mM benzaldehyde (42.8 to 45.5%). Beyond 1.2 mM benzaldehyde, both % ee and conversion decreased. However, the reason for low % ee of product with 0.6 mM benzaldehyde is not clear. Benzaldehyde concentration used in enantioselective synthesis of mandelonitrile using CLEA of different HNLs, varied with HNL source. Yildirim *et al* reported the use of 0.056 M benzaldehyde (100 µL of 1 M benzaldehyde) in the CLEA-*Pd*HNL catalyzed synthesis of (*R*)-mandelonitrile [18] while 0.2 M aldehyde has been used in case of CLEA-*Pa*HNL biocatalysis [21]. Alagöz *et al* reported CLEA-*Pd*HNL catalyzed synthesis of (*R*)-mandelonitrile with 0.056 M benzaldehyde [20]. Torrelo *et al* reported the recyclability of *Me*HNL-CLEA with 0.5 M benzaldehyde [22]. Stereoselective synthesis of cyanohydrins by CLEA-*Hb*HNL and CLEA-*Me*HNL was

reported with 0.5 M substrate concentration by Cabirol *et al* [15]. The enzyme concentration, used in all the above discussed CLEA-HNL biotransformations varies along with biocatalysis conditions. Thus a comparison of optimal substrate concentration between different CLEA-HNLs would be difficult.

5.5.4.3. Amount of enzyme

Enantioselective synthesis of mandelonitrile using different units of CLEA-*Bm*HNL was carried out with 0.4 and 1.2 mM benzaldehyde. These two benzaldehyde concentrations were selected based on the previous result (**Figure 5.6**) and 5 to 15 units of enzyme were used (**Figure 5.7**). The study showed the highest % ee with 7 U of CLEA-*Bm*HNL in both substrate concentrations. Between 0.4 and 1.2 mM benzaldehyde, highest % ee of product i.e. 92.7 was obtained in case of 1.2 mM benzaldehyde with 60.5% conversion. The % ee of product decreased with increased units of enzyme in both substrate concentrations. A possible explanation for this could be the dehydrocyanation of product. At higher enzyme concentration, cleavage of (*S*)-mandelonitrile could be active, apart from its synthesis. Since cleavage of cyanohydrin is a favorable reaction than its synthesis, this could be a reason for the decreased % ee.

5.5.4.4. Different organic solvents

Several HNL biocatalysis has been reported in biphasic system, as the usage of organic solvent helps in minimization of the spontaneous formation of racemic cyanohydrin and also in easy product extraction. Further, it facilitates the substrate solubility in biocatalysis [23] [12,14,20]. Effect of six organic solvents was studied in CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile. The biocatalysis was carried out in 40% v/v of six different

solvents as per section **5.3.11** which have been used in other HNL biocatalysis. The study revealed that % ee and conversion of the product was not improved in the tested organic solvents (**Figure 5.8**). In case of toluene, 96.9% ee of the product was achieved which was almost similar to the % ee of the product obtained in case of aqueous medium. However, % conversion decreased to 9.3% in toluene compared to 38% in aqueous system. Despite the poor conversion, we selected toluene as a cosolvent for the further experiment, especially to find out the effect of its % volume in the total reaction by varying its content. Biocatalysis with CLEA of other HNLs has been reported in biphasic system/organic medium with improved biocatalytic properties however, our study of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in a biphasic system did not show any improvement in % ee and conversion.

5.5.4.5. Ratio of organic solvent to buffer

To determine the effect of ratio of organic solvent in the biocatalysis, 30 to 78% v/v of toluene were used in CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile. Only 72.5% ee was obtained in 40% toluene which was the highest % ee among the tested ratio of toluene (**Figure 5.9**). Further increase in percentage of toluene decreased the % ee and conversion of product.

5.5.4.6. Buffer pH

It is well known that pH plays an important role in the enantioselective cyanohydrin syntheses. Higher pH leads to the spontaneous formation of racemic cyanohydrin by chemical reaction which decreases the enantiomeric yield of cyanohydrins [24]. To investigate the effect of pH in the CLEA-*Bm*HNL catalyzed synthesis of (*S*)-

mandelonitrile, the biocatalysis was carried out in 300 mM citrate buffer of pH from 3 to 6 (**Figure 5.10**). The CLEA-*Bm*HNL showed highest 98.76% ee of (*S*)-mandelonitrile and 50% conversion at pH 4.2 (**Figure 5.10** and **5.11**). Decreased % ee and conversion were observed with increased pH which results from racemization of product at high pH. A decreased % ee was also observed at pH less than 4.0 which could be due to enzyme instability at low pH. Dadashipour et al reported the optimum pH for *Bm*HNL catalyzed cyanohydrin cleavage as 5.0 [11]. However, they have carried out the enantioselective cyanohydrin synthesis with *Bm*HNL at pH 4.2 [11]. CLEA-*Bm*HNL in contrary showed optimum pH at 4.2. Additionally, CLEA-*Bm*HNL has improved % ee of (*S*)-mandelonitrile i.e. 98.76% compared to 54% by purified *Bm*HNL [11]. A similar observation of change in pH optima by CLEA has been reported [25,26]. CLEA of *Pichia pastoris* alcohol oxidase [27] and *Roystonea regia* peroxidase [25] has shown higher activity at low pH compared to their corresponding free enzymes.

5.5.5. Reusability of CLEA-*Bm*HNL

One of the goals of immobilization is to use the biocatalyst repeatedly in biocatalysis. Reusability of CLEA-*Bm*HNL was examined in the synthesis of (*S*)-mandelonitrile. It was used for 10 successive cycles (**Figure 5.12**). The process was carried out by separating the reaction mixture and enzyme via centrifugation after each cycle and reuse the CLEA-*Bm*HNL in (*S*)-mandelonitrile synthesis. A gradual decrease in % ee was observed with increasing cycles. Comparable % ee and conversion was observed in the first three cycles while a slight decrease in % ee was observed in the next two cycles. The % conversion was maintained between 59 and 72 from 1st to 8th cycles. Further increase in cycles decreased % ee as well as conversion of product. The gradual decrease in % ee with extended use of

immobilized enzyme could be due to leakage of the catalyst during each cycle in the operational process or enzyme inhibition by aldehyde. Nevertheless, this process has shown reusability of CLEA-*Bm*HNL for eight successive cycles without loss in conversion or product formation and five cycles with a little loss in % ee.

Recyclability of CLEA of other HNLs has also been reported. Torrelo *et al* reported the reuse of CLEA-*Me*HNL for seven cycles without wash. They observed >98% ee of product in first three cycles, with conversion of 98 to 95% while the conversion decreased up to 55% at the end of the 7th cycle, [22]. This is ~45% loss in % conversion of product at 7th cycle with CLEA-*Me*HNL while in the present study CLEA-*Bm*HNL showed nearly no loss in % conversion until 8th cycle. Recyclability of CLEA-*Pa*HNL has been tested for 10 successive cycles using 2-methyl benzaldehyde as substrate in its cyanohydrin synthesis that showed without any loss in % conversion. The enzyme was used in each cycle after washing with water [21]. CLEA-*Pd*HNL has been found to be used for eight cycles in the (*R*)-mandelonitrile synthesis in a biphasic system at 5 °C [20]. Although the % of ee of product was 99 at the end of the 8th cycle, but activity has been decreased to 29% of its original activity. Recyclability of CLEA-*Lu*HNL in the synthesis of (*R*)-2-butanone cyanohydrin at 30 °C has been reported for four cycles [17]. CLEA-*Lu*HNL showed 81% ee and 84% conversion in the first cycle which decreased to 78% ee and 56% conversion at the end of 4th cycle.

5.5.6. Synthesis of (*S*)-cyanohydrins using CLEA-*Bm*HNL

After optimizing above reaction parameters, CLEA-*Bm*HNL catalyzed synthesis of different (*S*)-cyanohydrin was carried out under optimal conditions. Enantioselective

synthesis of mandelonitrile using CLEA-*Bm*HNL resulted in 99.8% ee and 59.8% conversion while purified *Bm*HNL has been reported to synthesize (*S*)-mandelonitrile in 54% ee [11]. CLEA of *Bm*HNL has improved the % ee of product in this transformation. Earlier studies revealed that to improve the enantioselectivity of *Bm*HNL, the enzyme has been engineered [28]. *Bm*HNL-H103C-N156G catalyzed synthesis of (*S*)-mandelonitrile has been reported with 93% ee. Comparison of these results indicates that CLEA-*Bm*HNL has improved the enantioselectivity of *Bm*HNL even more than its engineered variant. The conversion of 3,5-dimethoxy benzaldehyde to (*S*)-**13** (**Chapter 3.A**) by CLEA-*Bm*HNL resulted in 91.4% ee and 12.5% conversion in 100 minutes (**Figure 5.14**) while the synthesis of the same using purified *Bm*HNL has been reported with 85% ee [11]. Apart from these two, other nine substrates (No **3** to **11**, **Table 5.2**) were first time tested with *Bm*HNL in the current study. The corresponding (*S*)-cyanohydrins from these nine aldehydes were synthesized using CLEA-*Bm*HNL for the first time. Among the nine, five aldehydes i.e. 2,4-dimethoxybenzaldehyde, 4-allyloxybenzaldehyde, 3-phenoxybenzaldehyde, *trans*-cinnamaldehyde, and 3-benzyloxybenzaldehyde (**Figure 5.22**) were converted to their corresponding (*S*)-cyanohydrins with very high % of ee i.e. 93 to 99 (S. No **3, 5, 8, 9** and **10**, **Table 5.2**). Three among these i.e. 3-phenoxy benzaldehyde (**Figure 5.20**), 4-allyloxybenzaldehyde (**Figure 5.17**) and 3-benzyloxybenzaldehyde (S. No **8, 5** and **10**, **Table 5.2**) have shown reasonable conversion i.e. 32 to 49% to their products. CLEA-*Bm*HNL converted three other aromatic aldehydes i.e. 2,5-dimethoxybenzaldehyde (**Figure 5.16**), 4-benzyloxybenzaldehyde (**Figure 5.19**) and 2-phenyl acetaldehyde (S. No **4, 6** and **7**, **Table 5.2**) to their respective (*S*)-cyanohydrins in 76.3, 88 and 75.6% of ee (**Figure 5.18**) respectively. Although the % ee

was moderate to high, the % conversion for these three substrates was very low i.e. 3 to 15% only. Among the eleven (*S*)-cyanohydrins syntheses reported here, eight of them (S. No. **2, 3, 4, 5, 6, 7, 10**, and **11**) have not been reported to be synthesized by any CLEA-HNL.

Synthesis of (*S*)-cyanohydrins has been described by CLEA-*Hb*HNL [15] and CLEA-*Me*HNL [14,29]. The CLEA-*Hb*HNL synthesis of (*S*)-mandelonitrile has been reported in 55% conversion and 67% ee in 72 h [15]. Cabirol *et al* synthesized (*S*)-*m*-phenoxybenzaldehyde or (*S*)-**10** cyanohydrin using CLEA-*Me*HNL in 81% conversion and 83% ee in 72 h. They also described the preparation of (*S*)-enantiomers of hexanal cyanohydrin in 92% conversion and 81% ee in 3 h, 2-furaldehyde cyanohydrin in 94% conversion and 94% ee in 30 min; and mandelonitrile in 96% conversion and 97% ee in 2 h [15]. Chmura *et al* showed CLEA-*Me*HNL catalyzed synthesis of three (*S*)-cyanohydrins in 55 to 99% ee and 86 to 96% conversion [14]. They also reported the preparation of cyanohydrins of two ketones i.e. acetophenone and 1-phenylpropanone with 99% ee and 7% conversion and 96% ee and 90% conversion respectively. Other HNLs as CLEA has also been reported to synthesize chiral cyanohydrins but in (*R*)-form [16,17,20].

5.6. Conclusions

- **Optimization of CLEA-*Bm*HNL preparation and characterization:** We have immobilized *Bm*HNL and prepared CLEA-*Bm*HNL for the first time. Studies were performed to optimize CLEA-*Bm*HNL preparation. Toward this optimization of different precipitants to prepare enzyme aggregates and amount of cross-linking agent

i.e. glutaraldehyde was optimized. CLEA-*Bm*HNL was prepared using optimal conditions at 4 °C and characterized by SDS-PAGE and SEM.

- The optimal conditions for CLEA-*Bm*HNL preparation are:
- Optimal precipitating agent: IPA
- Optimal glutaraldehyde ratio (v/v): IPA: glutaraldehyde: cell lysate = 9: 4: 1
- Time: 6 h

❖ **Standardization of reaction parameters for CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile:** The biocatalytic parameters of CLEA-*Bm*HNL biocatalysis were optimized using benzaldehyde as a standard substrate for the maximum production of (*S*)-mandelonitrile. The optimized conditions are as follows:

- Time of biotransformation: 20 minutes
- Amount of CLEA-*Bm*HNL: 7 U
- Substrate concentration: 1.2 mM (pre-dissolved in DMSO)
- pH of buffer: pH 4.2
- Organic solvent: addition of organic solvent did not improve in % ee of product
- Recyclability: 5 cycles with slight loss in enantioselectivity and 8 cycles without loss of % conversion of product.

❖ Using optimized conditions, benzaldehyde was converted to (*S*)-mandelonitrile in high (~99%) ee and ~60% conversion. CLEA of *Bm*HNL showed improved enantioselectivity in the synthesis of (*S*)-mandelonitrile compared purified or engineered *Bm*HNL that showed only 54 and 93% ee respectively.

- **Enantioselective synthesis of cyanohydrins using CLEA-*Bm*HNL:** CLEA-*Bm*HNL was used in the synthesis of different (*S*)-cyanohydrins under optimized conditions. CLEA-*Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins can be summarized as follow:
  - The % ee of (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile was improved by CLEA-*Bm*HNL synthesis i.e. 91.4% ee as compared to the reported 85% ee with purified *Bm*HNL.
  - (*S*)-Cyanohydrins of five substrates (No **3, 5, 8, 9** and **10** of **Table 5.2**) were synthesized using CLEA-*Bm*HNL with 93 to 99% ee while substrate no. 4, 5 and 7 were converted to their (*S*)-cyanohydrins in 76.3, 88 and 75.6% of ee.
  - Eleven different chiral cyanohydrins using CLEA-*Bm*HNL were synthesized. Among them, eight cyanohydrins have not been reported to be synthesized by any CLEA-HNL and nine substrates were tested first time with *Bm*HNL.

In conclusion, we have showed the preparation and characterization of a stable, robust and recyclable biocatalyst i.e. CLEA-*Bm*HNL. We demonstrated its biocatalytic application in the synthesis of different (*S*)-aromatic cyanohydrins.

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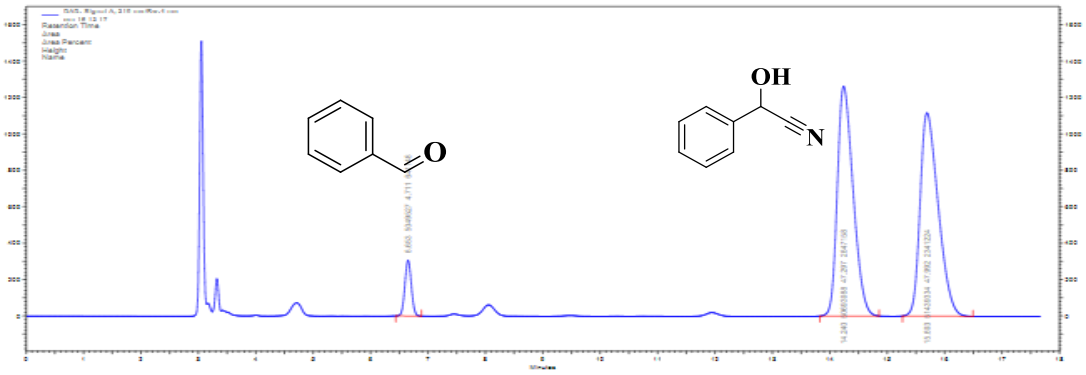
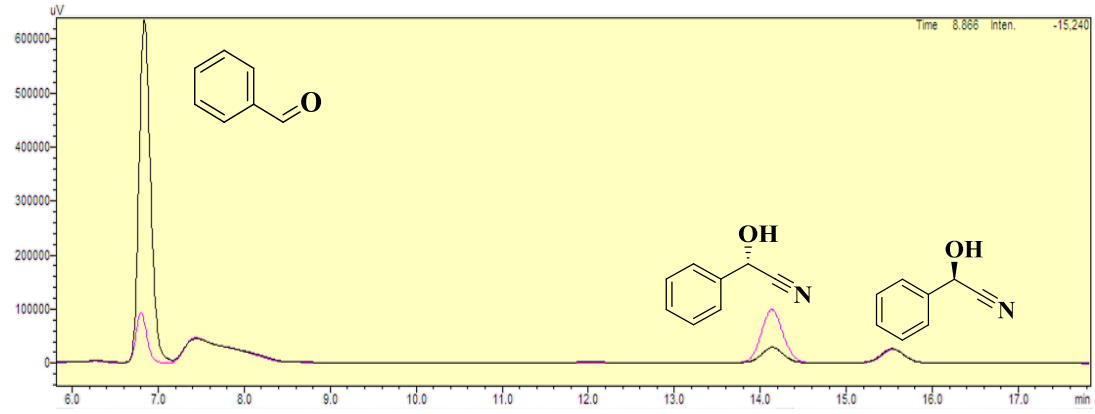
HPLC chromatograms

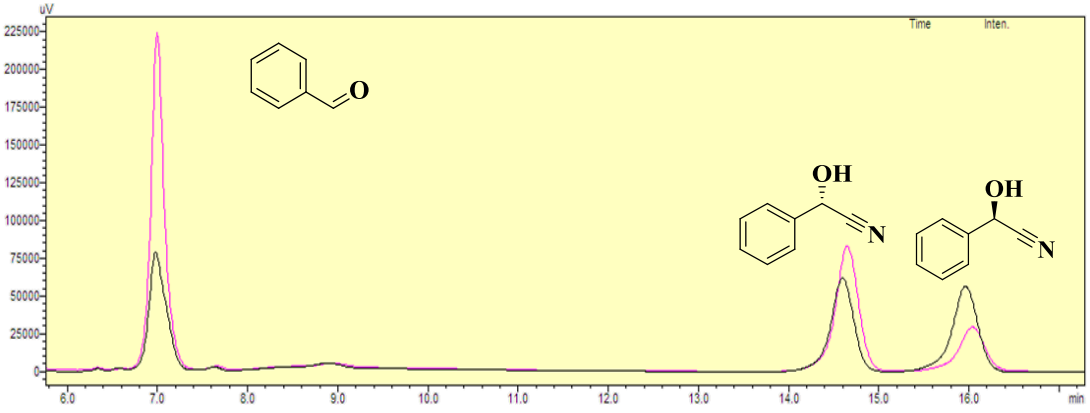
HPLC chromatogram of the chiral HPLC analysis of the biocatalysis product and control are given below. For the chiral HPLC profile of the corresponding racemic cyanohydrins please refer **Chapter 3B**.



(a)



(b)



(c)

Figure 5.13: (**a**): Racemic mandelonitrile; (**b**): HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile where buffer was used as control in place of enzyme and (**c**): HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile where CLEA-pCold1 (pCold1 vector in *E.coli* BL21(DE3) was used as control. Black: control; Pink: CLEA-*Bm*HNL.

Identification of (*S*)- and (*R*)-mandelonitrile in the HPLC chromatogram has been done by using standard (*R*)-mandelonitrile obtained by *At*HNL catalyzed synthesis and analyzed in the same chiral column by HPLC.

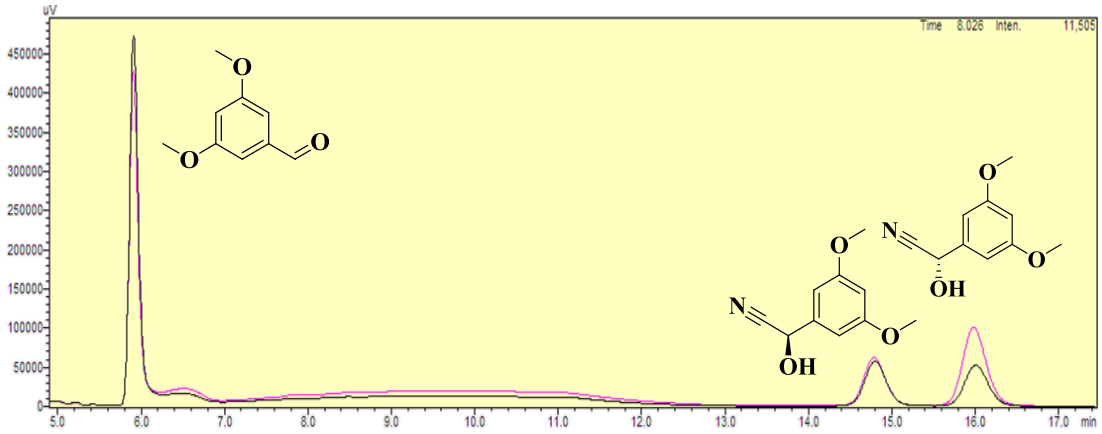


Figure 5.14: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl)acetonitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL.

Absolute configuration of the major enantiomer resulted from biotransformation was assigned based on the elution pattern of cyanohydrins in Chiralpak IB column as discussed in case of substrate number **6**, **8**, and **9**.

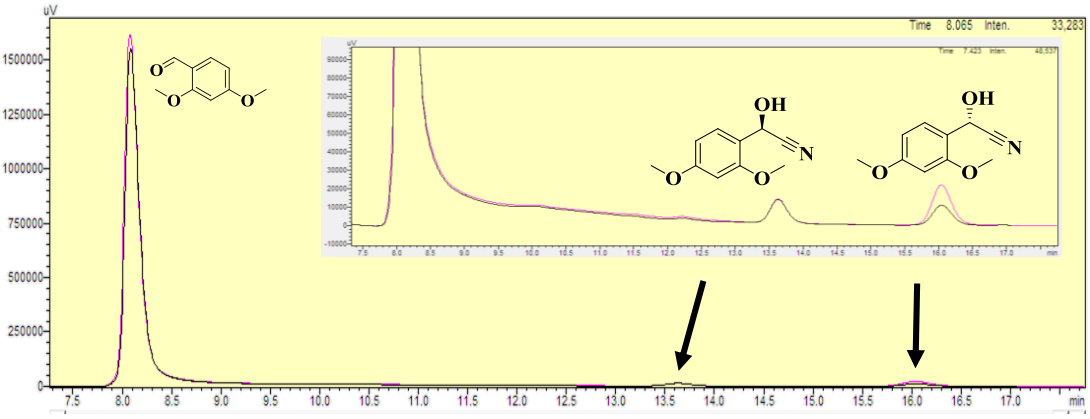


Figure 5.15: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(2,4-dimethoxyphenyl)acetonitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL.

Absolute configuration of the major enantiomer resulted from biotransformation was assigned based on the elution pattern of cyanohydrins in Chiralpak IB column as discussed in case of substrate number **6**, **8**, and **9**.

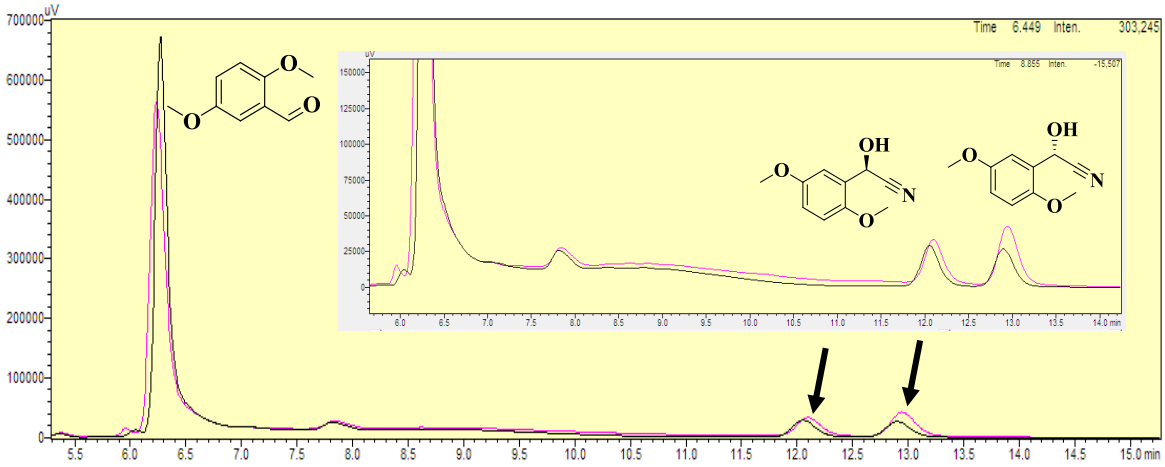


Figure 5.16: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(2,5-dimethoxyphenyl)acetonitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL

Absolute configuration of the major enantiomer resulted from biotransformation was assigned based on the elution pattern of cyanohydrins in Chiralpak IB column as discussed in case of substrate number **6**, **8**, and **9**.

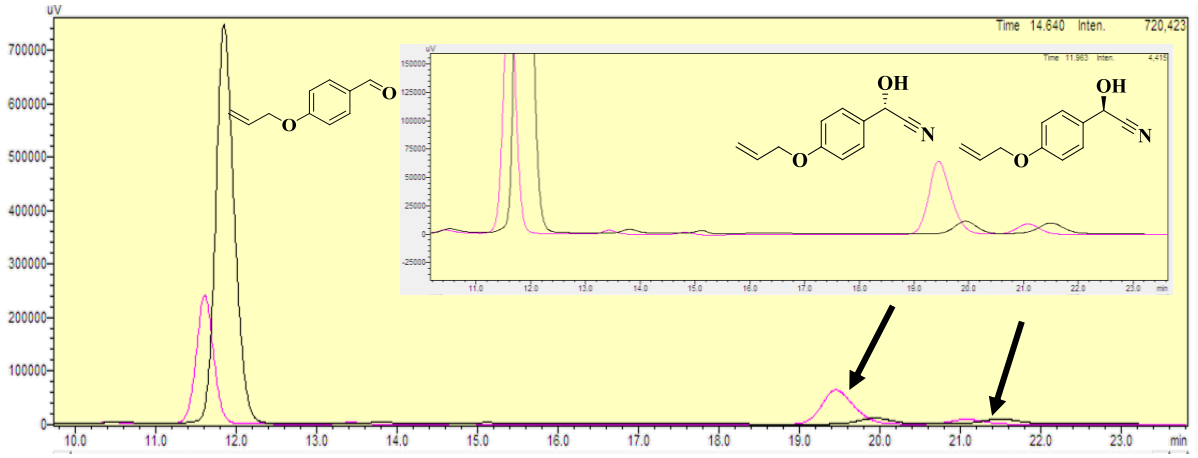


Figure 5.17: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-2-(4-(allyloxy)phenyl)-2-hydroxyacetonitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL.

Absolute configuration of the major enantiomer resulted from biotransformation was assigned based on the elution pattern of cyanohydrins in Chiralpak IE column as discussed in case of substrate number **1**.

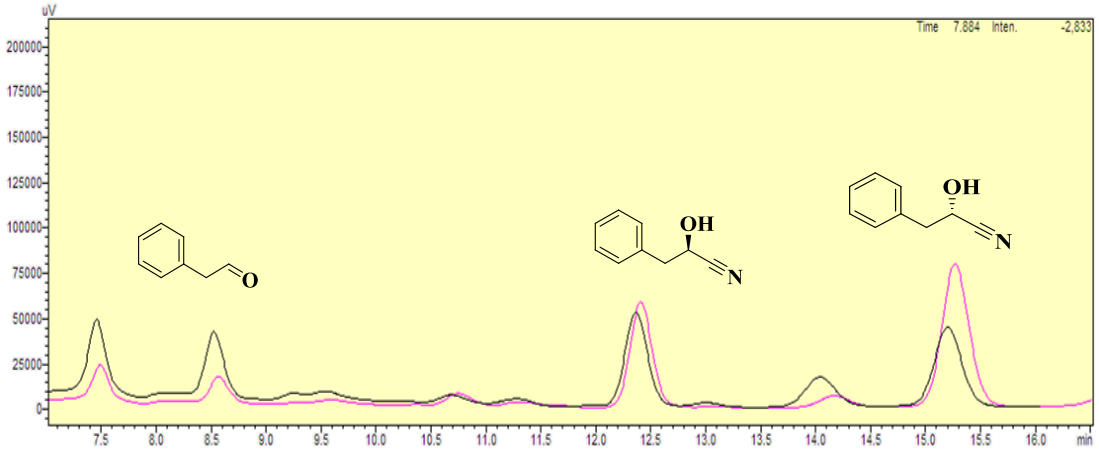


Figure 5.18: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-3-phenylpropanenitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL

The pattern of elution of the two enantiomers of **6** has been reported in Chiralcel-ODH column [30]. Since we have used chiralpak IB column in the present analysis which also consists of same CSP as Chiralcel-ODH, thus the later peak in the HPLC was assigned as (*S*)-enantiomer.

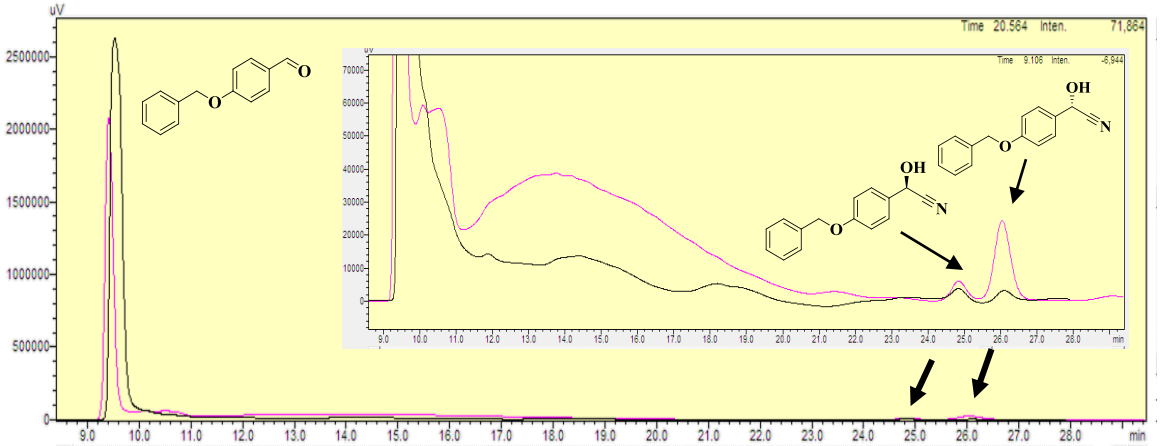


Figure 5.19: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(4-benzyloxyphenyl) acetonitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL

Absolute configuration of the major enantiomer resulted from biotransformation was assigned based on the elution pattern of cyanohydrins in Chiralpak IB column as discussed in case of substrate number **6**, **8**, and **9**.

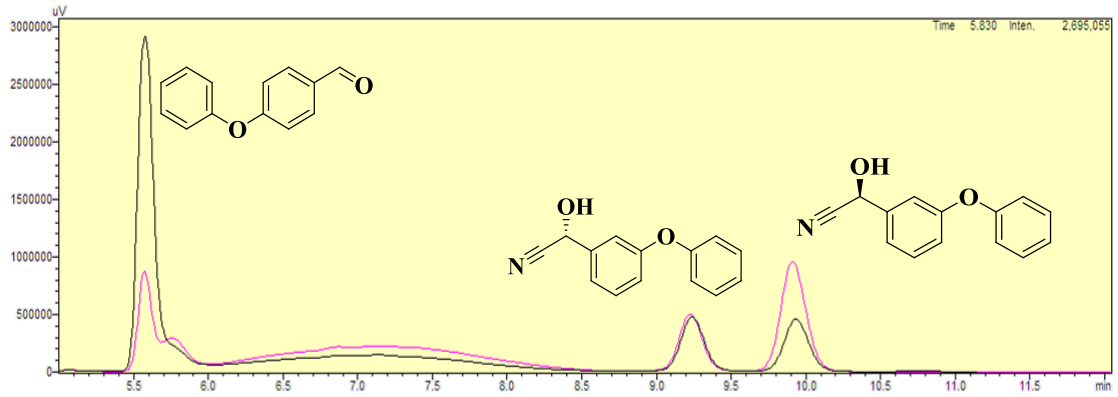


Figure 5.20: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL

The pattern of elution of the two enantiomers of **8** has been reported in Chiralcel-ODH column [30]. Since we have used chiralpak IB column in the present analysis which also consists of same CSP as Chiralcel-ODH, thus we have assigned the absolute configuration of the biotransformation products accordingly.

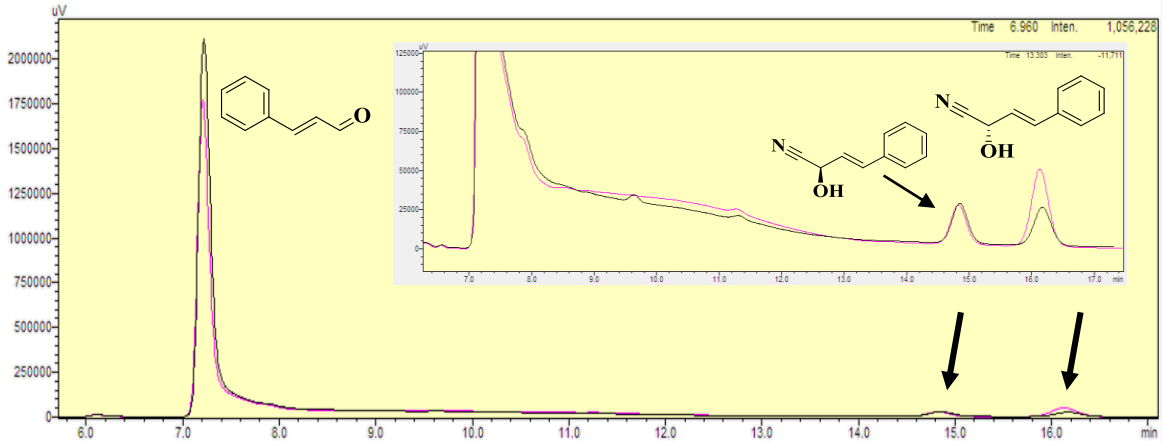


Figure 5.21: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-(*E*)-2-hydroxy-4-phenylbut-3-enenitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL.

The pattern of elution of the two enantiomers of **9** has been reported in Chiralcel-ODH column by Gerrits *et al* [31]. Since we have used chiralpak IB column in the present analysis which also consists of same CSP as Chiralcel-ODH, thus we have assigned the absolute configuration of the biotransformation products i.e. its (*S*)-enantiomer to the second peak.

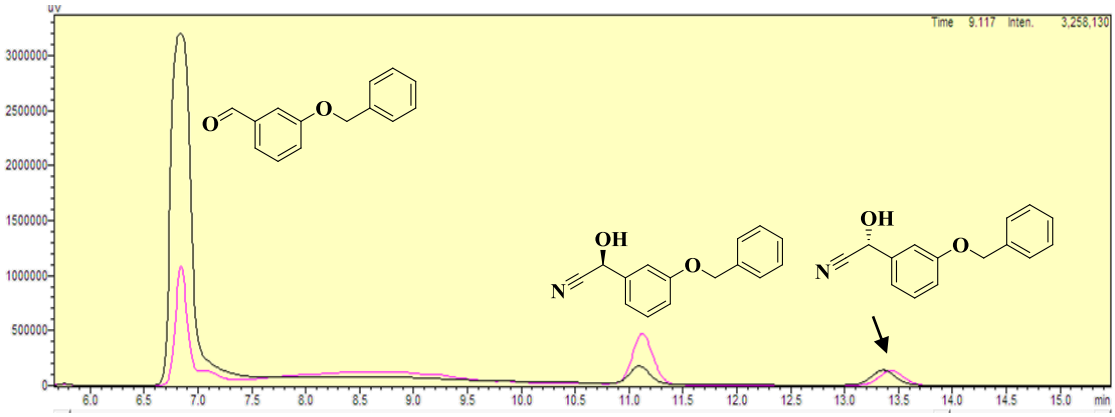


Figure 5.22: HPLC chromatogram of CLEA-*Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3-benzyloxyphenyl) acetonitrile. Black: using buffer as control in place of enzyme; Pink: using CLEA-*Bm*HNL

Absolute configuration of the major enantiomer resulted from biotransformation was assigned as (*S*), based on the assumption that *Bm*HNL catalyzed synthesis has resulted (S)-cyanohydrins in case of all other ten substrates mentioned in **Table 5.2.**

Chapter 6

A Study on increasing enzymatic stability and activity of Baliospermum montanum hydroxynitrile lyase in biocatalysis

6.1. Introduction

Hydroxynitrile lyases (HNLs) in nature catalyzes cyanogenesis, a process that involves cleavage of cyanohydrins to corresponding aldehyde/ketone and HCN. Reversibly they catalyze the addition of HCN to the carbonyl carbon of an aldehyde/ ketone [1–6]. It is a C-C bond formation that results in synthesis of chiral cyanohydrins. A few HNLs catalyze promiscuous nitroaldol reaction i.e. Henry reaction, and it's reverse i.e. retro Henry reaction, while some does ester hydrolysis when engineered [7,8]. Enantiopure cyanohydrins are important molecules with pharmaceutical, agrochemical and other industrial applications [2,9–12]. Demand of biocatalytic synthesis of chiral cyanohydrins has led to the discovery of several new HNLs [6,13–16]. One of the major limitations of HNL catalyzed synthesis of cyanohydrins is background chemical reaction i.e. non-enzymatic synthesis of racemic cyanohydrin which decreases the enantiopurity of the product [17]. In order to minimize this background reaction often HNL biocatalysis is carried out at (i) low pH and (ii) in presence of organic solvents [8,18]. However, stability of the enzyme in both the above conditions remains an issue. *Hevea brasiliensis* HNL (*Hb*HNL) and *Arabidopsis thaliana* HNL (*At*HNL), both members of α/β hydrolase fold superfamily have shown poor enzymatic stability at lower pH [17,19]. To optimize HNL

catalyzed synthesis of chiral cyanohydrin, it is required to investigate the stability and other biophysical parameters of the enzyme so that an appropriate reaction condition can be found where the background reaction is minimum. Among the reported α/β hydrolase fold HNLs, stability of *Hb*HNL and *Manihot esculenta* HNL (*Me*HNL) has been studied to help use them in biocatalysis [17,19–21]. *At*HNL's stability at lower pH has been improved by protein engineering and immobilization of it with a flavin-based fluorescent protein [22,23]. *Bm*HNL another member of α/β hydrolase fold family of HNLs has showed application in the synthesis of a broad range of chiral cyanohydrins [4,18,24]. Dadashipour *et al* have studied the effect of pH, temperature and additives on activity and stability of *Bm*HNL [4]. It showed optimum activity at pH 5.0, and was stable in broad pH range from 2.5 to 10.5. Further, it showed optimum activity at 20 °C while the enzyme was stable at broad range of temperature i.e. 10 to 60 ^{o}C. However, their stability study was limited to only 1 h incubation and majorly focused on finding optimal conditions for *Bm*HNL biocatalysis.

In the present study we investigated the effect of different parameters such as pH, temperature, buffer concentrations, presence of additives/inhibitors, stabilizers, substrate concentrations and organic solvents on the activity and stability of *Bm*HNL. We also investigated the effect of organic solvents and temperature over the secondary structure of *Bm*HNL by circular dichroism (CD) analysis. Our study has revealed that addition of polyols especially glycerol has improved the enantioselectivity of *Bm*HNL in the formation of (*S*)-mandelonitrile up to >99% ee compared to 75% ee in the absence of glycerol. Similarly, sucrose addition has improved *Bm*HNL's half-life at pH 3.5, more than five folds as compared to without stabilizer

6.2. Objectives

- To investigate the effect of different biocatalytic parameters on the activity and stability of *Bm*HNL.
- To explore the effect of stabilizers on the enantioselectivity of *Bm*HNL in the stereoselective synthesis of chiral cyanohydrins.

6.3. Materials and methods

*Bm*HNL (LOCUS: AB505969) synthetic gene was sub-cloned into pCold1 followed by expression and purification as per C**hapter 2**. Internal standards were synthesized as per **Chapter 3**. Mandelonitrile, metal salts and organic solvents used in the study were purchased from Sigma Aldrich, AVRA, SRL and Alfa-Aesar. HPLC grade solvents were obtained from RANKEM, Molychem, FINAR, and SRL. Different stabilizers e.g. sorbitol, glycerol, lactose and sucrose were purchased from SRL.

6.3.1. Expression and protein purification

*Bm*HNL crude extract obtained and purified by Ni-NTA agarose resin as per section **2.4.13** (**Chapter 2**).

6.3.2. HNL assay via mandelonitrile cleavage

HNL activity was measured by monitoring the formation of benzaldehyde due to cleavage of mandelonitrile, in a microtitre plate using UV-Visible spectrophotometer (Thermo Fisher Scientific, No. 1510-02398C) as per **Chapter 2** (Section **2.4.14**). One unit is defined as the amount of the enzyme which converts 1 μmol of mandelonitrile to benzaldehyde in one minute under standard conditions. All reactions were performed in triplicates.

6.3.3. Influence of biophysical parameters on *Bm*HNL stability and activity

To determine stability and activity of *Bm*HNL under different conditions, various parameters were studied. The enzyme was incubated in different conditions such as different pH, temperature, buffer concentration, additives, substrate inhibition and stabilizers and their effect on stability and activity of *Bm*HNL was studied. For finding specific activity, all reactions involved cleavage of racemic mandelonitrile as given in **6.3.2.** In case of control reactions, the corresponding buffer was used instead of the enzyme. All measurements were performed in triplicates.

6.3.3.1. Effect of pH

On stability: The effect of pH on stability of *Bm*HNL was studied in 50 mM citrate-phosphate buffer of different pH e.g. 3.5, 4, 4.5, 5, 5.5, 6 and 6.5. A 0.5 mL of enzyme in 20 mM KPB pH 7.0 found on purification was subjected to buffer exchange with 50 mM citrate-phosphate buffer of corresponding pH i.e. 3.5 to 6.5 and was stored at room temperature. HNL activity of the buffer exchanged enzyme was determined, which considered as initial activity. Further activity was measured at different time intervals until the half-life of *Bm*HNL is reached.

On activity: Specific activity of *Bm*HNL was measured as described in **6.3.2**, except the assay buffer was replaced by 50 mM citrate-phosphate buffer of pH 3.5 to 6.5 in separate experiments.

6.3.3.2. Effect of temperature

On stability: Influence of temperature on the stability of *Bm*HNL was studied by incubating 0.5 mL of enzyme in 20 mM KPB pH 7.0 at different temperature ranging from 10 to 80 ^{o}C in a thermo shaker. Initial activity was measured using HNL assay at room temperature

(as given in 6.3.2). Activity of enzymes, incubated at different temperature, was measured at multiple time points until the half-life of the enzyme was reached. A control experiment was also performed under similar conditions where the enzyme was replaced by its corresponding buffer.

On activity: Specific activity of *Bm*HNL was measured as described in **6.3.2**, except separate experiments were carried out for measuring the activity by incubating the reaction mixture at different temperature i.e. 10 to 80 ^{o}C in a thermomixer using 50 mM citrate-phosphate buffer pH 5.5.

6.3.3.3. Effect of buffer concentrations

On stability: To find out the effect of buffer concentrations on stability of *Bm*HNL, the purified enzyme was stored in different concentrations of citrate-phosphate buffer pH 5.5. The different concentrations of citrate-phosphate buffer pH 5.5 used were 50, 100, 200, 300 and 400 mM. The enzyme was buffer exchanged as described in section **6.3.3.1**. The initial activity was measured after buffer exchange. The enzyme was stored in room temperature and activity was measured until half-life reached.

On activity: The specific activity of *Bm*HNL was measured as per protocol described in section **6.3.2** using citrate-phosphate buffer pH 5.5 of different concentrations (50, 100, 200, 300 and 400 mM) instead of only 50 mM citrate-phosphate pH 5.5.

6.3.3.4. Effect of addition of organic solvents

On stability: Different organic solvents such as toluene, hexane, *n*-butyl acetate (*n*-BA), acetonitrile (AcN), *tert*-butyl methyl ether (TBME) and *di*-isopropyl ether (DIPE) were selected for this study because these are commonly used in HNL catalyzed cyanohydrin synthesis. The stability of *Bm*HNL was studied using different organic solvent in 10% v/v.

After the addition of organic solvent to an enzyme solution it was stored at room temperature and half-life was measured.

On activity: The specific activity of *Bm*HNL with different organic solvents was calculated. 10% v/v of each organic solvent was added to the reaction mixture and the assay was carried out as described in **6.3.2** using 50 mM citrate-phosphate buffer pH 5.5.

6.3.3.5. Effect of stabilizers

On stability: To improve the stability of *Bm*HNL at low pH, different stabilizers were added to 0.5 mL of enzyme solution (50 mM citrate-phosphate buffer pH 3.5) and enzyme was stored at room temperature. Different sugars and polyols were selected as stabilizers for this study. Sucrose, sorbitol, and glycerol were taken in 50, 100, 200 and 400 mg/mL concentration while lactose was tested in 50 and 100 mg/mL. After proper mixing, the initial activity was measured as mentioned in section **6.3.2** and subsequently activity was measured at different time intervals until half-life has reached.

On activity: The specific activity of *Bm*HNL with different stabilizers was calculated. The stabilizers were added to the reaction mixture. The additives were properly mixed and the assay was carried out as described in **6.3.2** using 50 mM citrate-phosphate buffer pH 5.5.

6.3.3.6. Influence of benzaldehyde concentration

On stability: To investigate the effect of benzaldehyde over stability of *Bm*HNL, various concentration of benzaldehyde was added to 0.5 mL of *Bm*HNL in 20 mM KPB pH 5.5 and stored at room temperature. Benzaldehyde concentrations were varied from 0, 5, 10, 15, 20, 25, 30, 35, and 40 mM. After addition of benzaldehyde into enzyme solution, the initial activity was measured towards cleavage of racemic mandelonitrile. The activity was

measured until half-life of enzyme has reached. Controls also carried the respective benzaldehyde concentrations.

On activity: The specific activity of *Bm*HNL with different concentrations of benzaldehyde was calculated. Various concentration of benzaldehyde was added to the reaction mixture and the assay was carried out as described in **6.3.2** using 50 mM citrate-phosphate buffer pH 5.5.

6.3.3.7. Effect of addition of chemical additives

On stability: Effect of different chemical additives/inhibitors on the stability of *Bm*HNL was elucidated. Selection of the inhibitors/additives and their concentration was based on an earlier report [4]. $ZnSO_4$, $AgNO_3$, metal chelator e.g. EDTA and PMSF of 1 mM final concentration were added into the enzyme solution, while 2-mercaptoethanol in 10 mM and acetone and acetone cyanohydrin (AcCN) in 50 mM were added. The enzyme solution with additives/inhibitors was incubated at room temperature and HNL activity was measured using mandelonitrile cleavage assay as described in section **6.3.2**.

On activity: The specific activity of enzyme in presence of inhibitors were also measured by adding them into the reaction mixture. Mandelonitrile cleavage assay was carried out for using 50 mM citrate-phosphate buffer pH 5.5 to measure the activity. Enzyme solution without inhibitors was used as a control for the study.

6.3.4. Circular dichroism (CD) analysis

The study of effect of temperature and organic solvent on the secondary structure of *Bm*HNL was carried out by a CD spectrophotometer. A 0.1 mg/mL of enzyme solution was loaded in the CD spectrophotometer at different temperature range starting from 10 to

80 ^{o}C. The spectra recorded from 195 to 300 nm were analyzed by CDNN software. In case of organic solvents, 10% v/v of DIPE and *n*-BA were added to 0.1 mg/mL of *Bm*HNL solution separately. The enzyme solution in organic solvent was subjected to the CD analysis at 25 ^{o}C.

6.3.5. Kinetic study of *Bm*HNL

Kinetic parameters of *Bm*HNL were determined using mandelonitrile cleavage assay. Two sets of kinetic experiments were carried out. In first, enzyme concentrations were varied by keeping the substrate concentration fixed. In the second experiment, the best enzyme concentration of the first experiment was selected and different substrate concentrations were used for the kinetic study. Different concentrations of *Bm*HNL i.e. 1.92, 0.96, 0.48, 0.24 and 0.12 mg/mL were selected for the first i.e. enzyme kinetics with a fixed substrate volume i.e. 20 µL of 70 mM mandelonitrile in 5 mM citrate-phosphate buffer pH 3.15. The reaction mixture contained 175 µL of 50 mM citrate-phosphate buffer pH 5.5, 5 µL of pure *Bm*HNL (various concentrations) and 20 µL of 70 mM mandelonitrile. The highest activity was observed in 0.24 mg/mL enzyme concentration. For the second, i.e. substrate varied enzyme kinetics, concentration of mandelonitrile was varied from 0.1 to 30 mM (0.01 to 3 mM final concentration) because with 3 mM final concentration of mandelonitrile we observed the saturation in assay. The assay was performed as mentioned with different enzyme concentrations. Increase in benzaldehyde was measured at 280 nm.

Kinetic studies of *Bm*HNL with the addition of sucrose was also performed using mandelonitrile as a substrate. 400 mg/mL of sucrose was added to *Bm*HNL which was

stored in 50 mM citrate-phosphate buffer pH 3.5. It was used in enzyme kinetics using protocols described above.

6.3.6. Effect of benzaldehyde concentration in synthesis of (*S*)-mandelonitrile

The effect of benzaldehyde concentrations in synthesis of (*S*)-mandelonitrile was studied by varying benzaldehyde concentrations from 0.8 to 20 mM. The reaction mixture of 1 mL total contained 4 U of purified *Bm*HNL, 40 µL of benzaldehyde of 20 to 500 mM stock solution in dimethyl sulphoxide (DMSO) equivalent to a final concentration 0.8 to 20 mM, 100 µL of 1 M KCN in double distilled water and 820 µL of 300 mM citrate buffer pH 4.2. Biocatalysis was carried out in a thermomixer by incubating the reaction mixture at 22 ^{o}C, 1000 rpm. After 5 min, 1 mL of hexane: IPA (90:10) was added to it. The organic extract was analyzed by chiral HPLC in a Chiralpak IE column using hexane: IPA. The % conversion and ee of mandelonitrile synthesized were calculated from the HPLC chromatograms as per literature [24].

6.3.7. Effect of polyols in synthesis of (*S*)-mandelonitrile

Effect of polyols was studied in enantioselective synthesis of mandelonitrile. In order to pursue this, we have selected a condition where *Bm*HNL is less stable, so that any improvement in stability can be suitable studied. The enzyme stored in 50 mM citrate-phosphate buffer pH 3.5 was used in the biocatalysis. To investigate the effect of polyols, glycerol and sorbitol were selected in 50 and 200 mg/mL concentration respectively. A reaction mixture of 40 µL of purified *Bm*HNL (4 U) stored in 50 mM citrate-phosphate buffer pH 3.5, 40 µL of 20 mM benzaldehyde in DMSO, 100 µL of 1M KCN in double distilled water, and 820 µL of 300 mM citrate buffer pH 4.2 along with 50 mg of glycerol/200 mg sorbitol were taken. Biocatalysis and product monitoring was carried out

as per **6.3.6**. A control experiment was performed in the same manner without addition of polyols.

We further studied the effect of polyols in the *Bm*HNL catalyzed cyanohydrin synthesis where the enzyme stored in 20 mM potassium phosphate buffer pH 7.0 was used. The rest of the protocol is the same as described above. Effect of glycerol was also studied toward *Bm*HNL catalyzed synthesis of (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile i.e. (*S*)-**10** (**Chapter 3.A**) and (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile i.e. (*S*)-**13** (**Chapter 3.A**) under the same condition where *Bm*HNL was stored in 20 mM potassium phosphate buffer pH 7.0.

6.4. Results

6.4.1. Protein purification and HNL assay

*Bm*HNL was purified by affinity chromatography in a one-step purification with Ni-NTA agarose column. Different fractions obtained during purification were analyzed by SDS-PAGE (**Figure 6.1**). Presence of a clear band of ~29 kDa for the purified fraction confirms the presence of *Bm*HNL. Further, its HNL activity was confirmed by mandelonitrile cleavage assay. The specific activity was found to be 42.4 U/mg in comparison to 49.3 U/mg reported earlier [4].

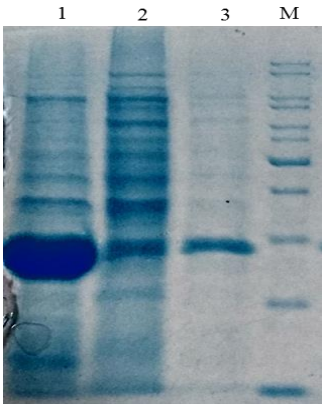


Figure 6.1: SDS-PAGE of different fraction of *Bm*HNL purification (1: Cell pellet of *E.coli* BL21 (DE3) pCold1-*Bm*HNL syntgene 1, 2: Cell lysate, 3: Pure *Bm*HNL, M: Marker)

6.4.2. Biophysical parameters

6.4.2.1. Effect of buffer pH on stability and activity

The stability and activity of *Bm*HNL were studied in different pH ranging from 3.5 to 6.5, by incubating it in citrate-phosphate buffer of the corresponding pH. Below pH 3.5, the enzyme may denature while beyond pH 6.5, spontaneous formation of racemic cyanohydrin increases.The half-life of *Bm*HNL was measured using HNL assay (**Figure 6.2**). The enzyme showed higher half-life and hence increased stability with an increase in pH from 4.5 till 6.5. At pH 6.5 its half-life was ~990 h, at pH 6 it was ~794 h, and at pH 5.5 it was 554 h. At pH 3.5, *Bm*HNL inactivated fast with least stability i.e. ~6 h, which is almost 160 fold less than its stability at pH 6.5. *Bm*HNL showed half-life of ~47 h, at pH 4.5 while its half-life at pH 5.0 was ~238 h. Along with stability, specific activity of *Bm*HNL in buffer ranging from 3.5 to 6.5 was also studied. The highest specific activity i.e. 41.82 U/mg was observed at pH 5.5 while it was 2.3 U.mg at pH 3.5. The enzyme

showed 9 and 28 U/mg at pH 4 and 4.5 respectively. Subsequently, specific activity of 33 and 30 U/mg were observed at pH 6 and 6.5 respectively.

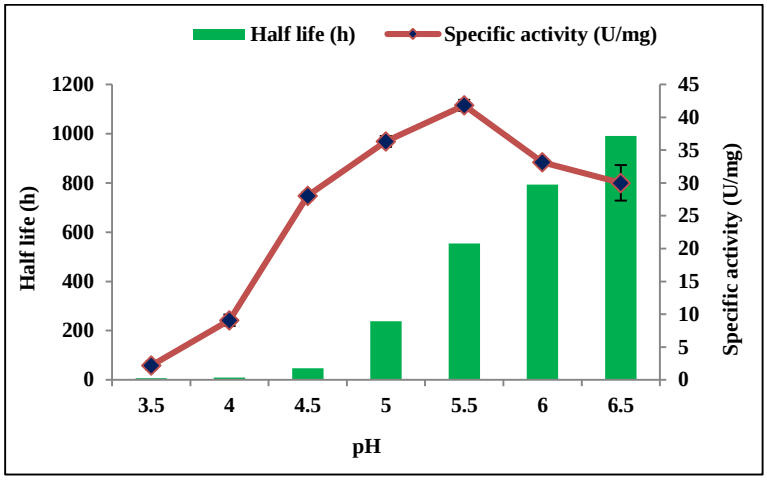


Figure 6.2: Half-life and specific activity of *Bm*HNL in different pH

6.4.2.2. Effect of reaction temperature

Study of stability and activity of *Bm*HNL at various temperature (**Figure 6.3**), showed that half-life of *Bm*HNL was decreased with increase in temperature. The enzyme was highly stable at lower temperature e.g. highest half-life of 831 h was observed at 10 ^{o}C. Enzyme's half-life at 20 ^{o}C and 30 ^{o}C were 686 and 503 h respectively. The half-life decreased to 0.2 h with an increase in temperature i.e. 80 °C. The specific activity of *Bm*HNL was also measured at different temperatures ranging from 10 to 80 ^{o}C (**Figure 6.3**). It showed maximum activity i.e. 43.4 U/mg at 20 ^{o}C. It has reduced to 50% at 50 ^{o}C while beyond that it decreased further.

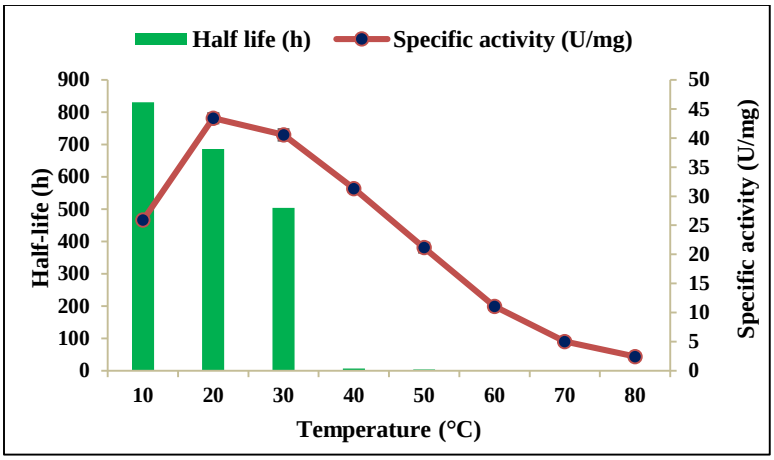


Figure 6.3: Half-life and specific activity of *Bm*HNL at different temperature

6.4.2.3. Effect of different buffer concentration

The stability and activity of *Bm*HNL different concentrations of citrate-phosphate buffer were studied. The half-life of *Bm*HNL was increased with an increase in buffer concentration (**Figure 6.4**). The enzyme showed the highest half-life of ~1399 h at room temperature in 400 mM of citrate-phosphate buffer. The half-life of *Bm*HNL in 300 mM citrate-phosphate buffer was ~973 h whereas in 50, 100 and 200 mM citrate-phosphate buffer, the half-life was 534.5 h, 689.9 h, and 751 h respectively.

The specific activity of *Bm*HNL was also measured in different citrate-phosphate buffer concentrations (**Figure 6.4**). The highest activity was observed in 100 mM citrate-phosphate buffer i.e. 42.4 U/mg. The specific activity decreased gradually with increased buffer concentration. In 200 mM citrate-phosphate buffer, the activity was 33.9 U/mg whereas the lowest was observed in 400 mM buffer concentrations i.e. 15.6 U/mg.

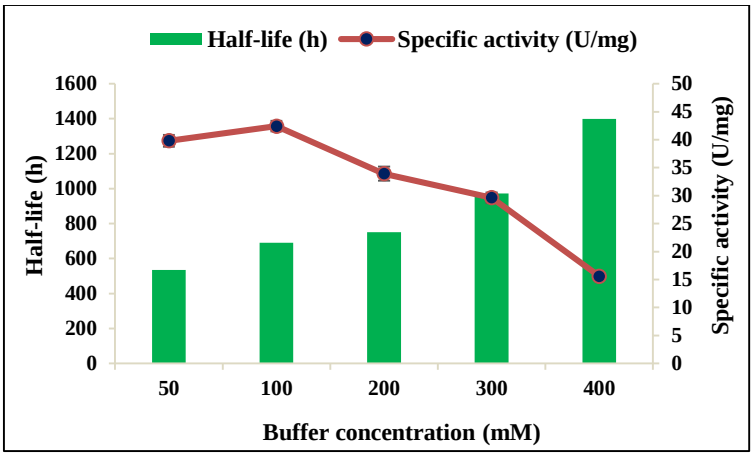


Figure 6.4: Half-life and specific activity of *Bm*HNL in different buffer concentration

6.4.2.4. Effect of organic solvents

The stability and activity of *Bm*HNL were studied in organic solvents by incubating the enzyme solution in 10% v/v selected organic solvents as mentioned in section 6.3.3.4. Among the organic solvents, the highest half-life of 196 min was observed in DIPE, followed by 122 min in TBME (**Figure 6.5**). The half-life of *Bm*HNL in hexane, AcN, *n*-BA, and toluene were 22, 32, 37 and 48 min respectively.

The specific activity of *Bm*HNL was investigated in different organic solvents (**Figure 6.5**). Among all solvents highest activity was observed in *n*-BA i.e. 0.71 U/mg. The enzyme showed 0.6 U/mg specific activity in DIPE while in case of toluene it was 0.5 U/mg. In acetonitrile and TBME, the specific activity of enzyme was the lowest i.e. 0.28 and 0.25 U/mg.

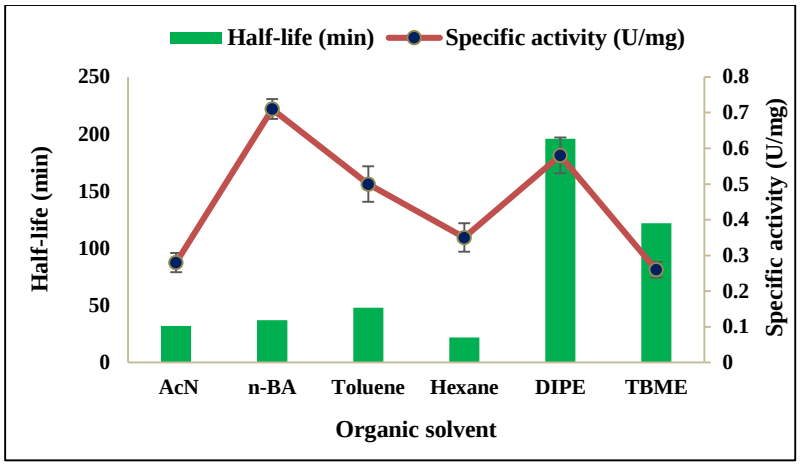


Figure 6.5: Half-life and specific activity of *Bm*HNL in organic solvents

6.4.2.5. Effect of stabilizers

In order to investigate the effect of stabilizers, we have selected a condition where *Bm*HNL showed poor stability i.e. the enzyme was taken in 50 mM citrate-phosphate buffer pH 3.5. Addition of sucrose of high concentrations e.g. 400 mg/mL has improved the half-life of *Bm*HNL at pH 3.5, more than five folds (1980 min) as compared to without stabilizer (371 min) (**Figure 6.6**). The stability increased 3, 3.6 and 4.8 fold with 50, 100 and 200 mg/mL of sucrose respectively which was 1147, 1357 and 1794 min respectively. Improved enzymatic stability was observed with all the four tested stabilizers. Addition of 50 and 100 mg/mL of lactose increased *Bm*HNL's stability 2.75 fold (1020 min) and 3.27 fold (1214 min) respectively as compared to without stabilizer. Addition of 50, 100, 200 and 400 mg/mL of sorbitol has improved the stability by 1.26, 1.64, 1.9 and 2.67 fold compared to without addition.

The effect of stabilizers on *Bm*HNL's specific activity was also studied. All four stabilizers tested in various concentrations have increased the HNL activity (**Figure 6.6**). Highest activity i.e. 30.11 U/mg was observed with sorbitol (200 mg/mL) and glycerol (50 mg/mL)

while with other additives the activity was in the range of 25-29 U/mg vs 3.4 U/mg in case of no additive. Thus there is ~ 9 fold increase in specific activity observed due to the addition of sorbitol or glycerol.

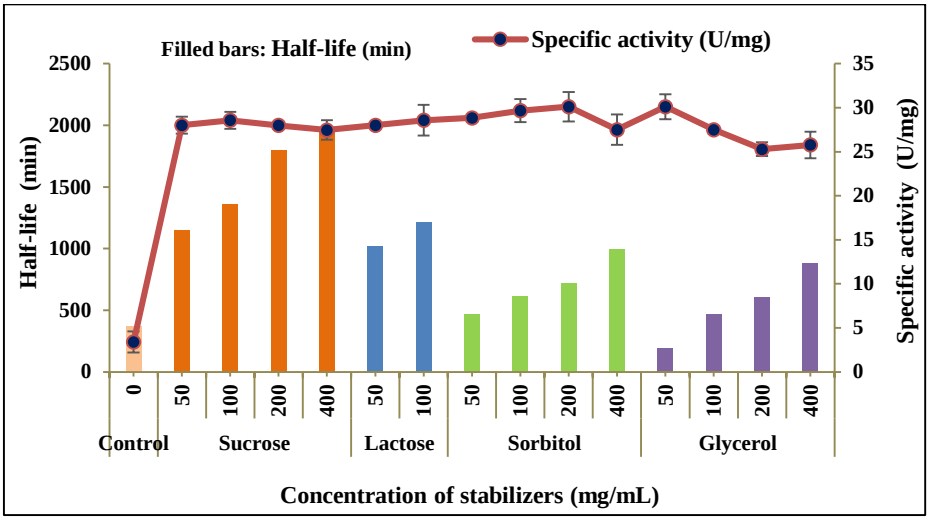


Figure 6.6: Half-life and specific activity of *Bm*HNL at stabilizers

6.4.2.6. Effect of benzaldehyde concentrations

Effect of benzaldehyde concentrations over the stability of *Bm*HNL was studied by measuring the half-life of the enzyme in different benzaldehyde concentrations (**Figure 6.7**). With increased benzaldehyde concentration, *Bm*HNL's stability decreased significantly. The half-life of enzyme decreased drastically with the addition of 5 mM benzaldehyde i.e. 379 min. It decreased up to 17 min in 35 mM benzaldehyde.

The specific activity in the presence of different benzaldehyde concentrations was also measured. The enzymatic activity decreased to 5.5 U/mg with 5 mM benzaldehyde as compared to 41 U/mg without benzaldehyde (**Figure 6.7**). The enzyme showed specific

activity of 4.7, 3.4, 2.5 and 1.9 U/mg with 10, 15, 20 and 30 mM benzaldehyde. The specific activity decreased to 1.4 U/mg with the addition of 40 mM benzaldehyde.

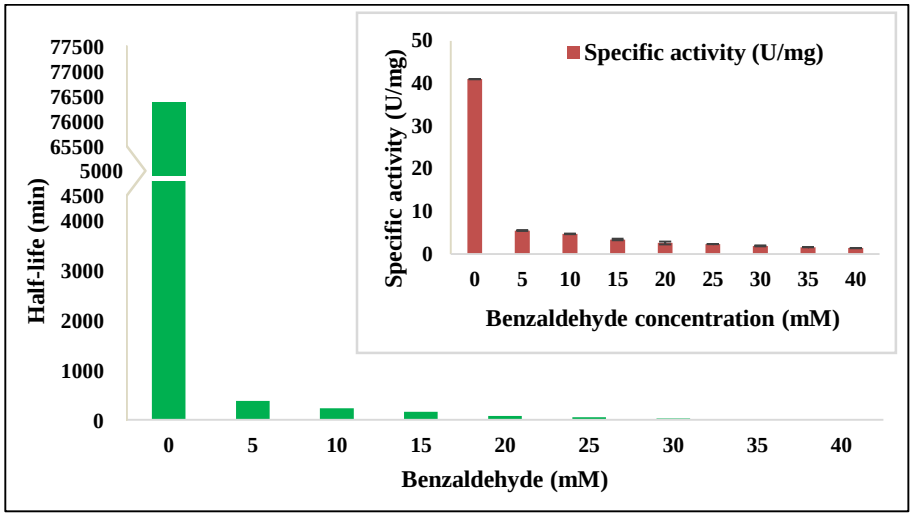


Figure 6.7: Half-life and specific activity of *Bm*HNL with different benzaldehyde concentration

6.4.2.7. Effect of chemical additives/inhibitors

The effect of various inhibitors over stability and activity of *Bm*HNL was studied. *Bm*HNL's half-life in the presence of 2-mercaptoethanol was 112 min while the enzyme activity was 14 U/mg (**Figure 6.8**). *Bm*HNL in absence of any inhibitor showed a half-life of 67 days and specific activity 42.8 U/mg. EDTA addition has little or no change in enzyme's activity as well as stability. The half-life of enzyme was 4651 min and specific activity was 37.71 U/mg in the presence of EDTA. In the presence of acetone, the half-life and residual activity were 226 min and 14.17 U/mg and in presence of AcCN they were 245 min and 9.68 U/mg respectively. In case of PMSF addition, 27% loss of enzyme activity and low stability i.e. 196 min half-life was observed. In the presence of $AgNO_3$, the activity and stability were 6.35 U/mg and 40 min respectively. The enzyme lost the activity by 27.59 U/mg in case of $ZnSO_4$ while the half-life was 389 min.

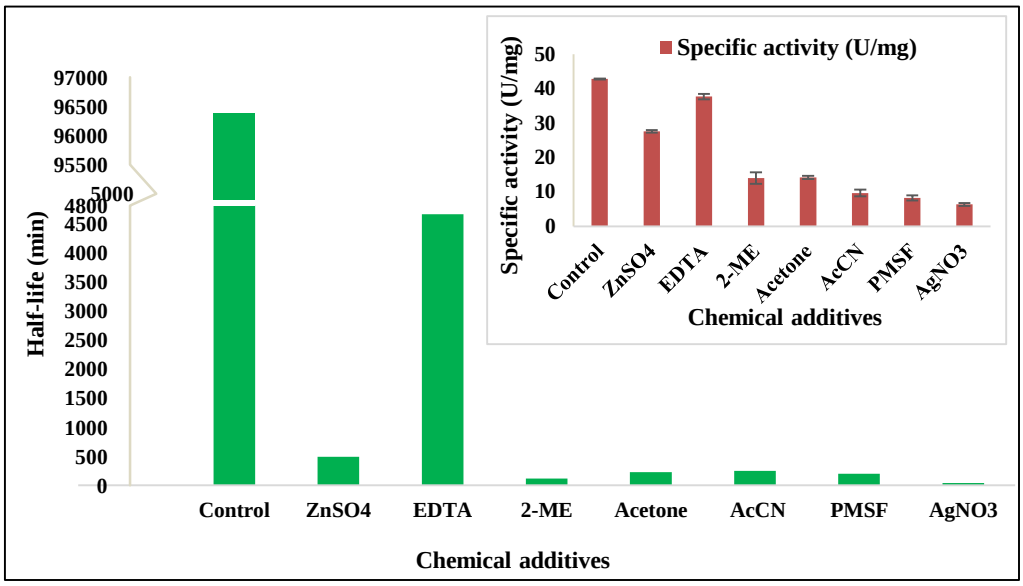


Figure 6.8: Half-life and specific activity of *Bm*HNL in presence of different chemical additives

6.4.3. Secondary structure study by CD analysis

6.4.3.1. CD analysis of *Bm*HNL at different temperatures

The effect of temperature on the secondary structure of *Bm*HNL was investigated in a circular dichroism spectrophotometer over a range of 195 to 255 nm. The spectra were recorded on different temperatures e.g. from 10 to 70 ^{o}C (**Figure 6.9**). After incubating the enzyme at different temperature ranges there was change observed in both α-helix and β-sheets. The random coil content of the enzyme did not change to that extent with an increase in temperature (**Table 6.1**). The percentage of helix was decreased from 38.3 to 28.8% with the increase in temperature, whereas the percentage of β-sheets increased from 12.8 to 22.9%. At 25 °C the α-helix decreased from 38.3 to 34.8% while β-sheets increased 12.5 to 15.4%.

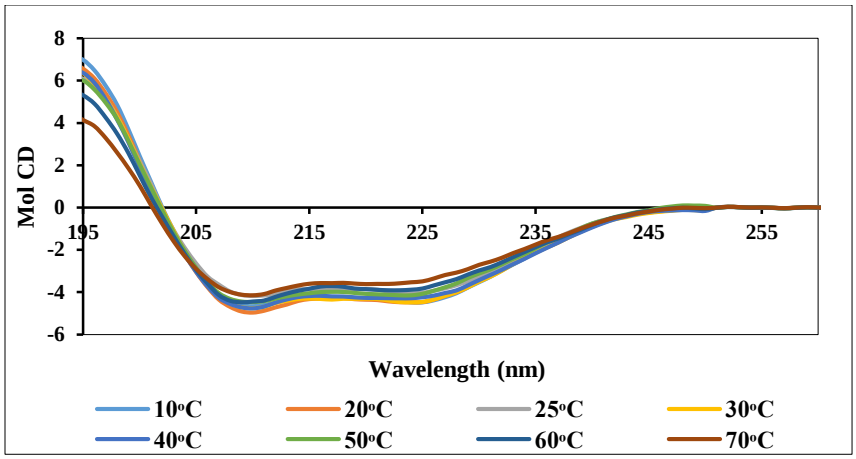


Figure 6.9: CD spectra of *Bm*HNL at different temperatures

Table 6.1: Secondary structure elements of *Bm*HNL at different temperatures

Temp (°C)	% *α*-Helix	% *β*-sheets	% Random coils
10	38.3	12.8	48.9
20	38.2	13	48.8
25	34.8	15.4	49.8
30	33.8	16.3	49.9
40	33.4	16.8	49.8
50	32.2	18	49.8
60	31	19.7	49.3
70	28.8	22.9	48.3

6.4.3.2. CD analysis of *Bm*HNL in different organic solvents

The influence of organic solvents on the structure of *Bm*HNL was also investigated by circular dichroism spectrophotometer over a range of 195 to 255 nm. The spectra were recorded in two different organic solvents i.e. *n*-BA and DIPE (**Figure 6.10**). The enzyme was most stable in DIPE whereas the activity of enzyme was the highest in *n*-BA as

mentioned in section **6.4.2.4**. After incubating the enzyme in organic solvents, there was change observed in both α-helix and β-sheets. The random coil did not change the enzyme to that extent in both the organic solvents (**Table 6.2**). The percentage of α-helix was decreased from 38.3 to 28.4% in 10% v/v DIPE, whereas the percentage of β-sheets increased from 15.4 to 22%. In *n*-BA, percentage of α-helix was decreased from 38.3 to 32.8%, whereas the percentage of β-sheets increased from 15.4 to 17.2%.

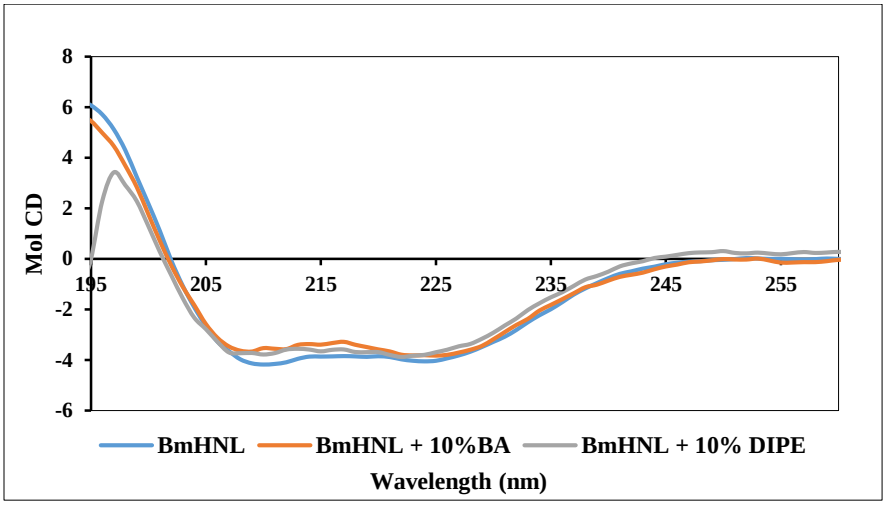


Figure 6.10: CD spectra of *Bm*HNL in organic solvents

Table 6.2: Secondary structure elements of *Bm*HNL in presence of different organic solvents

Sample	% α-Helix	% β-sheets	% Random coils
*Bm*HNL	34.8	15.4	49.8
*Bm*HNL+10% *n*-BA	32.8	17.2	50
*Bm*HNL+10% DIPE	28.4	22	49.6

6.4.4. Kinetic study of *Bm*HNL

*Bm*HNL's kinetic behavior was studied by determining its kinetic parameters using mandelonitrile cleavage assay. For kinetic study, two sets of experiments were conducted simultaneously. In first set of experiments, the enzyme concentrations were varied with a fixed substrate concentration to obtain the highest activity. In the second experiment, different substrate concentrations were used with a fixed enzyme concentration (**Figure 6.11, left**). The kinetics was performed with 0.24 mg/mL *Bm*HNL and substrate concentration was varied from 0.1 to 30 mM (0.01 to 3 mM final concentration). K_M value for the enzyme was found to be 0.05 mM and V_{max} was 32.04 U/mg (**Table 6.3**). The enzyme showed k_{cat} value of 897.12 min^{-1} and catalytic efficiency (k_{cat}/K_M) of 17942.4 $min^{-1}mM^{-1}$. The kinetic parameters were calculated as per Michalis-Menten equation.

Further, we studied the kinetic behavior of *Bm*HNL at low pH after addition of stabilizer using the same procedure as above (**Figure 6.11, right**). Enzyme with 400 mg/mL of sucrose was selected for the study because it provided the highest stability to the enzyme at low pH (section **6.4.2.5**). K_M value for the enzyme was found to be 0.20 mM and V_{max} 28.09 U/mg (**Table 6.3**). The enzyme showed k_{cat} value of 786.52 min^{-1} and catalytic efficiency (k_{cat}/K_M) of 3932.6 $min^{-1}mM^{-1}$.

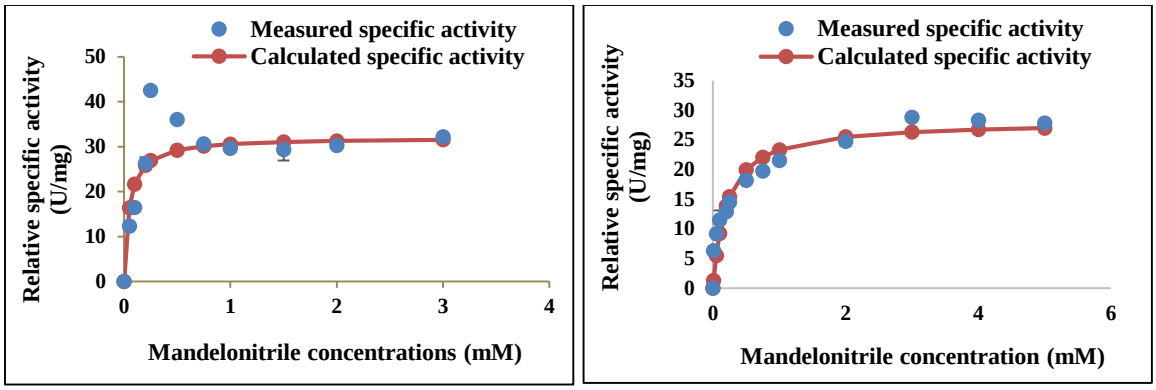


Figure 6.11: Left: Kinetic study of purified *Bm*HNL, stored in 20 mM KPB pH 7.0; Right: Kinetic study in presence of sucrose at 50 mM citrate-phosphate buffer pH 3.5

Table 6.3: Kinetic parameter of *Bm*HNL

Enzyme	K_M (mM)	V_{max} (U/mg)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}mM^{-1}$)
*Bm*HNL in pH 7.0	0.05	32.02	897.12	17942.4
*Bm*HNL in pH 3.5	0.20	28.09	786.52	3932.6

6.4.5. Effect of benzaldehyde concentration in the synthesis of (*S*)-mandelonitrile

Effect of different benzaldehyde concentrations in the *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was investigated (**Figure 6.12**) by performing the biotransformation for 5 minutes as per **6.3.6**. Benzaldehyde concentration was varied from 0.8 to 20 mM in different biocatalysis. In case of 0.8 mM benzaldehyde, *Bm*HNL showed highest i.e. 75.6% ee and 58.8% conversion. With increasing concentrations, decreased % ee was observed. With 5 mM benzaldehyde, 65.5% ee and 76.3% conversion was observed while with 10 mM benzaldehyde, it was 55.5% ee and 44% conversion respectively.

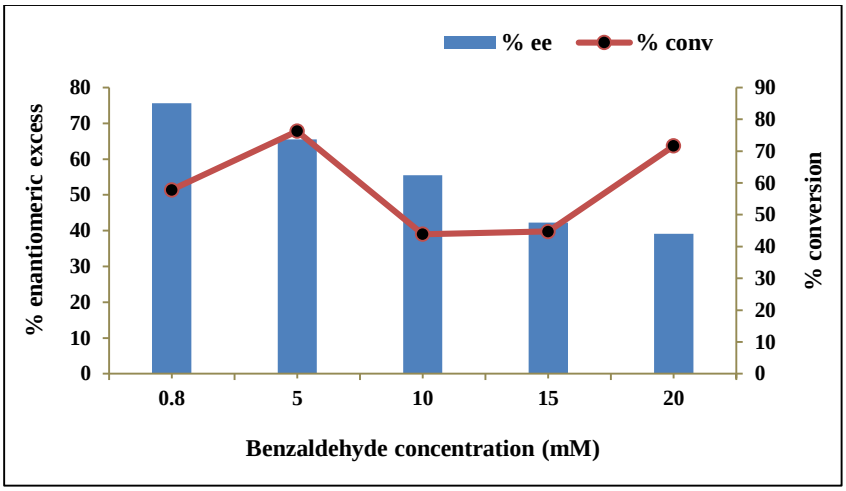


Figure 6.12: Effect of benzaldehyde concentration in the synthesis of (*S*)-mandelonitrile

6.4.6. Effect of polyols in the synthesis of (*S*)-mandelonitrile

As polyol addition to *Bm*HNL has increased its half-life at low pH, we aimed to understand the effect of polyols in the *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile by using glycerol and sorbitol in the concentration of 50 and 200 mg/mL respectively. The enzyme was stored in 50 mM citrate-phosphate pH 3.5 and biocatalysis was performed by adding glycerol and sorbitol separately. In case of the sorbitol addition, the biocatalysis has produced 97.6% ee and 56.2% conversion of (*S*)-mandelonitrile in 10 minutes while in presence of glycerol it showed highest i.e. 99.2% ee and 47.7% conversion in 15 minutes (**Figure 6.13, 6.14** and **6.16**). A control experiment with purified enzyme having no polyol showed ~75% ee and ~33% conversion in 5 minutes (**Figure 6.13, 6.14** and **6.15**). The % ee of product in the biocatalysis having pure enzyme without polyol has decreased from ~75 to 32% with an increase in reaction time. A similar trend is observed in case of sorbitol and glycerol added biocatalysis. In case of sorbitol added biocatalysis, the % ee of product

decreased to 93% in 15 minutes and 36.2% in 20 minutes, however, highest conversion i.e. 65.6% was found in 20 minutes.

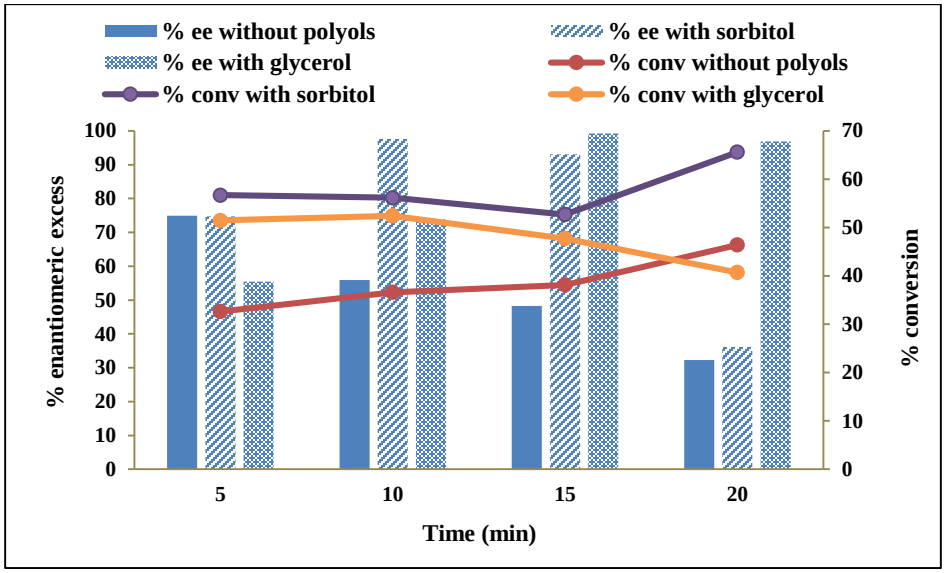


Figure 6.13: Synthesis of (*S*)-mandelonitrile by purified *Bm*HNL stored in pH 3.5 buffer, with addition of polyols

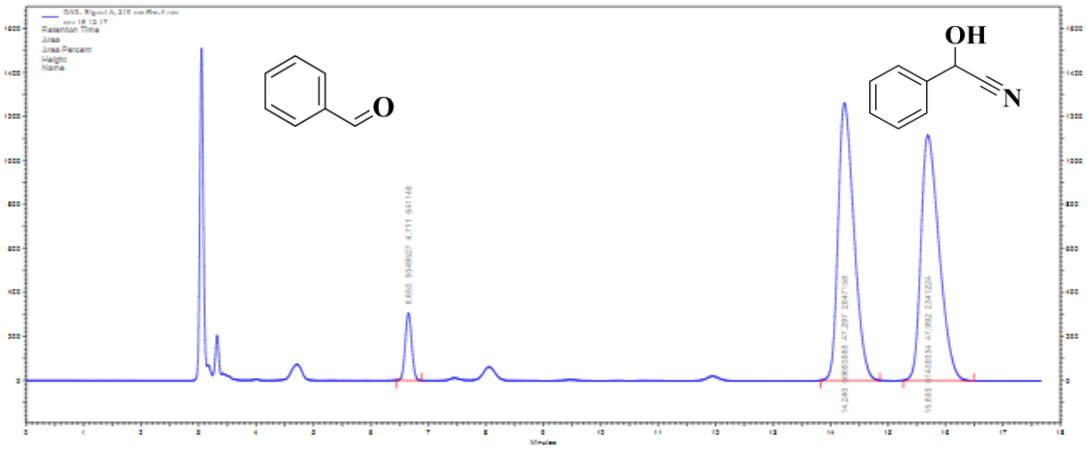


Figure 6.14: HPLC chromatogram of racemic mandelonitrile

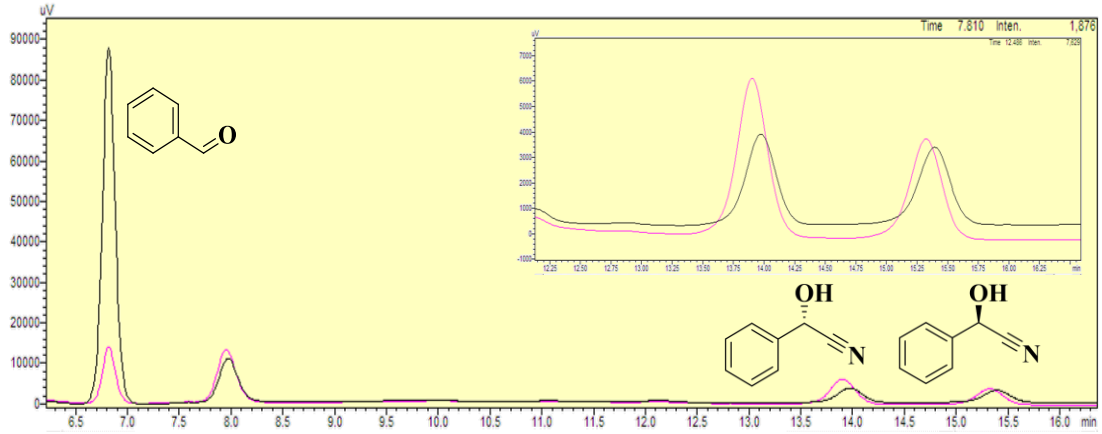


Figure 6.15: HPLC chromatogram of *Bm*HNL (stored in pH 3.5) catalyzed synthesis of (*S*)-mandelonitrile with 0.8 mM benzaldehyde without any polyol. Black: using buffer as control in place of enzyme; Pink: *Bm*HNL

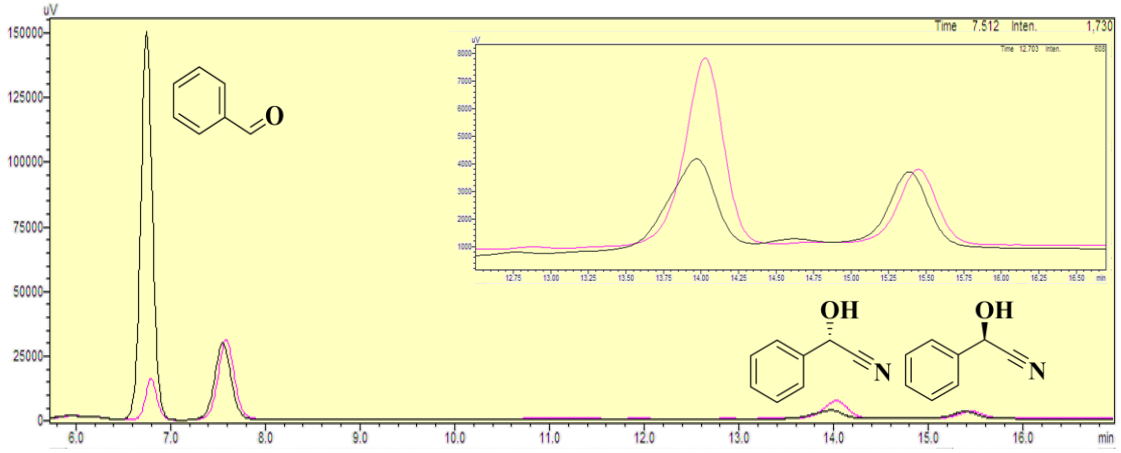


Figure 6.16: HPLC chromatogram of *Bm*HNL (stored in pH 3.5) catalyzed synthesis of (*S*)-mandelonitrile with 0.8 mM benzaldehyde in presence of glycerol. Black: using buffer as control in place of enzyme; Pink: *Bm*HNL

Having achieved very high % ee of product by polyol addition to *Bm*HNL at low pH, we performed another study where the enzyme used was stored in 20 mM KPB pH 7.0 instead of 50 mM citrate-phosphate pH 3.5 and the biocatalysis was carried out as described in

section 6.3.7. In the *Bm*HNL catalyzed biocatalysis with glycerol, benzaldehyde concentration was varied from 0.8 to 20 mM (**Figure 6.17**). The control experiment was performed without the addition of glycerol. The enzyme showed 95% ee and 54.3% conversion with 0.8 mM benzaldehyde in the presence of glycerol while 75.6% ee and 54.3% conversion was observed without the addition of glycerol in 5 min. In 5 mM benzaldehyde, biocatalysis with glycerol showed 96.2% ee and 28.2% conversion while 65.5% ee and 76.3% conversion was observed without glycerol in 5 min. Even in case of 10 mM benzaldehyde, high i.e. 95% ee was observed in the presence of glycerol compared to only 55.5% ee in the absence of glycerol in 5 min.

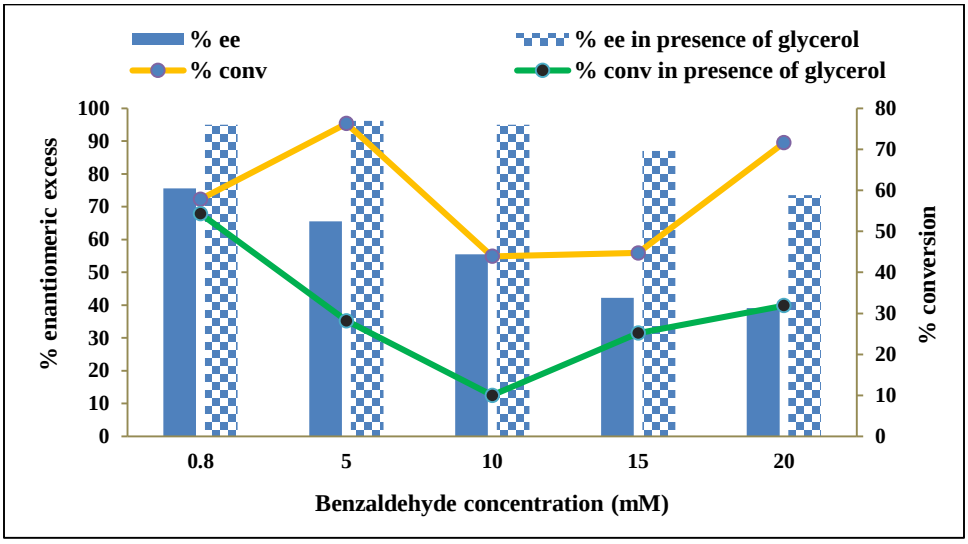


Figure 6.17: Synthesis of (*S*)-mandelonitrile by purified *Bm*HNL stored in pH 7.0, in presence of glycerol and with different benzaldehyde concentration

As we observed 95 to 96% ee in the biocatalysis with 5 and 10 mM benzaldehyde in presence of glycerol, so we further investigated the effect of glycerol and sorbitol in the synthesis of (*S*)-mandelonitrile at different time intervals using both 5 and 10 mM benzaldehyde (**Figure 6.18**). With 5 mM benzaldehyde, enzyme without polyol showed 71% ee and 51% conversion (**Figure 6.19**), in presence of sorbitol 71% ee and 50%

conversion while glycerol addition has improved the % ee to 99.3% in 5 minutes (**Figure 6.20**). However, the later showed decreased % conversion i.e. 35.8% compared to 51% without glycerol. Decreased % ee was observed with increased reaction time in all the cases with and without polyols. In case of 10 mM benzaldehyde, enzyme without polyol gave 65.2% ee and 55.2% conversion, in presence of sorbitol 73% ee and 54% conversion while glycerol addition has improved the % ee to 99.85% ee but again the % conversion decreased to 38% in 5 minutes. In this case also, decreased % ee was observed with increased reaction time. Sorbitol did not improve the % enantiomeric excess of the purified enzyme in this study.

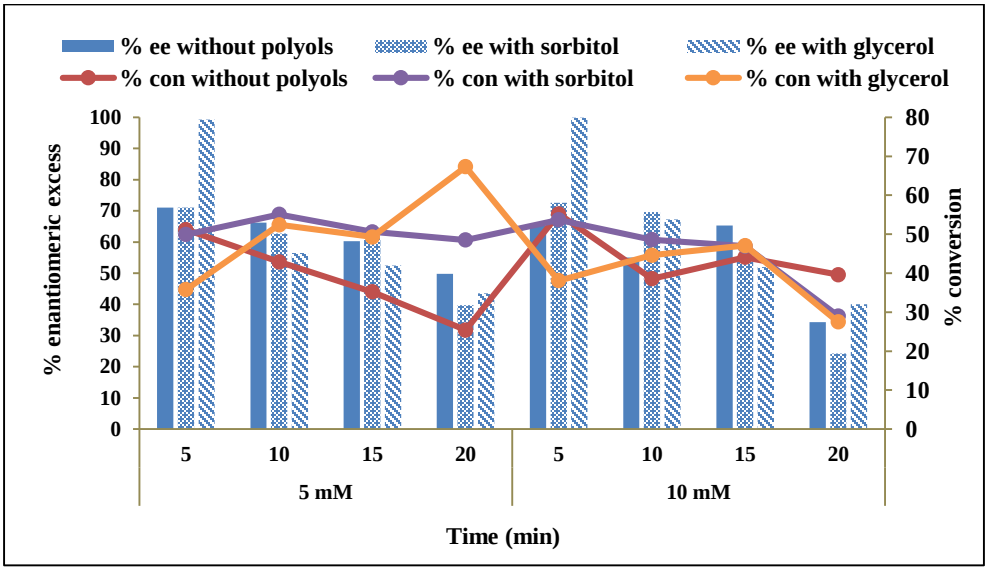


Figure 6.18: Synthesis of (*S*)-mandelonitrile by purified *Bm*HNL (pH 7.0) with addition of polyols

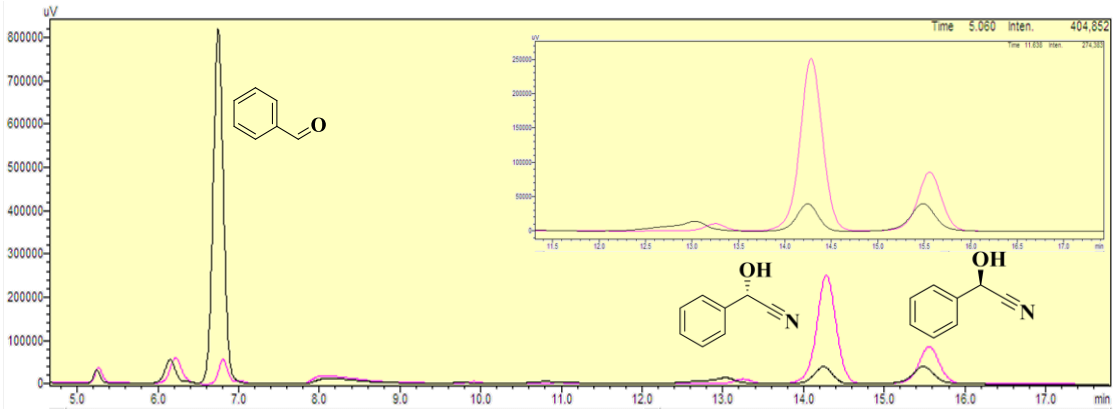


Figure 6.19: HPLC chromatogram of *Bm*HNL (stored in pH 7.0) catalyzed synthesis of (*S*)-mandelonitrile with 5 mM benzaldehyde without any polyol. Black: using buffer as control in place of enzyme; Pink: *Bm*HNL

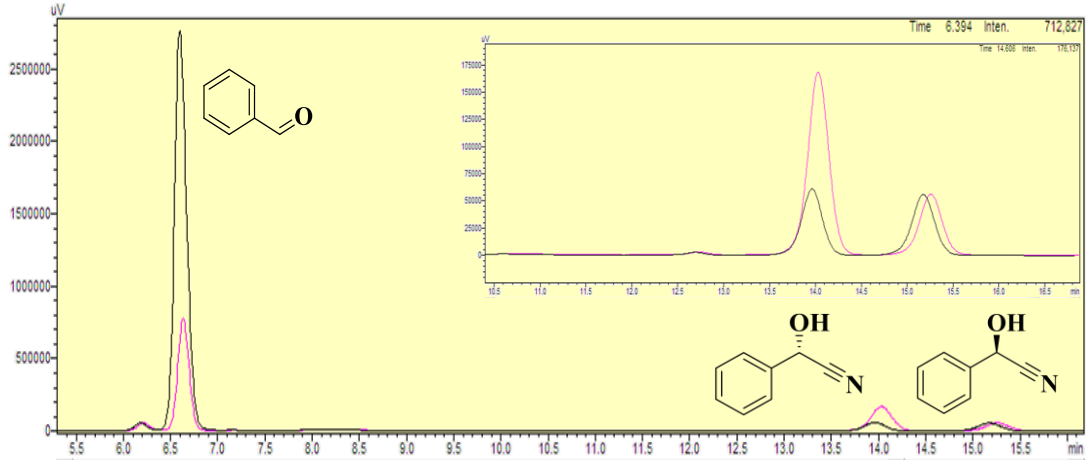


Figure 6.20: HPLC chromatogram of *Bm*HNL stored in pH 7.0, catalyzed synthesis of (*S*)-mandelonitrile with 5 mM benzaldehyde with glycerol. Black: using buffer as control in place of enzyme; Pink: *Bm*HNL

Note: Identification of (*S*)- and (*R*)-mandelonitrile in the HPLC chromatogram has been done by using standard (*R*)-mandelonitrile obtained by *At*HNL catalyzed synthesis and analyzed in the same chiral column by HPLC.

We studied *Bm*HNL catalyzed synthesis of (*S*)-**10** (**Chapter 3.A**) in the presence of glycerol. The biotransformation was carried out by varying substrate concentration from 0.8 to 10 mM (**Figure 6.21**). *Bm*HNL with 0.8 mM substrate concentration showed improved % ee i.e. 89.5% in the presence of glycerol compared to 54% without glycerol. However, % conversion of product was decreased to 25.8% compared to 70.41% without glycerol. In 5 mM substrate concentration, the biocatalysis with glycerol has shown the 86.4% ee and 13.4% conversion of product compared to 55% ee and 22.4% conversion in the absence of glycerol. In case of 10 mM substrate concentration, ~60% ee and 13.4% conversion was observed in the presence of glycerol while enzyme showed ~44% ee and 13.5% conversion without glycerol.

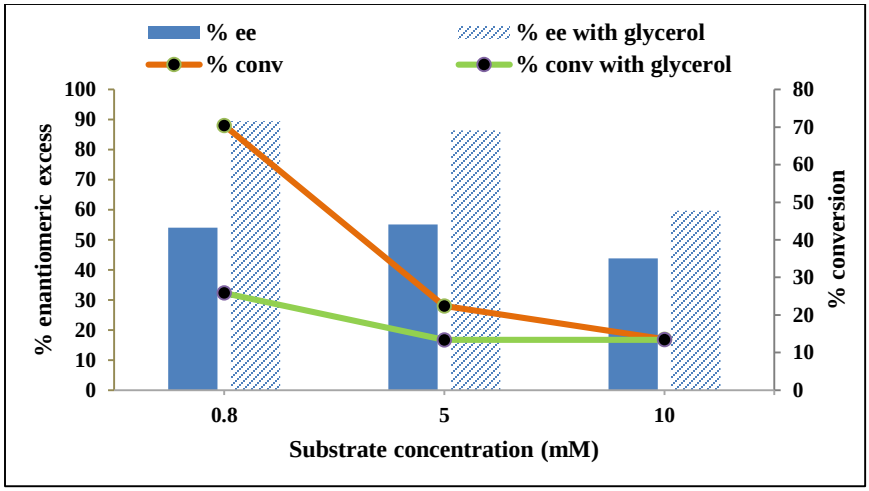


Figure 6.21: Synthesis of (*S*)-2-hydroxy-2-(3-phenoxyphenyl) acetonitrile by purified *Bm*HNL (pH 7.0) with addition of glycerol.

*Bm*HNL catalyzed synthesis of (*S*)-**13** (**Chapter 3.A**) was carried out in the presence of glycerol (**Figure 6.22**) while the study with addition sorbitol was excluded due to negligible increase in % ee by it (**Figure 6.18**). The biocatalysis was carried out with 10 mM substrate concentration. Glycerol added *Bm*HNL biocatalysis has showed improved

% ee i.e. 75% and ~29% conversion compared to 39% ee and ~19% conversion in absence of glycerol in 15 minutes. While the % ee of product in the glycerol added biocatalysis decreased with time, % conversion of product was not affected much.

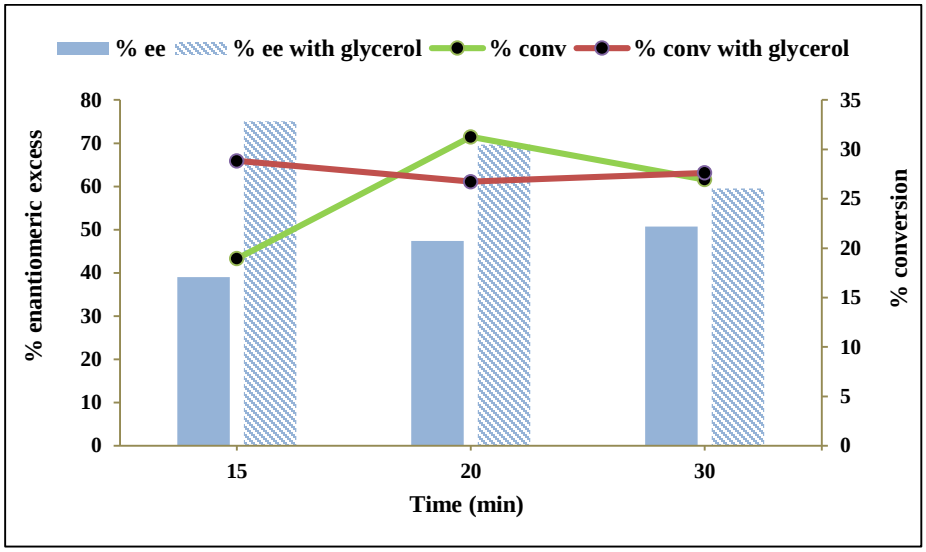


Figure 6.22: Synthesis of (*S*)-2-hydroxy-2-(3,5-dimethoxyphenyl) acetonitrile by purified *Bm*HNL (pH 7.0) with addition of glycerol

6.5. Discussion

6.5.1. Effect of buffer pH on stability and activity

pH is an important factor in HNL catalyzed cyanohydrin synthesis as well as cyanogenesis. At higher pH, spontaneous formation of racemic cyanohydrin (or formation of benzaldehyde in case of cyanogenesis) occurs at higher rate which results in decreased enantiomeric excess of product. To overcome this limitation, HNL mediated reactions are usually carried out at pH 5 or below. However, in order to carry out HNL biocatalysis, the knowledge of stability and activity of the enzyme are very important. The stability and activity of *Bm*HNL were studied in different pH. The half-life of *Bm*HNL was measured using HNL assay (**Figure 6.2**). The stability of enzyme increased with increase in pH from

4.5 till 6.5. The half-life of enzyme at pH 6.5 was ~990 h while it was ~794 h in pH 6 buffer. At lower pH unfolding of 3D structure of enzyme occurs that leads to denaturation of enzyme. At extreme pH, enzyme inactivation could be due to irreversible ionizations leading to the unfolding of the protein while enzyme stability increases with increase in pH [25]. This study suggests that *Bm*HNL can be used even at low pH such as 4.5 and 5.0 for ~47 and ~238 h respectively. In a similar study, Bauer *et al* checked the stability of *Hb*HNL at different pH and observed that increase in pH has increased the stability of the enzyme [17]. The half-life of *Hb*HNL in 5 mM citrate buffer at pH 3.5, 5.0 and 6.5 were 7, 250 and 2250 min respectively. However, *Hb*HNL's stability in 20 mM citrate-phosphate buffer at different pH ranging from 3.5 to 5.5 was found to be higher compared to the corresponding stability in 5 mM citrate buffer of same pH [20]. In 20 mM CPB at pH 4.5, 5 and 5.5, its half-life were 3.33, 24.17 and 83.33 h respectively [20]. At pH 3.5 *Hb*HNL's half-life was only 3 min while at pH 4.5, it was 3.33 h. The stability was more at higher pH. The highest half-life was 83.33 h in pH 5.5. Guterl *et al* compared the effect of pH in two different HNLs i.e. *At*HNL and *Me*HNL [21]. The (*R*)-selective *At*HNL showed half-life up to 50 h in citrate-phosphate buffer pH 6.0, while the (*S*)-selective *Me*HNL showed half-life of 50 h at both pH 5.0 and 6.0. The half-life of *At*HNL decreased drastically at pH 5.0, while the same happened to *Me*HNL at pH 4, suggesting that *Me*HNL is more stable at lower pH than *At*HNL.

The highest activity of *Bm*HNL was observed at pH 5.5 i.e. 41.82 U/mg. The activity decreased below and above pH 5.5. The activity at pH 5.5 was 20 fold higher than its activity at pH 3.5. At lower pH unfolding of the secondary and tertiary structure of enzyme occurs that leads to denaturation of enzyme [19]. At higher pH, spontaneous degradation

of substrate was more which resulted in lower activity. *Hb*HNL biocatalysis is carried out at pH 5, the highest activity of *At*HNL was between pH 5.75 to 6.5 while *Me*HNL has in citrate-phosphate buffer pH 5.75 [21]. Among all α/β hydrolase fold hydroxynitrile lyase enzyme, *Bm*HNL showed the highest half-life at its optimum pH i.e. 554.46 h, at pH 5.5. Stability of *Bm*HNL at its optimum pH 5.5 was found to be ~56% than its stability at pH 6.5.

6.5.2. Effect of reaction temperature

Study of stability and activity of *Bm*HNL at various temperature was decreased with increase in temperature (**Figure 6.3**).The highest half-life of 831 h was observed at 10 ^{o}C. Its half-life decreased drastically at higher temperatures e.g. ~7 and ~4 h at 40 °C and 50 ^{o}C respectively. The increased stability at low and decreased stability at high temperature could be due to slow or fast inactivation of enzyme respectively [26]. The specific activity of *Bm*HNL was also measured at different temperatures. Maximum activity of 43.4 U/mg was observed at 20 ^{o}C which is similar to that reported [4]. The enzymatic activity decreased with temperature.

In the comparative study of stability and activity between *At*HNL and *Me*HNL at different temperature, *At*HNL showed maximum activity at 35 ^{o}C while *Me*HNL at 60 ^{o}C [21]. *At*HNL was stable for 96 h in the temperature range of 0 to 10 ^{o}C, but its stability was low at higher temperature. Although its half-life at its temperature optima i.e. 35 ^{o}C is not reported but at 30 and 37 ^{o}C it was 33 and 6.6 h respectively. Similarly *Me*HNL was stable up to 96 h in the temperature range of 0 to 20 ^{o}C, however at its temperature optima i.e. 60 ^{o}C it was stable for only 30 minutes. The exact half-life of both the enzymes was not

reported as it was mentioned as >96 h. The stability of both enzymes decreased with increase in temperature. The stability of *At*HNL at 60 °C was 9.6 minutes only. Bauer *et al* studied the influence of temperature on stability of *Hb*HNL [17]. They observed its highest half-life of 2315 minutes i.e. 38.6 h at 30 °C in 5 mM citrate buffer and it decreased up to 7 min at 70 °C [17]. Thus comparison of the half-life of all the four α/β hydrolase fold HNLs at their corresponding temperature optima indicates that *Bm*HNL has highest among them all.

6.5.3. Effect of different buffer concentration

The concentration of the buffer used in biotransformation has been known to play an important role in enzyme stability. Study of stability of *Bm*HNL in different concentrations of citrate-phosphate buffer showed that with an increase in buffer concentrations, enzyme stability has increased (**Figure 6.4**). The enzyme showed maximum half-life of ~1399 h in 400 mM of citrate-phosphate buffer. The higher the concentration of the buffer system, the higher is its capacity to stabilize the pH, because it increases ionic strength and hence stabilizes the protein structure [25]. The highest specific activity of 42.4 U/mg was observed in 100 mM citrate-phosphate buffer. The specific activity decreased with increased buffer concentration. The reason for this decrease in activity at high buffer concentrations is not clear at the moment, however, it could be due to different charge distributions in enzyme by different buffer concentrations. Bauer *et al* have studied the stability of *Hb*HNL in not only different buffer concentrations but also of different buffers. They also made a similar observation of increased stability of enzyme with increased buffer concentration [17]. The half-life of *Hb*HNL determined at 40 ^{o}C, in 50 and 100 mM citrate buffer pH 6.5 was 600 and 700 min respectively. In contrary, in 50 and 100 mM buffer,

the half-life of *Bm*HNL was 534.46 h and 689.9 h respectively. In case of *Me*HNL and *At*HNL, a similar study has not been reported, although four different buffers e.g. citrate phosphate, potassium phosphate, glutamate and acetate buffer were used [21].

6.5.4. Effect of organic solvents

HNL-catalyzed cyanohydrin synthesis is usually carried out in biphasic system to suppress the background reaction [18,27–33]. *Bm*HNL's stability in organic solvents has not been studied earlier and we have investigated the same here especially in a biphasic system. *Bm*HNL was incubated in six different organic solvents, selected based on our earlier reported [18]. The enzyme showed lower stability in organic solvents compared to aqueous system (**Figure 6.5**). Among the organic solvents, DIPE was observed as the best solvent with the highest half-life of 196 min, followed by 122 min in TBME. Presence of organic solvents alter the hydrophobicity which could make conformational change of enzyme and may cause destabilization of its native structure or protein denaturation [34–36]. The aqueous environment is one of the dominant contributors to protein folding and stability.

The influence of biphasic system on the activity was also studied. There was a tremendous decrease in enzyme activity upon addition of organic solvents. *Bm*HNL showed the highest activity of 0.71 U/mg in *n*-BA among all the organic solvents, while 43.2 U/mg without any organic solvent. Bauer *et al* reported the stability of *Hb*HNL in biphasic system [20]. It showed the highest half-life in the mixture of MTBE and hexane in 40:60, however, this solvent system has shown ~55% relative activity. The highest activity of *Hb*HNL was seen in hexane while rapid inactivation of the enzyme was observed in it.

6.5.5. Effect of stabilizers

Addition of stabilizers such as polyols and sugars increases the stability of proteins at extreme pH [10,17,19,37–41]. The stability of enzyme was improved excellently by the addition of 400 mg/mL sucrose, as compared to without stabilizer (**Figure 6.6**). *Bm*HNL's stability as well as specific activity increased in case of addition of all the tested sugars and polyols in all the mentioned concentrations. Enhancement in enzyme stability by addition of sugars and polyols has been reported previously. Addition of sugars and polyols to an enzyme solution is assumed to improve the hydrophobic interactions among nonpolar amino acids and hence provide resistant to the enzyme to unfold [17,37,42,43]. Increase in *Bm*HNL's stability by sorbitol is comparatively low than sucrose and lactose. This may be due to the less number of hydroxyl groups in the sorbitol structure than lactose and sucrose. Glycerol, the other polyol tested with the lowest number of –OH groups among the four has shown the least increase in half-life of *Bm*HNL compared to the four stabilizers. Addition of 100, 200 and 400 mg/mL of glycerol has shown 1.26, 1.61, and 2.36 fold increased the stability of *Bm*HNL. Stability of *Hb*HNL in presence of different additives e.g. saccharose, sorbitol and different concentrations of *Hevea* extract has been reported [19]. Addition of these additives into the enzyme in 20 mM potassium phosphate buffer pH 3.75 has increased the half-life of *Hb*HNL by 3.84 to 58 fold as compared to without additives. *Hb*HNL's stability has also been tested by adding *Hevea* and *Nephrolepis* extract into the enzyme in 10 mM sodium-citrate buffer pH 3.75. Between the two, *Nephrolepis* extract addition has shown no loss of activity within 60 min after addition to enzyme solution while *Hevea* extract showed half-life of only 20 min. Another study on *Hb*HNL's stability has been reported where different concentrations e.g. 50 to 400 mg/mL of sorbitol, sucrose, lactose, and glycerol were added into the enzyme solution in 5 mM glutamate

buffer pH 3.5 at 30 °C [17]. Among all the tested stabilizers, sorbitol in 400 mg/mL concentration showed six fold improved stability of *Hb*HNL while all others could show a maximum of 3 fold increased stability.

6.5.6. Effect of benzaldehyde concentrations

Study of *Bm*HNL's stability in different benzaldehyde concentrations showed that decreased stability was observed with increased benzaldehyde concentration (**Figure 6.7**). Even with 5 mM benzaldehyde, the half-life of enzyme decreased i.e. 379 min. The reason for lower stability is due to inhibition of *Bm*HNL with increase in benzaldehyde concentration [4]. Similar kind of study was also reported by Bauer *et al* with *Hb*HNL [20]. They performed the stability of *Hb*HNL in the presence of varying concentrations of benzaldehyde in 20 mM citrate-phosphate buffer pH 5.0 at 20 ^{o}C. They observed that the half-life of *Hb*HNL without the addition of benzaldehyde was 450 min but after addition of benzaldehyde, it decreased drastically. In the presence of 2 mM benzaldehyde, the half-life decreased up to 230 min. The half-life was 130 min in 5 mM benzaldehyde while in 10 mM, it was only 75 min. In 40 mM benzaldehyde, half-life of *Hb*HNL was less than <20 min.

The effect of benzaldehyde concentrations over activity was also studied. Similar to stability, decrease in activity with increasing benzaldehyde concentration was observed (**Figure 6.7**). The highest activity of 41 U/mg was observed without the addition of benzaldehyde. The reason for decreasing the specific activity of *Bm*HNL is due to its inhibition by benzaldehyde [4].

6.5.7. Effect of chemical additives

The study of effect of stability and activity of *Bm*HNL in addition of various chemical additives was performed. In the presence of studied chemical additives both the stability and activity of *Bm*HNL decreased, compared to control having no additive (**Figure 6.8**). *Bm*HNL in the absence of any inhibitor was stable up to 67 days. Addition of 2-mercaptoethanol has decreased its enzymatic activity as well as half-life possibly because of the disruption of the homo-dimer of enzyme complex. The half-life and activity of enzyme with the addition of EDTA was 4651 min and 37.71 U/mg. *Bm*HNL is not a metal-dependent enzyme hence EDTA addition has caused the least change to its activity and stability. Addition of acetone and AcCN has decreased *Bm*HNL's activity and stability to a greater extent because of their inhibitory effect. Similar observations were earlier made by Dadashipour *et al* [4]. In case of PMSF addition, 27% loss of enzyme activity and low stability was observed. This is probably due to binding of *Bm*HNL's active site serine (Ser80) with PMSF. It was observed that $AgNO_3$ completely inhibited *Bm*HNL's activity and stability both which were 6.35 U/mg and 40 min respectively. Dadashipour *et al* made a similar observation in their study of *Bm*HNL's activity in the presence of PMSF, $AgNO_3$, $ZnSO_4$, acetone, and AcCN [4].

6.5.8. Secondary structure study by CD analysis

6.5.8.1. CD analysis of *Bm*HNL at different temperatures

Investigation of the secondary structure of *Bm*HNL on different temperature revealed that the enzyme retained some secondary structure at high temperature that might be the reason

for broad temperature stability/activity of the enzyme even at high temperatures although the activity was less.

6.5.8.2. CD analysis of *Bm*HNL in different organic solvents

Secondary structure of *Bm*HNL in organic solvents i.e. *n*-BA and DIPE was also investigated and it was observed that the structure of enzyme was not destabilized to that extent with the above two solvents.

6.5.9. Kinetic study of *Bm*HNL

The current study for kinetic parameters of *Bm*HNL showed less K_M value of the enzyme toward mandelonitrile i.e. 0.05 mM, k_{cat} i.e. 897.12 min^{-1} or 14.95 s^{-1} and V_{max} of 32 U/mg as mentioned in **Table 6.3**. The kinetic parameters of pure *Bm*HNL catalyzed mandelonitrile cleavage has been earlier studied by Dadashipour *et al* [4]. They have reported the K_M, V_{max}, k_{cat} and k_{cat}/K_M as 1.05 mM, 52.5 Umg^{-1}, 25.9 s^{-1} and 24.6 $mM^{-1}\,s^{-1}$ respectively. Comparison of our kinetic parameters with that reported by Dadashipour et al indicates that we observed low K_M and k_{cat}. The probable reason for this could be due to the degree of purity of enzyme used in our experiment is different than that of they used. *Bm*HNL's kinetic parameters at low pH in presence of 400 mg/mL sucrose were found to be K_M of 0.2 mM, k_{cat} of 786.52 min^{-1} or 13.1 s^{-1} and V_{max} of 28.1 U/mg towards mandelonitrile cleavage (**Figure 6.11**, **right** and **Table 6.3**). These kinetic parameters are almost comparable with *Bm*HNL's kinetic parameters obtained at pH 7.0. Comparison of this specific activity i.e. 28.1 U/mg with *Bm*HNL's specific activity at pH 3.5 i.e. 2.3 U/mg (**Figure 6.2**) clearly indicates that sucrose addition to the enzyme has increased its activity

which could be due to the fact that it might have prevented enzyme's denaturation at low pH as explained in 5.5.

6.5.10. Effect of benzaldehyde concentration in the synthesis of (*S*)-mandelonitrile

*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was performed in different benzaldehyde concentrations (**Figure 6.12**). The highest % ee was observed in 0.8 mM benzaldehyde which was 75.6% ee while the % conversion was obtained 58.8. The decrease in % ee with increasing benzaldehyde concentration could be due to the inhibition of *Bm*HNL [4]. Hanefeld *et al* reported a similar trend with *Hb*HNL catalyzed cyanohydrin syntheses [44]. With increasing concentration of benzaldehyde (5 to 16 mM), *Hb*HNL inactivation and decrease in % ee of product were observed [44].

6.5.11. Effect of polyols in the synthesis of (*S*)-mandelonitrile

Synthesis of (*S*)-mandelonitrile with the addition of glycerol and sorbitol was performed using purified *Bm*HNL stored in 50 mM citrate-phosphate pH 3.5. The biocatalysis with glycerol addition showed the highest % ee of 99.2 in 15 minutes while 97.6% ee and 56.2% conversion of (*S*)-mandelonitrile was produced in the presence of sorbitol (**Figure 6.13**). The enzyme showed ~75% ee in absence of any polyol. This clearly indicates that polyol addition has increased the % ee of product.

*Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in the presence of polyols was also carried out using purified enzyme stored in 20 mM KPB pH 7.0 due to enhanced enantiomeric excess observed with enzyme at low pH. The enzyme showed excellent % ee (95-96.2) with 0.8, 5 and 10 mM benzaldehyde in the presence of glycerol as compared to biocatalysis without polyol (**Figure 6.17**). Further increase in benzaldehyde concentration

resulted in decreased % ee both in the presence and absence of glycerol which could be due to the inhibitory effect of benzaldehyde on *Bm*HNL [4].

Study of *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile in the presence of glycerol and sorbitol with 5 and 10 mM benzaldehyde at different time points was carried out based on the above results. The biocatalysis with glycerol showed improved % ee of 99.8 in both substrate concentration while in the presence of sorbitol, the % ee remained almost the same (**Figure 6.18**).

The excellent % ee of (*S*)-mandelonitrile observed in the *Bm*HNL catalyzed biocatalysis with glycerol is unique because this is the first report where mere addition of a polyol into an HNL has improved the % ee of product. Purified *Bm*HNL has been reported to synthesize (*S*)-mandelonitrile in 54% ee [4] while the double mutant *Bm*HNL-H103C-N156G improved the % ee to 93% [45]. In another study, we observed 99.8% ee of (*S*)-mandelonitrile with immobilized *Bm*HNL [24]. However, findings in this study reveal that a simple addition of glycerol to the enzyme could produce very high % ee of product. This result is comparable with the results of *Bm*HNL engineering or immobilization. Further comparison of substrate concentration between this biocatalysis and that reported by Dadashipour *et al* shows that we used 10 mM benzaldehyde vs. 2 mM benzaldehyde by them.

The cyanohydrin of 3-phenoxybenzaldehyde (cyanohydrin **10**) is an intermediate in the synthesis of pyrethroids. *Bm*HNL catalyzed synthesis of (*S*)-**10** (**Chapter 3.A**) in the presence of glycerol resulted in enhanced % ee of 89.5 as compared to 54% ee without glycerol (**Figure 6.21**). With an increase in substrate concentration, we observed a similar trend i.e. decrease in both the % ee and conversion of product. Jangir *et al* reported

synthesis of (*S*)-**10** (**Chapter 3.A**) by crude enzyme with 0.8 mM substrate concentration in a biphasic system that produced only 7.4% ee of the product [18]. CLEA-*Bm*HNL catalyzed synthesis of this (*S*)-cyanohydrin has also been reported that produced 97.6% ee of the product [24].

In case of *Bm*HNL catalyzed synthesis of (*S*)-**13** (**Chapter 3.A**), the biocatalysis in the presence of glycerol has improved % ee of product. Biocatalysis with glycerol produced 75% ee and 29% conversion (**Figure 6.22**). A decrease in % ee was observed with time. Purified *Bm*HNL catalyzed synthesis of (*S*)-**13** (**Chapter 3.A**) was already reported by Dadashipour *et al* with 85% ee [4]. They have used purified enzyme in the biotransformation. Jangir *et al* also reported the synthesis of the same cyanohydrin by using 6 U of crude *Bm*HNL in a biphasic system that resulted in 28% ee and 9.8% conversion of the product [18]. Immobilized *Bm*HNL catalyzed synthesis of (*S*)-**13** (**Chapter 3.A**) produced the product in 91.4% ee and 12.5% conversion in 100 minutes [24].

6.6. Conclusions

Effect of different biocatalytic parameters i.e. pH, temperature, buffer concentrations, presence of stabilizers, organic solvents and chemical additives on the stability of *Bm*HNL was studied. *Bm*HNL at its optimum pH 5.5, temperature 20 ^{o}C, and in optimal buffer concentration (100 mM citrate-phosphate pH 5.5) showed half-life of 554 to 690 h. These are the highest half-life among all the α/β hydrolase fold HNLs of their corresponding parameters, which suggests that *Bm*HNL can be used for several days without loss of activity. Polyol addition to *Bm*HNL at low pH has increased the enzyme's stability and activity. Biocatalysis of *Bm*HNL with sucrose has fivefold increased its half-life while the addition of sorbitol or glycerol increased ~ 9 fold specific activity. Importantly, glycerol

added *Bm*HNL biocatalysis has showed >99% ee of (*S*)-mandelonitrile from benzaldehyde. To our knowledge, this is the first observation of increase in % ee in the stereoselective cyanohydrin synthesis by any HNL. This opens the opportunity to explore the catalytic potential of an HNL not only to enhance its stability but also in the chiral cyanohydrin synthesis by polyol addition. However, this assumption needs to be tested to verify the expected success with other HNLs or at least among the α/β hydrolase fold HNLs.

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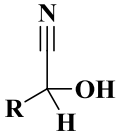
Chapter 7

Conclusions and future prospects

The major objective of the present work was to synthesize optically pure cyanohydrins by enantioselective C-C bond formation using *Baliospermum montanum* hydroxynitrile lyase. Towards this, two synthetic genes of *Bm*HNL i.e. *Bm*HNL syntgene-1 and 2, were sub-cloned into pET28a and pCold1 expression vectors. Between the two expression vectors, high protein expression was observed in pCold1 while in case of pET28a vector, protein expression was negligible. Further, protein purification via affinity chromatography by Ni-NTA agarose resin was carried out followed by enzymatic activity was measured using mandelonitrile cleavage assay. Specific activity of *Bm*HNL syntgene-1 and 2 were found to be 43.2 and 17.4 U/mg respectively. *Bm*HNL syntgene-1 was selected for further study due to its higher specific activity.

As the main focus of the thesis was *Bm*HNL catalyzed synthesis of chiral cyanohydrin hence to analyze the biocatalytic products, eighteen racemic cyanohydrins were synthesized using three methods with KCN, TMSCN and acetone cyanohydrin as cyanide sources. These racemic cyanohydrins were used as internal standards in HPLC chiral analysis.



KCN as cyanide source: R= 2,4-*di*$MeOC_6H_3$, 2,3,4-*tri*$MeOC_6H_2$, 3,4,5-*tri*$MeOC_6H_2$, 2-Naphthyl, *trans*-PhCH=CH and 4-CH_2=CH-CH_2OPh

TMSCN as cyanide source: R= 9-Anthranyl, Ph-CH_2, Ph-CH(CH_3), 3-PhO-C_6H_4, 3-$PhCH_2O$-C_6H_4 and 3-C_5H_4N

Acetone cyanohydrin as cyanide source: R= 3,5-*di*$MeOC_6H_3$, 2,5-*di*$MeOC_6H_3$, 4-$PhCH_2O$-C_6H_4, 4-BrC_6H_4, 3-OHC_6H_4 and 4-OHC_6H_4

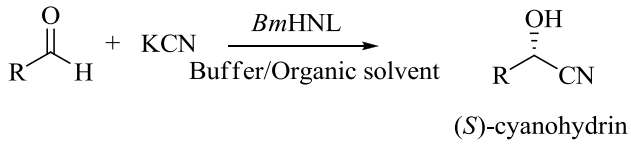
Among the three methods, two have been reported for synthesis of racemic cyanohydrins while the third method i.e. use of acetone cyanohydrin as a cyanide source has been reported for the synthesis of α-amino nitriles. We have modified this method and used it for the synthesis of racemic cyanohydrins.

Six racemic cyanohydrins, synthesized using KCN as a cyanide source have resulted in 22-64.9% yields. Another six racemic cyanohydrins were synthesized using TMSCN as a cyanide source in the presence of LiCl as catalyst and the products resulted in 24-68% yields. Similarly, another six racemic cyanohydrins were synthesized using acetone cyanohydrin as a cyanide source and 5% w/v aqueous $NaHCO_3$ solution that resulted in 20-76.4% yield of the corresponding products.

Enantiomeric separation of eighteen racemic cyanohydrins was carried out in chiral stationary phases, amylose tris-3,5-dichlorophenylcarbamate (Chiralpak IE) and cellulose tris-3,5-dimethylphenylcarbamate (Chiralpak IB) by varying ratio of hexane and 2-propanol with 1 mL/min flow-rate at 210 nm wavelength. Chiral separation of thirteen racemic cyanohydrins (**1-5**, **7**, **8**, **10**, **11** and **13-16**, **Table 3B.1**, **Chapter 3B**) was achieved in Chiralpak IB while three cyanohydrins (**6**, **17** and **18**, **Table 3B.1**, **Chapter 3B**) were

resolved in Chiralpak IE column. Enantiomers of two cyanohydrins (**9** and **12**, **Table 3B.1**, **Chapter 3B**) could not be resolved in either of the columns. A good separation was achieved in all sixteen cyanohydrins as the value for separation factor (α) >1 and resolution factor (R_s)>1.5 was observed for each case.

In order to improve the % enantiomeric excess of products, the *Bm*HNL catalyzed synthesis of chiral cyanohydrins was carried out in a biphasic system (**Scheme 7.1**). The reaction conditions such as different organic solvents, ratio of organic solvents (% v/v), substrate concentration (benzaldehyde and HCN), pH, and temperature were optimized using benzaldehyde as a standard substrate to get maximum % ee and conversion of (*S*)-mandelonitrile. The biotransformations were carried out using crude *Bm*HNL. Optimal results were found in 10% *n*-butyl acetate, 0.8 mM benzaldehyde, 10 min reaction time, pH 4.2, 22 °C and substrate to KCN ratio of 1:125.



Scheme 7.1: *Bm*HNL catalyzed synthesis of (*S*)-cyanohydrins

Under optimized conditions, seventeen different aldehydes were converted into their corresponding (*S*)-cyanohydrins. Among them, fourteen (*S*)-cyanohydrins were synthesized by *Bm*HNL for the first time and among them, eight have not been synthesized by any other (*S*)-selective HNL except the present study.

To improve biocatalytic properties of *Bm*HNL, immobilization of *Bm*HNL was carried out using cross-linking enzyme aggregate (CLEA) approach. To pursue this, optimization of

preparation of CLEA-*Bm*HNL was performed. After characterization of the CLEA-*Bm*HNL by its enzymatic activity, SDS-PAGE and SEM, it was used in the synthesis of (*S*)-cyanohydrins. Various biocatalytic parameters were optimized toward the synthesis of (*S*)-mandelonitrile as a standard product, to get maximum % ee and conversion. The optimized biocatalytic conditions attained were 20 minutes of reaction time, 7 U of CLEA-*Bm*HNL, 1.2 mM substrate, and 300 mM citrate buffer pH 4.2. Under optimal conditions, CLEA-*Bm*HNL showed highest 99% ee and 60% conversion of (*S*)-mandelonitrile, while only 54% ee was reported by purified *Bm*HNL and 93% ee by *Bm*HNL-H103C-N156G for the same product synthesis. Using the optimized biocatalytic conditions, eleven different chiral cyanohydrins were synthesized. Among them, ten (*S*)-cyanohydrins were obtained in 75.6-99.8% ee and 1.3-59.8% conversion while (*S*)-4-hydroxymandelonitrile was obtained in only 18.3% ee and 14.3% conversion. Among the selected substrates, nine (S. No. **3-11, Table 5.1, Chapter 5**) were converted into their corresponding (*S*)-cyanohydrins for the first time using CLEA-*Bm*HNL and eight cyanohydrins (S. No. **2, 3, 4, 5, 6, 7, 10**, and **11, Table 5.1, Chapter 5**) have not been synthesized by any CLEA-HNL. The reusability of CLEA-*Bm*HNL was also explored for the synthesis of (*S*)-mandelonitrile. The immobilized enzyme could be reused for eight successive cycles without loss of conversion and five cycles with a little loss in enantiomeric excess.

Biophysical characterizations of *Bm*HNL was studied in order to understand the stability and activity of the enzyme. Various parameters i.e. pH, temperature, buffer concentrations, presence of stabilizers, organic solvents, benzaldehyde, and chemical additives were selected for the study. The activity and stability of *Bm*HNL were measured by mandelonitrile cleavage assay. *Bm*HNL showed half-life of 554 h at its optimum pH 5.5,

686 h at its optimum temperature 20 ^{o}C, and 690 h in optimal buffer concentration (100 mM citrate-phosphate pH 5.5) which was highest among all the α/β hydrolase fold HNLs. The stability of *Bm*HNL at low pH was studied by the addition of stabilizers. We observed that the addition of 400 mg/mL of sucrose increased enzyme's half-life more than five folds and activity of the enzyme increased after addition of all the selected stabilizers. The effect of two polyols, glycerol, and sorbitol in *Bm*HNL catalyzed synthesis of (*S*)-mandelonitrile was studied. Sorbitol did not improve % ee and conversion of (*S*)-mandelonitrile while the addition of glycerol has resulted in >99% ee of (*S*)-mandelonitrile with 5 mM benzaldehyde. Subsequently, we extended this study using 3-phenoxy benzaldehyde and 3,5-dimethoxy benzaldehyde to synthesize corresponding cyanohydrins. In both, the cases improved % ee was found as compared to free enzyme. The inhibitory effect of benzaldehyde was studied in the synthesis of (*S*)-mandelonitrile which showed that % ee as well as conversion was decreased with increase in benzaldehyde concentrations. Secondary structural study of *Bm*HNL investigated in the presence of organic solvent and different temperatures using CD-spectrophotometer showed that the enzyme's structure is least affected.

Future prospects:

- A series of optically pure bulky cyanohydrins synthesized by *Bm*HNL can be used as precursors in the synthesis of appropriate pharmaceuticals, agrochemicals, and useful fine chemicals.
- We proved CLEA-*Bm*HNL as a robust catalyst in the synthesis of chiral cyanohydrins, therefore it has the potential to be used as an attractive biocatalyst in the industrial process. However preparative scale synthesis and practical

application of CLEA-*Bm*HNL to synthesize enantiopure cyanohydrins still remain a challenge because of the low substrate concentration used here. Such scale-up synthesis can be further studied.

- Increased % ee of (*S*)-cyanohydrins was observed by CLEA immobilization of *Bm*HNL. The exact reason behind this observation has not been explored by us. This needs a further study which can be extrapolated to other HNLs of at least α/β hydrolase fold.
- The available information of enantiomeric separation of eighteen cyanohydrins using Chiralpak IB and IE can be used for the analysis of biocatalytically produced cyanohydrins.
- The present study showed improved % ee of *Bm*HNL catalyzed (*S*)-cyanohydrins by addition of glycerol into biocatalysis. This is the first observation of increase in % ee in the stereoselective cyanohydrin synthesis by any HNL. The study opens the opportunity to explore the catalytic potential of a HNL not only to enhance its stability but also in the chiral cyanohydrin synthesis by polyol addition. However, this assumption needs to be tested to verify the expected success with other HNLs or at least among the α/β hydrolase fold HNLs.
- Effect of polyol addition in the synthesis of chiral cyanohydrins using CLEA-*Bm*HNL can also be studied as both the factors contributed to the increase in % ee of *Bm*HNL's catalysis in the present thesis.

LIST OF PUBLICATIONS

a) In Refereed international journals

1. **Nisha Jangir**, Dheeraj Sangoji, and **Santosh Kumar Padhi**, *Baliospermum montanum* hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins in a biphasic solvent. *Biocatal Agric Biotechnol.*, **2018**, *16*, 229-236.

2. **Nisha Jangir**, **Santosh Kumar Padhi**, Immobilized *Baliospermum montanum* hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins. *Bioorg. Chem.*, **2019**, *84*, 32-40.

3. **Nisha Jangir**, Preeti, **Santosh Kumar Padhi,** A Study on Increasing Enzymatic Stability and Activity of *Baliospermum montanum* Hydroxynitrile lyase in Biocatalysis. *Process Biochemistry* (*Communicated*).

b) Presentations in conferences

1. **Nisha Jangir**, Sheetal Uikey, and Santosh Kumar Padhi, "Biocatalytic studies of an immobilized Hydroxynitrile lyase" - Presented in "BioQuest 2015" School of Life Sciences, University of Hyderabad, Hyderabad, India from 23rd -24th September 2015 (Poster P-41).

2. Asha Jakhar, **Nisha Jangir**, Mohammed Ahmad, and Santosh Kumar Padhi, "Screening and purification of new Hydroxynitrile lyases from a cyanogenic glycoside containing plants"- Presented in "BioQuest 2015" School of Life Sciences, University of Hyderabad, Hyderabad, India from 23rd -24th September 2015 (Poster P-4).

3. T. Revathi, **Nisha Jangir**, and Santosh Kumar Padhi, "Cross linked enzyme aggregates of Hydroxynitrile lyase and its biocatalytic studies"- Presented in "BioQuest 2016" School of Life Sciences, University of Hyderabad, Hyderabad, India from 20th – 21st October 2016 (Poster P-48).

4. K. Mohammad, **Nisha Jangir**, and Santosh Kumar Padhi, "Biocatalytic studies of an immobilized Hydroxynitrile lyase" - Presented in "BioQuest 2016" School of Life Sciences, University of Hyderabad, Hyderabad, India from 20th – 21st October 2016 (Poster P-19).

5. **Nisha Jangir**, Ashwini Khaladkar, Dheeraj Sangoji, and Santosh Kumar Padhi, "Hydroxynitrile lyase catalyzed enantioselective synthesis of 2-hydroxy-2-(4-phenoxyphenyl) acetonitrile" - Presented in "BioQuest 2017" School of Life Sciences, University of Hyderabad, Hyderabad, India from 12th –13th October 2017 (Poster P-32).

6. **Nisha Jangir** and Santosh Kumar Padhi "Immobilized *Baliospermum montanum* hydroxynitrile lyase catalyzed synthesis of chiral cyanohydrins" - Presented in "International Conference on Life Science Research & its Interface with Engineering and Allied Sciences 2018 (LSRIEAS-2018)" BITS Pilani, Pilani, Rajasthan, India from 1st– 3rd November 2018 (Poster: BBS-8).

Baliospermum montanum hydroxynitrile lyase catalyzed stereoselective synthesis of chiral cyanohydrins

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